THE PHYSIOLOGY, BIOCHEMISTRY AND MOLECULAR CLONING OF A *PSEUDOMONAS* LIPASE.

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ABSTRACT.

THE PHYSIOLOGY, BIOCHEMISTRY AND MOLECULAR CLONING OF A *PSEUDOMONAS* LIPASE.

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The physiological regulation of lipase production by a newly-isolated strain of *Pseudomonas aeruginosa* (strain EF2) was investigated during growth in batch, fed-batch and continuous cultures. Lipase activity (measured as the rate of olive oil hydrolysis) was strongly induced by long-chain fatty acyl esters (especially Tween 80), weakly induced by carbon and/or energy limitation, and repressed by long-chain fatty acids, including oleic acid. Lipase production by *Ps. aeruginosa* EF2 was optimised with respect to temperature and pH in a Tween 80-limited continuous culture using statistical response surface analysis at a fixed dilution rate of 0.05 h^{-1} (optimum values 35.5°C, pH 6.5). Maximum activities were obtained under these conditions at a dilution rate of 0.04 h^{-1} (39 LU [mg cells]$^{-1}$; where 1 LU equalled 1 µmol titratable fatty acid released min^{-1}). These were over eight-times greater than the maximum activities detected following growth on Tween 80 (8.3 LU mg $cells^{-1}$), and over forty-times greater than growth on glucose (< 1 LU mg $cells^{-1}$) in batch cultures. Esterase activities (measured as both the rate of hydrolysis of both *p*-nitrophenyl acetate and Tween 80) varied approximately in parallel with lipase activities under nearly all growth conditions, suggesting that a single enzyme catalysed both activities.

Lipase was isolated from a high activity continuous culture of *Ps. aeruginosa* EF2. The enzyme was purified to homogeneity (99.5 % pure from SDS-PAGE analysis) by ultrafiltration of the culture supernatant, followed by anion-exchange and gel-filtration FPLC. The lipase was composed of a single subunit protein which appeared to aggregate variably under non-dissociating conditions. The enzyme was a true lipase that exhibited some esterase activity towards *p*-nitrophenyl acetate and Tween 80. The enzyme preferentially hydrolysed the 1,3-oleyl residues of radiolabelled triolein, was relatively stable at moderate temperatures and very stable to freezing and thawing. The enzyme was only weakly inhibited by the serine-active reagent 3,4-dichloroisocoumarin, and not inhibited by the chelating agent EDTA. The N-terminal amino acid sequence of the *Ps. aeruginosa* EF2 lipase showed a significant homology with those of other *Pseudomonas* lipases.

The lipase gene of *Ps. aeruginosa* EF2 was identified by Southern analysis of restricted chromosomal DNA using a synthetic oligonucleotide probe based on the N-terminal amino acid sequence of the purified lipase. The hybridizing *Eco*R I fragments were ligated into the plasmid vectors pKT230 (pJG1) and pUC18 (pJG5 and pJG31). These constructs were used to transform the lipase-negative *E. coli* JM109 and the resultant clones (JG1, JG5 and JG31) were able to grow on Tween 80. pJG5 was further analysed by restriction with various endonucleases and Southern blotting. Sub-clones of pJG5 were constructed (containing either a 4.3 kbp *Bam*H I, 2.5 kbp *Sal* I or 3.2 kbp *Sph* I fragment), but these failed to confer the lipase-positive phenotype to *E. coli* JM109. Esterase activity (measured as *p*-nitrophenyl acetate hydrolysis) of *E. coli* JG5, unlike that of *Ps. aeruginosa* EF2, was not subject to substrate-induction/end-product repression and was very low.

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Thanks.

ABBREVIATIONS.

A	alanine
cAMP	cyclic adenosine monophosphate
AMP	adenosine triphosphate
BSA	bovine serum albumin
CTAB	hexadecyl trimethylammonium bromide
D	aspartic acid
DCI	3,4-dichloroisocoumarin
DMF	*N,N*-dimethyl formamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
DOT	dissolved oxygen tension
E	glutamic acid
EDTA	ethylenediamine tetra acetic acid
FPLC	fast protein liquid chromatography
g	relative centrifugal force
G	glycine
GTE	glucose-Tris-EDTA buffer
H	histidine
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
I	isoleucine
IPTG	isopropyl-β-D-thiogalactopyranoside
kbp	kilobase pairs
L	leucine
LU	lipase unit(s)
M	methionine
MOPS	3-(*N*-morpholino) propane sulphonic acid
M_r	relative molecular mass
OD	optical density
P	proline
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol (average molecular mass shown as a subscript)
Q	glutamine
RNA	ribonucleic acid

RNAase	ribonuclease A Type II-A
rpm	revolutions per minute
S	serine
SE	standard error
SDS	sodium dodecyl sulphate
T	threonine
TAE	tris-acetate-EDTA buffer
TCA	trichloroacetic acid
TE	tris-EDTA buffer
TEMED	*N,N,N',N'*-tetramethyl ethylene diamine
TNE	Tris-NaCl-EDTA buffer
Tris	tris(hydroxymethyl)aminomethane
UV	ultra violet light
V	valine
X	unspecified amino acid
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactoside
Y	tyrosine

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CHAPTER 1.

GENERAL INTRODUCTION.

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1.1 *The definition of lipases.*

Lipases (EC 3.1.1.3) are glycerol ester hydrolases that catalyse the hydrolysis of triacylglycerols into fatty acid, partial acylglycerols and glycerol. Their natural substrates are generally long chain fatty acyl esters that are insoluble in aqueous solutions. Lipases therefore differ from other enzymes in one important aspect; their reactions occur in a heterogeneous system, where two and often four or more distinct domains are present (Patton & Carey, 1979). Similarly, the phospholipases (EC 3.1.1.4) and cholesterol esterases (EC 3.1.1.13), which also act on structurally similar insoluble substrates, are also often described as lipases. They are all encompassed by the definition of Desnuelle (1961), as esterases that act specifically on insoluble esters at the oil-water interface.

1.2 *The kinetics of lipolysis.*

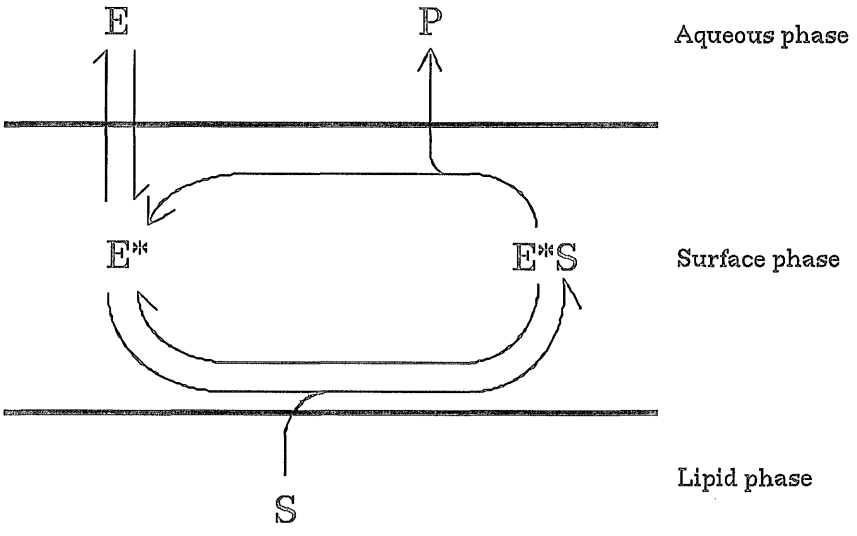
An important feature of lipases is their apparent activation in the presence of lipid interfaces. This was first recognised by Holwerda *et al.* in 1936 and Schønheyder and Volqvartz in 1945 (see Verger 1980), although it was not until 1958 that this phenomenon was actually quantified by Sarda & Desnuelle. They demonstrated that porcine pancreatic lipase preferentially hydrolysed triacetin when it was present in an emulsified, rather than a monomeric form. This was unlike horse liver esterase, which did not discriminate between the two states, and therefore exhibited normal Michaelis-Menten kinetics. Entressangles and Desnuelle (1968) further demonstrated this interfacial activation using triacetin and tripropionin present as micelles in an isotropic system (*ie.* at concentrations above the critical micellar concentration, but below the level of saturation where emulsification occurs). In addition, Brockman *et al.* (1973) reported a

thousand-fold increase in the rate of hydrolysis of tripropionin by porcine pancreatic lipase in the presence of siliconised glass beads, a phenomenon also demonstrated by Chapus *et al.* (1976) who attributed it to a conformational change in the enzyme.

The presence of an interface for the effective hydrolytic activity of lipases has meant that simple Michaelis-Menten kinetics do not apply. Benzonanna and Desnuelle (1965) calculated values of K_M and V_{max} for the hydrolysis of emulsified triacylglycerols, when the substrate concentration was considered in terms of per unit surface area, rather than per unit mass. The first model taking into account the adsorption of the soluble enzyme on to the interface was proposed by Verger *et al.* (1973). They derived rate equations which accounted for the reversible penetration (this term was used preferentially over adsorption) of the enzyme into the interface, prior to the formation of the enzyme-substrate complex and subsequent catalysis. A simplified diagram of this process is shown in Figure 1.1. Penetration was assumed to confer a conformational change on the enzyme which was responsible for its activation.

A variety of different methods have been used to detect lipolytic activity. These are generally based on the continuous (*eg.* a pH-stat) or discontinuous titration of released fatty acid from a lipid substrate, or the measurement of a chromogenic anion from a synthetic ester (*eg.* *p*-nitrophenyl esters); for reviews see Jensen (1983) and Erdmann *et al.* (1991). Whilst these are often sufficient to detect and quantify activities, there are inherent limitations in their use to study the kinetics of lipolysis. In particular, an increase in the amount of bulk emulsion in a heterogeneous system does not necessarily increase the effective substrate concentration (molecules per unit surface area), whilst the properties of the interface may vary appreciably. These considerations have led to the development of novel techniques which,

Figure 1.1 *Schematic diagram of the lipolytic reaction.*



E, enzyme; E*, activated enzyme; S, substrate; P, product.

although often utilising sophisticated experimental equipment, significantly reduce the number of variables, therefore enabling reactions to occur under more defined conditions. Examples include an oil drop tensiometer method (based on the variation in the interfacial tension of a single, large oil drop due to the action of a lipase; Nury *et al.*, 1991), an automated optical particle sizer linked to a pH-stat which enables the interfacial area of a lipid emulsion to be calculated, and a hollow fiber membrane reactor (Kloosterman *et al.*, 1991). The application of monolayer techniques (for reviews see Verger, 1980; Brockman, 1984) have, however, been extensively used. Although the reaction takes place at the air-water interface where the denaturation of many lipases is known to occur, the method is sensitive and enables the properties of the interface, such as the density (hence the concentration), orientation, charge, and fluidity of the substrate molecules to be varied.

This method has been successfully refined by Verger and co-workers who designed a 'zero order' trough, consisting of a separate substrate and reaction chamber connected by a narrow surface canal (see Verger & de Haas, 1976; Verger, 1980). The action of a lipase on the monolayer results in the formation of soluble products, which effectively lowers the interfacial tension. This is compensated for by the movement of a barrier across the substrate trough to maintain a constant surface pressure. Enzyme activity is therefore measured mechanically and is often expressed as a percentage of the remaining substrate. A major advantage of this method is that it results in linear kinetics, unlike a single ('first order') trough, where the decrease in substrate is exponential. The regulatory effects of non-substrate components, such as proteins, which are often present along-side lipases *in vivo* (*eg*. in the small intestine in the case of pancreatic lipase) may also be determined using this method (Verger *et al.*, 1991).

It has been appreciated for some time now that lipase activity is not only regulated by diffusional constraints imposed on the enzyme, substrate and products (*ie.* the rate of penetration and binding of the enzyme to the substrate to form the catalytic complex and the removal of inhibitory products), but also by the 'quality of the interface' (Verger *et al.*, 1973). Recently Brockman and Muderhwa (1991) demonstrated that non-substrate lipids play a key role in the regulation of carboxylester lipase activity. This was performed using monolayers containing a substrate fatty acid (13,16-*cis,cis*-docosadienoic acid; DA) and a non-substrate surface-active component (1-palmitoyl-2-oleoyl-phosphatidylcholine; POPC). Enzyme activity was measured as the extent of ^{18}O exchanged between the carboxyl oxygen of DA and water over a standard incubation time. This approach was unique, insomuch as it enabled lipase activity to be quantified under precise conditions, without the accumulation of detrimental products at the interface which would otherwise occur using a lipid substrate (Muderhwa *et al.*, 1991). Lipase activity was shown to be dependent upon the mole fraction ratios of DA:POPC, increasing from <10 % to >90 % over a very narrow range. It was suggested that at low substrate concentrations, DA formed distinct domains within the POPC matrix to which the enzyme bound. As the concentration of DA was increased, a 'percolation'-type transition between DA and POPC occurred which profoundly affected the availability of substrate to the enzyme (Muderhwa & Brockman, 1991). Although far from complete, these novel experiments further indicate the complexity of the lipolytic reaction.

1.3 *The structure of lipases.*

In addition to the above techniques, group-specific inhibitors have been used to identify certain amino acid residues that contribute to the binding and catalytic properties of various lipases (for a short review see Lóokene & Sikk, 1991). As early as 1960, Desnuelle and co-workers demonstrated that lipase activity was irreversibly inhibited following incubation of the enzyme with the serine-active reagent diethyl *p*-nitrophenyl phosphate (E_{600}). Unfortunately this was erroneously attributed to the binding site, rather than the catalytic site of the enzyme, by Chapus and Sémériva (1976). However, since then many independent studies have confirmed the presence of a catalytic serine (see for example Kordel & Schmid, 1991; Kordel *et al.*, 1991 b; Hadváry *et al.*, 1991, for *Pseudomonas* and pancreatic lipases respectively) and lipases are now recognised as serine hydrolases. More recently, advances in genetic manipulation techniques and nucleotide sequencing, have revealed striking similarities between lipases from many unrelated species. The complete nucleotide sequences of a number of mammalian, fungal and bacterial lipase genes are now known, and all suggest that the enzymes contain a highly conserved pentapeptide sequence around the active site serine (- G X S X G -), although it is interesting to note that Dartois *et al.* (1991) recently reported the sequence - A H S M G - in a *Bacillus subtilis* lipase. This sequence homology is identical to that of the serine proteases, whose catalytic mechanism has been well characterised (see, for example, Stryer, 1988).

A knowledge of the primary structure has been used to predict the secondary structure of some lipases (*eg.* Götz *et al.*, 1987). Kordel *et al.* (1991 a) analysed only the pentapeptide consensus sequences of 21 lipases from different sources, and concluded that most were buried beneath the surface of the enzyme and formed a turn structure. Kloosterman *et al.* (1991)

adopted an alternative approach and modelled the active site of a *Pseudomonas fluorescens* lipase based on a knowledge of its stereoselective catalysis of various compounds.

Only recently have precise active site models been constructed of mammalian and fungal lipases, based on X-ray crystallographic studies. Detailed structures of human pancreatic, *Rhizormucor miehei* (previously *Mucor miehei*) and *Geotrichum candidum* lipases have been published (Winkler *et al.*, 1990; Brady *et al.*, 1990; Schrag *et al.*, 1991, respectively). All are α/β-type structures containing a central β sheet. Although they share no sequence homology (apart from the conserved pentapeptide), the human pancreatic and *R. miehei* lipases both possess a -D···H···S- trypsin-like catalytic triad. In contrast, the *G. candidum* lipase contains a -S···H···E- triad. These confirm earlier observations using site-specific inhibitors, that lipases are indeed serine hydrolases. The active sites are buried within the protein and a similar mechanism to that involved in peptide cleavage by serine proteases has been proposed. Refinement of the human pancreatic model (Gubernator *et al.*, 1991) has identified an oxyanion hole and two plausible hydrogen bond-donating groups required to stabilise the oxyanion of the tetrahedral intermediate; a calcium-binding site was also suggested. The similarity between lipases and serine proteases led to the proposition by Brenner (1988), that they have descended from a single cysteine-containing ancestral enzyme.

The unique feature of these structures is the presence of a surface flap or loop covering the active site. This takes the form of a single tryptophan-containing α-helix in both the human pancreatic and *R. miehei* lipases, whilst the active site of the *G. candidum* lipase is covered by two parallel α-helices. Hydrophobic interactions predominate between the surface of the loop and the active site, whilst polar side chains are present on the aqueous

side, thus effectively blocking access of any substrate. Recently, Brzozowski *et al.* (1991) proposed a model depicting movement of the loop to reveal the active site, thus explaining the supposed activation of lipases at lipid/water interfaces. This was determined from X-ray crystallography of the *R. miehei* lipase complexed with the irreversible inhibitor *n*-hexachlorophosphonate ethyl ester. The active site was shown to be exposed by rotation of the loop. The hydrophilic side, which was previously in contact with the aqueous solution became buried within a pre-formed polar cavity, whilst the hydrophobic portion, previously in contact with the active site, became exposed. This therefore increased the non-polar surface around the active site, enabling binding to the lipid substrate. In addition, a putative tetrahedral substrate/enzyme intermediate was proposed, similar to the chymotrypsin complex (Stryer, 1988). However, unlike serine proteases, the oxyanion hole was thought to be formed by the movement of the helical loop. The depth of the buried active site within the protein structure is thought to account for the specificity of many lipases for long chain fatty acyl esters. Preliminary data of X-ray crystallographic studies of *Pseudomonas* lipases have been reported, but have yet to be refined and structural models assigned (Cleasby, 1991; Kordel *et al.*, 1991 b; Sarma *et al.*, 1991); although the *Pseudomonas* lipase investigated by Genencor, is known to contain a -S···D···H- triad (Bott, 1990). Similar structural models have recently been proposed for phospholipases (snake and bee-venom phospholipases A_2; Blow, 1991), therefore it will be interesting to discover whether a surface helical loop is also common to these bacterial lipases, and is therefore a property of lipases in general.

1.4 *The application of lipases.*

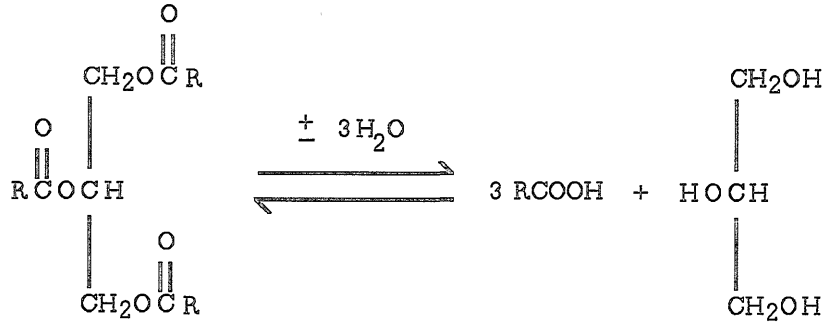
Lipases are interesting enzymes in that they not only exhibit specificities for for long/short or saturated/unsaturated fatty acyl residues, but also a positional specificity for either the 1 (3) or 1,2 (2,3) positions of a triacylglycerol molecule (Figure 1.2; Macrae, 1983). In addition, they also remain active in a variety of organic solvents where they can catalyse various transformations other than the hydrolytic reaction by which they are defined (see, for example, Schuch & Mukherjee, 1989). The versatility of lipases is therefore being exploited industrially, either to replace existing processes, or to produce a variety of different compounds previously not deemed possible.

The world market for industrial enzymes has been estimated at approximately US $ 600 million, with lipases comprising approximately US $ 20 million (Arbridge & Pitcher, 1989). The successful application of recombinant DNA technologies (Boel *et al.*, 1991), protein engineering (Nosoh & Sekiguchi, 1990) and novel processes has enabled lipases to effectively compete with other well-established chemical technologies (Björkling *et al.*, 1991). Until recently, the advantages of enzymic processes were offset by high production and purification costs and the general instability of the enzyme. However, recent environmental considerations (such as energy expenditure, the composition of effluent waste) and the quality of the product has tended to favour the use of enzymes.

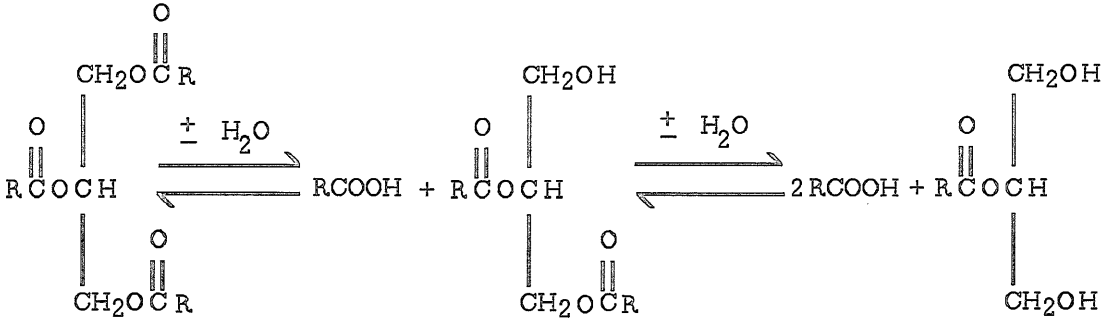
Lipases have been applied to the degreasing of animal hides, as components of chewing gums, dentifrices, cosmetics and digestive aids (Seitz, 1974), to de-block drains in fast food outlets ('Biofree'; Interbio) and to remove troublesome pitch build-up during paper manufacture (Björkling *et al.*, 1991; Fischer & Messner, 1991). Although useful, these applications

Figure 1.2 *Positional specificity of lipases.*

Non-specific lipase reaction.



1,3-Specific lipase reaction.



only comprise a minute fraction of the total lipase market. Currently, lipases are restricted to three major industries, as described below:

1.4.1 *The food industry.*

Lipases have been exploited indirectly for many centuries, where they are produced *in situ* during the fermentation and ripening of various vegetable and meat products (Seitz, 1974). In particular, the selective hydrolysis of milk lipids by various lipolytic microorganisms during the maturation of cheeses is directly responsible for their distinctive flavours (Stead, 1986). More recently, lipases have been employed directly in the preparation and processing of various foods (recently reviewed by Whitaker, 1990). This can be exemplified by the development of a commercial lipase preparation ('Flavor Age'; Chr. Hansen's Laboratory, Inc.), which, when added to milk curds during the early stages of production substantially reduced the ripening time required to produce the mature Cheddar cheese (Arbridge *et al.*, 1986). In addition to flavours produced *in situ*, lipases have also been used to produce so-called 'natural' flavours, such as the simple esters described by Gillies *et al.* (1987). This reflects a trend away from the use of chemical-based processes, which may employ potentially hazardous chemicals and detrimental reaction conditions (Malley, 1983).

The use of lipases within the oil and fats industry is currently receiving considerable interest, either for the total hydrolysis, or the selective modification of various triacylglycerols.

(i). *The hydrolysis of triacylglycerols.* In the past, the use of lipases to completely hydrolyse triacylglycerols has been considered uneconomical compared to conventional chemical methods (Rattray, 1988). These processes involve the use of high temperatures and pressures (50-60 atm), which although obtaining a 98-99 % conversion rate, often result in the partial

breakdown of the products (Nielsen, 1985 a; Derksen *et al.*, 1991). As 1.6 million tons of fatty acids are produced annually (Pronk *et al.*, 1988) for uses as surfactants, in cosmetics, lubricants, resins, enamels, varnishes *etc.*, a large market already exists where the introduction of a lipase-based process would be potentially advantageous. For this to be feasible, however, the use of thermostable enzymes, and the development of novel processes to ensure maximum interfacial contact between enzyme and triacylglycerol would be required. Examples include a hydrophilic membrane reactor (Pronk *et al.*, 1988) and reversed micelles (Han & Rhee, 1986).

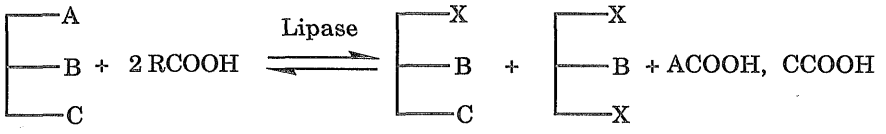
(ii) *The modification of triacylglycerols.* Over the last thirty years there has been a general global trend away from the use of saturated (generally animal-based) triacylglycerols towards the use of mono- and poly-unsaturated forms (*eg.* soya, sunflower, rape seed and palm oils; for a review see Rattray, 1988). These are used primarily for human consumption, and reflect an increasing awareness of the quality of our diet. Extensive research has been carried out to produce plant varieties which not only yield elevated levels, but also produce oils with novel fatty acyl compositions. In addition, the use of oleaginous microorganisms to produce speciality triacylglycerols (so-called 'single cell oil') has been assessed (Ratledge, 1984), but unfortunately economical considerations limit these at present.

Recently lipases have been effectively applied to modify the fatty acyl moieties of various triacylglycerols for specific applications by interesterification reactions (Figure 1.3; Kalo *et al.*, 1989; for reviews see Macrae, 1983; Macrae, 1985; Macrae & Hammond, 1985; Harwood, 1989; Macrae, 1989). These occur in organic (often water immiscible) solvents where the hydrolytic reaction is suppressed and the stability of the enzyme is enhanced (although small amounts of water are required to activate the catalyst; Deetz & Rozzell, 1988). The chemical modification of oils has been

Figure 1.3. *Examples of lipase-catalysed reactions other than hydrolysis.*

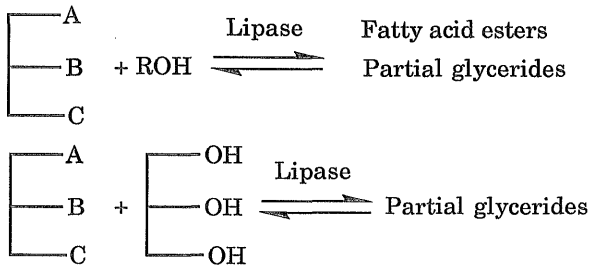
i. Acidolysis.

The reaction between triglyceride and fatty acid;



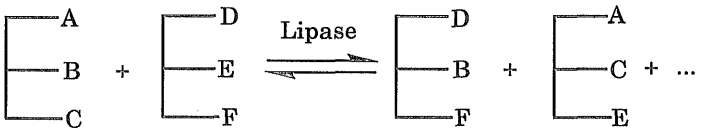
ii. Alcoholysis.

The reaction between triglyceride and alcohol;



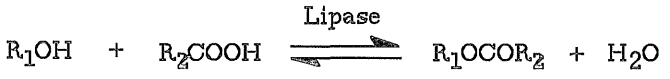
iii. Interesterification.

The exchange of fatty acyl groups between triglycerides.



iv. Ester synthesis.

The synthesis of esters other than glycerides

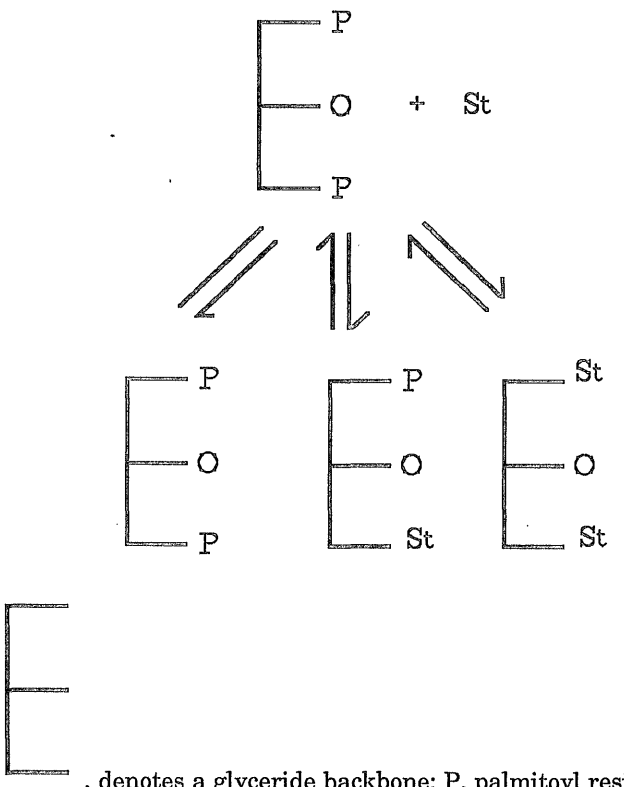


used in the production of margarines, where the random migration of fatty acyl residues is promoted by inorganic catalysts (such as sodium hydroxide or sodium metal). The specificity of lipases enables a greater degree of control over the final product, therefore enabling the production of specific ('tailored') triacylglycerols which would otherwise not be possible by chemical means. Thus, a large market exists with values of enzymatically-produced fatty acyl esters and triacylglycerols estimated at £ 1-10 kg^{-1} at production rates of between 500-50,000 tonnes per annum (Macrae, 1989)

Cocoa butter (which is used primarily in confectionery) is very expensive and has led to the development of various processes to produce alternatives. 1,3-specific lipases have been successfully exploited to produce cocoa butter substitutes from relatively inexpensive palm oil. This involves the selective replacement of a palmitate residue at either the 1- or 3-position of palm oil mid fraction with a stearate residue to yield a mixture of triacylglycerols in similar ratios to those present in cocoa butter (Macrae, 1985; Harwood, 1989; Figure 1.4). The development of these lipase-catalysed systems has required the use of a variety of immobilisation techniques (see the above reviews and Wisdom *et al.*, 1984; Linko & Linko, 1985) to enable re-use of the enzyme and maximum interfacial contact with the substrate. In addition, the stability of the enzyme is also increased, enabling use at high temperatures (70°C) for long periods of time (Harwood, 1989). Processes have either been carried out in a batch stirred tank reactor, or in continuous mode using packed bed reactors.

Novo Industri A/S market a 1,3-specific *Rhizormucor miehei* (previously *Mucor miehei*) lipase (Lipozyme) immobilised onto a macroporous ion exchange resin (Eigtved & Hansen, 1985; Hansen & Eigtved, 1985), specifically for interesterification reactions (half-life at 60°C is 1800 h; Björkling *et al.*, 1991). This has been subject to extensive toxicological

Figure 1.4. *Production of cocoa butter substitutes from palm oil by 1,3-specific lipases.*



, denotes a glyceride backbone; P, palmitoyl residue; O, oleoyl residue; St, stearoyl residue or stearic acid.

Approximate composition of cocoa butter and the enzymatically-produced equivalent (Macrae, 1985):

Triglyceride	Amount in cocoa butter (%)	Amount in enzymatically produced fat (%)
POP	16	16
POSt	41	39
StOSt	27	29

studies, and is deemed safe for use in the preparation of foods (Jensen & Eigtved, 1990).

1.4.2 *The speciality chemicals industry.*

The ability of lipases to catalyse a variety of different reactions in organic media is currently receiving considerable interest from both academia and industry (for reviews see Lilley & Woodley, 1985; Deetz & Rozzell, 1988; Zaks *et al.*, 1988). The stereo-specific nature of many lipases enables the production of optically pure stereoisomers or the resolution of racemic mixtures (Kircher *et al.*, 1985; Zaks *et al.*, 1988; Spreitz *et al.*, 1991), which would otherwise require complex chemical methods (Sheldon *et al.*, 1985). This has far-reaching implications within the pharmaceutical and agrochemical industries, where often only one stereoisomer of a compound confers the desired properties; the corresponding racaemate may either be inactive or detrimental (*eg.* the birth defects caused by Thalidomide were due soley to the *S*-enantiomer). Examples include (*R*)-phenoxypropionic acids used as herbicides (Kloosterman *et al.*, 1991), (*S*)-aryloxypropanolamine used as β-blockers (Kloosterman *et al.*, 1988) and an optically pure secondary alcohol used in the preparation of insecticidal pyrethroids (Mitsuda *et al.*, 1988).

Lipases have also been applied to the production of unusual compounds, such as carbohydrate esters (so-called 'green' chemicals which have potential as surfactants; Björkling *et al.*, 1991; Janssen *et al.*, 1991) and as alternatives to proteases in the synthesis of peptides (Harwood, 1989). The unique conditions present in either low/non-aqueous environments have led to the development of novel techniques to either improve the catalytic properties or stability of the enzyme. These have included non-covalent

modifications (*eg.* Wu *et al.*, 1990) or covalent complexing with, for example, PEG (Takahashi *et al* ., 1985; Inada *et al.*, 1986; Inada *et al.*, 1988).

1.4.3 *The detergents industry.*

The use of enzymes in laundry products dates back to 1913, when Röhm incorporated animal pancreatic extracts into a pre-soaking agent 'Burnus' (see Towalski, 1986, 1987). Altenberg was probably the first person to describe the use of a lipase in a German patent in 1930 (see Towalski, 1986, 1987). The shortage of fats (from which soaps were made) during World War II initiated interest in the use of enzymes as alternatives. This ultimately resulted in the development and production of 'Bio 40' in 1959 (a preparation containing a neutral protease), then 'Alcalase' (an alkaline protease from *Bacillus licheniformis*; Novo Industri A/S) formulated into 'Bio 40' and 'Bio-Tex', followed shortly by 'Maxatase' (Gist-Brocades). By 1966 detergent formulations containing enzymes comprised 2-3 % of the market, and growth continued into the 1970's (Towalski, 1986, 1987). When a correlation between skin allergies and the use of enzyme-based detergents became evident, demand fell sharply. This resulted in the development of dust-free formulations by encapsulating the enzyme in a variety of different coatings (van Tilburg, 1984) or by adsorption on to inert particles (Gandelot & Talkes, 1985). Unfortunately, public concern still persisted, such that when Unilever introduced New System Persil Automatic in 1983 it was forced to re-introduce the original non-biological Persil within a year (Abrahams, 1988). More recently, the trend towards the use of 'green' detergents (such as Ecover) which actively avoid the use of enzymes, has somewhat impeded the growth of the enzyme-based market. Despite this, the demand for detergents containing enzymes is increasing, due primarily to the following reasons:

(i) a decrease in the average European wash temperature from 60°C to 40°C, resulting from an increased awareness of energy conservation and an increase in the use of synthetic fabrics (van Tilburg, 1984) and;

(ii) a change in the composition of detergents, resulting from legislation regulating phoshate emissions and the development of liquid detergents (Maase, 1983; van Tilburg, 1984).

For an enzyme to be successfully incorporated into a detergent formulation it must retain its activity at alkaline pH values (up to pH 10) in the presence of a variety of different chemicals, which include:

(i) non-ionic and anionic surfactants, ranging from 1-30 % (w/w);

(ii) builders, which act to soften the water, reduce corrosion within washing machines and remove dirt by forming ion complexes. These range from 1-45 % (w/w) and include phosphates, carbonates, citrates, nitriloacetates, carboxymethylsuccinates, zeolites and polyacetalcarboxylates;

(iii) bleaching agents and associated activators, such as sodium perborate (1-35 % w/w);

(iv) fluorescent materials, latherboosters, foam supressors, anti-corrosion agents, soil-suspending agents, anti-soil redeposition agents, perfumes and dyes;

(v) other enzymes, such as proteases, amylases, oxidases and cellulases (Starace, 1983; Van Tilburg, 1984; Thom *et al.*, 1986; Towalski, 1986, 1987; Stinson, 1987).

In addition, stability over a period of months in the presence of these agents is imperative if the product is to perform according to the manufacturers' claim.

The patent literature describes many examples of microbial lipases deemed suitable for incorporation in to laundry detergents (see, for example, Nielsen, 1985 b; Thom *et al.*, 1986; Enomoto & Riisgard, 1987; Farin *et al.*, 1987; Huge-Jensen & Gormsen, 1988; Andreoli *et al.*, 1989; Pierce *et al.*, 1990; Batenburg *et al.*, 1991). These are usually assessed under simulated wash conditions using standard pieces of cloth or wood spotted with known amounts of triacylglycerols; the efficiency of cleaning being either expressed as a percentage of the remaining oil, or of the reflectance of the test material (Andree *et al.*, 1980; van Tilburg, 1984).

As lipases are surface-active enzymes (see above), the effects of a variety of surfactants may profoundly affect their catalytic activity. Their relative instability in the presence of detergent components has so far restricted their widespread application, unlike the inherently stable *Bacillus*-derived proteases. One recent success, however, regards the *Humicola lanuginosa* lipase 'Lipolase' produced by Novo Industri A/S. This performs well under various wash conditions although, ironically, activity is substantially reduced at and above 45 % (w/w) moisture (Boel *et al.*, 1991). The enzyme is produced commercially in a high expression *Aspergillus oryzae* host system and was incorporated in 1988 in to a Japanese detergent in conjunction with the Lion corporation (Cookson, 1989). Further applications of protein and genetic engineering technologies will inevitably lead to the introduction of new, better lipases, in response to a growing environmental concern.

1.5 *The production of microbial lipases.*

As outlined above, the application of lipase-based processes has been restricted, due to either an inherent instability of the enzyme, or high production and purification costs, making them economically unattractive compared to traditional chemical methods. Molecular cloning techniques have helped alleviate production costs, although a fundamental understanding of the physiological regulation of microbial lipase production is poorly understood. This probably stems from the failure of many researchers to adopt a logical, systematic approach when investigating lipase production. Reports abound in the literature on the optimisation of growth media for lipase production, which are based purely on empirical observations. Quite often these involve growth on complex substrates where the effects of individual components cannot be identified. Unfortunately lipase activities are often expressed on a volumetric basis; the effect of substrate on cellular growth, hence the variation in specific lipase activities, being frequently overlooked. As the natural substrates of lipases are, by definition, insoluble in aqueous solutions, this has undoubtedly compounded the problem.

The effects of a wide range of carbon and nitrogen sources, micronutrients, oxygen and surfactants on microbial lipase production has been well documented (see for example the reviews by Brockerhoff & Jensen, 1974; Macrae, 1983; Bloquel & Veillet-Poncet, 1984; Sugiura, 1984). Many growth substrates have been inferred to stimulate lipase production, although they have often not been assigned a more precise physiological role (such as whether they act as true inducers). In general, microbial lipase production requires growth on triacylglycerols and/or fatty acids, however, the nature of the substrate is often critical.

1.6 *The metabolism of* Pseudomonas aeruginosa.

Lipases function to hydrolyse exogenous triacylglycerols to yield fatty acid and ultimately glycerol, as sources of carbon for both cellular biosynthesis and energy. *Pseudomonas aeruginosa* has been well documented as a nutritionally versatile chemoorganotroph, capable of catabolising a very wide range of organic compounds (Palleroni, 1984). Glycerol, a relatively small hydrophilic molecule, probably enters the bacterial cell by facilitated diffusion, where it is modified prior to entry in the central Entner-Doudoroff pathway. Fatty acids are degraded into acetyl-CoA by the high energy yielding ß-oxidation system (Palleroni, 1984). Transport of long chain fatty acids in *Escherichia coli* K12 has been implicated to be mediated by specific membrane carrier proteins (Nunn, 1989), however, no such mechanism has been reported in *Pseudomonas* species.

Interestingly, a correlation between lipase production and hydrocarbon utilisation was noted in *Acinetobacter lwoffii* O16 and *Pseudomonas aeruginosa* NRCC 5005 (Breuil *et al.*, 1978). Metabolism of n-alkanes often occurs *via* the ß-oxidation pathway, following terminal oxidation to the corresponding fatty acid (Watkinson & Morgan, 1990; Witholt *et al.*, 1990). It is possible that some of the strategies adopted by various bacteria to aid hydrocarbon uptake may also facilitate fatty acid transport, such as the production of surfactants which have been widely reported amongst pseudomonads (Persson & Molin, 1987; Goswami & Singh, 1991)

A wide range of organic and inorganic nitrogen-containing compounds can be utilised as sources of nitrogen. Nitrate is specifically assimilated aerobically following its reduction to ammonia (Palleroni, 1984).

As most *Pseudomonas* species are metabolically versatile, it is hardly surprising that many strains are suspected lipase producers, especially

those belonging to the rRNA homology group I (Palleroni, 1984). This is often reported on the basis of tributyrin (see for example, Lawrence *et al.*, 1967a) or Tween 80 hydrolysis (Sierra, 1957).

1.7 *Aims of the thesis.*

(i) To investigate the physiological regulation of lipase production by a novel strain of *Pseudomonas aeruginosa* isolated under strongly selective conditions (designed to mimic detergent wash conditions) using well-established cultivation methods;

(ii) to purify and assess some of the biochemical properties of the lipase and;

(iii) to clone and express the lipase gene in a heterologous host with a view to further investigating some of the control mechanisms which regulate lipase synthesis, and hence to enable over-production of the recombinant lipase.

These are described in chapters 3, 4 and 5 respectively, each of which has a more-specific introduction. The results are discussed with reference to other *Pseudomonas* lipases.

CHAPTER 2.

MATERIALS AND METHODS.

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2.1 *Isolation of organism.*

This was carried out at Shell Research by S. Davidson (1987). Soil and water samples from various sites rich in oil and grease were screened as potential sources of lipase-producing microorganisms. They were selected by their ability to grow aerobically at pH 8.5, 50°C, in minimal medium (Section 2.4) supplemented with KCl (1.1 g l^{-1}) and using 1 % (v/v) olive oil (Nucross) as the sole source of carbon and energy. This was initially carried out by repeated sub-culturing in batch culture, and subsequently by growth on the same medium in nitrate-limited continuous culture (dilution rate, D 0.05 h^{-1}) supplemented with 0.05 % (w/v) nitriloacetic acid as a chelating agent and 0.004 % (w/v) dobane PT sulphonate as a non-biodegradable surfactant. Several pure cultures were isolated in this way.

2.2 *Identification of organism.*

Strain EF2 was identified using API 20 NE tests (API-Biomerieux) as a strain of *Pseudomonas aeruginosa* (94.8 % probability) by the Department of Microbiology, Eastbourne District General Hospital, Eastbourne, UK. This strain was deposited in the National Collection of Industrial and Marine Bacteria, Aberdeen, UK as *Ps. aeruginosa* NCIMB 30024, and was used in all subsequent work.

2.3 *Maintenance of* Ps. aeruginosa *EF2.*

Stock cultures were stored either as a cell suspension in 20 % (v/v) sterile glycerol at -20°C, or on a nutrient agar solid medium slope (Section 2.4) at 4°C.

2.4 *Growth of* Ps. aeruginosa *EF2 on solid media.*

Cultures were grown at 37°C on either;

(i) a nutrient agar medium consisting of 1.3 % (w/v) Oxoid nutrient broth solidified with 1.5 % (w/v) Oxoid technical agar; or

(ii) an olive oil (Nucross) emulsion (1% [v/v] prepared by sonication) added to a minimal-salts solution and solidified with 1.5 % (w/v) Oxoid technical agar. The minimal-salts medium contained (l^{-1}): KNO_3, 2.7 g; Na_2HPO_4, 2.0 g; $MgSO_4.7H_2O$, 0.37 g; $CaCl_2$, 0.022 g; ferric citrate, 0.013 g; trace elements solution, 2.5 ml. The trace elements solution contained (l^{-1}): $MnSO_4.7H_2O$, 55.7 mg; $ZnSO_4.7H_2O$, 72 mg; $CuSO_4.5H_2O$, 62.5 mg; $CoCl_2.6H_2O$, 29.7 mg; $NaMoO_4.2H_2O$, 31.2 mg; KI 20.7 mg; H_3BO_4, 7.3 mg. The medium was adjusted to pH 8.5 with a solution of Na_2CO_3 prior to autoclaving. In order to avoid precipitation problems the sodium phosphate, ferric citrate, trace elements and olive oil were autoclaved separately, cooled and then added to the basal medium.

2.5 *Growth of* Ps. aeruginosa *EF2 in liquid media.*

Ps. aeruginosa EF2 was grown aerobically at 37°C, pH 8.5 in the minimal-salts medium described above (Section 2.4), except where indicated.

2.5.1 *Growth in batch culture.*

A minimal-salts medium (125 ml) supplemented with either Tween 80 (polyoxyethylene sorbitan monooleate) or various other carbon sources (final concentration 3.85 g l^{-1}) was inoculated with 5 ml of an overnight culture to an initial cell density of approximately 0.1 mg dry cell mass ml^{-1}. Cultures (130 ml) were grown in 500 ml baffled flasks, aerated by rotary shaking at

approximately 200 rpm and sampled (1 to 3 ml) during the subsequent 8 to 12 hours. Cultures were assayed for cell density, lipase and/or esterase activities (Sections 2.6, 2.9.1 and 2.9.2). The effect of nitrogen source was investigated by replacing KNO_3 with either ammonium sulphate or urea (30 mM), using glucose (3.85 g l^{-1}) as the carbon source.

2.5.2 *Growth in fed-batch culture.*

A minimal-salts medium (usually 125 ml) was inoculated with washed cells (to an initial cell density of approximately 0.1 mg cells ml^{-1}), recovered from an overnight culture by centrifugation at 10,000 *g* in an MSE High Speed centrifuge for 10 min at 20°C and resuspended in warmed minimal medium minus the growth-limiting nutrient (approximately 5 ml). Cultures were grown as for batch cultures, except that one component was omitted from the medium (*eg.* carbon source or nitrate) and was added continuously using a constant-speed peristaltic pump at a rate sufficient to produce a specific growth rate (μ) at the time of harvesting of approximately 0.09 h^{-1}. Oxygen-limited growth was obtained in an un-baffled flask by increasing the volume of growth medium to 390 ml and decreasing the shaking speed to approximately 150 rpm. Optical density determinations during oxygen-limited growth required dispersal of the culture samples by repeated pipetting through a Gilson tip, as the cells tended to form filamentous strands.

2.5.3 *Growth in continuous culture.*

A minimal-salts medium (approximately 850 ml) was inoculated with 130 ml of an overnight batch culture grown on Tween 80 (Section 2.5.1). Cultures were grown in a 1 l laboratory fermentation vessel (LH Engineering, 500 series) at a reduced agitation rate (DOT < 10 %) in batch mode until cell densities of approximately 1 mg ml^{-1} were attained.

Continuous culture conditions were then employed by switching on a Pharmacia peristaltic pump connected to the medium reservoir. The flow rate was calibrated using a 10 ml glass pipette by measuring the volume change of medium (ml) per unit time (min). The pump setting was then adjusted according to the desired dilution rate (h^{-1}), which was calculated as the total flow rate (ml h^{-1})/ culture volume (assumed to be 1,000 ml). The culture was deemed to be in steady-state following the passage of at least 6 culture volumes. The minimal-salts medium was similar to that used in batch and fed-batch determinations, except that Na_2HPO_4 was replaced by K_2HPO_4 (0.75 g l^{-1}) and the ferric citrate (0.26 g l^{-1} acidified with 2 ml concentrated HCl l^{-1}) was added at a flow rate of approximately 2.5 ml h^{-1}. A silicon-based antifoam (20 % [v/v] Dow Corning RD emulsion, continually dispersed by agitation on a magnetic stirrer) was periodically dispensed by a Watson-Marlow pump at a rate just sufficient to prevent foaming. For Tween 80-limited growth the concentration of Tween 80 was 6.76 g l^{-1}, and for nitrate-limited growth the concentrations of Tween 80 and nitrate were 13.52 and 0.87 g l^{-1} respectively. The pH of the medium was maintained at the desired value by the automatic addition of either 0.5 M HCl or 2 M KOH. The DOT was measured using a lead/silver galvanic electrode and maintained at > 20 % air saturation by the automatic control of the air supply.

2.6 *Determination of cell density.*

Cell density (mg dry cell mass ml^{-1}) was calculated by determining the optical density of cell suspensions at 600 nm (OD_{600}) using a Pye-Unicam SP600 spectrophotometer (3 ml cuvette, 1 cm path length) and multiplying it by a conversion factor of 0.7. This was determined for glucose and Tween 80-grown cells in batch culture as follows: Exponentially-growing cells were

harvested by centrifugation, washed and re-suspended in distilled water. Dilutions of the cell suspensions were performed (in distilled water) and the OD_{600} determined. Samples (2 ml) were placed on pre-weighed aluminium weigh boats and dried at 80°C to constant mass. A plot of OD_{600} against cellular dry mass was linear and yielded a conversion factor of 0.7.

2.7 *Determination of growth rate.*

The OD_{600} of cultures was determined at various time intervals; a plot of the logarithm of the OD_{600} against time enabled the doubling time (t_d) to be calculated. The specific growth rate of batch cultures was calculated from the relationship μ (= μ_{max} during the exponential growth phase) = $\frac{0.693}{t_d}$ (Section 3.2.2 Equation. 6). For fed-batch cultures, μ ($<\mu_{max}$) was the instantaneous growth rate at the time of harvesting, calculated as the tangent to the curve and was therefore only an approximate value. For continuous cultures, μ was equal to the dilution rate.

2.8 *Preparation of cell suspensions and culture supernatants.*

Cultures were harvested by centrifugation in an MSE high-speed centrifuge at 10,000 *g* for 15 min, and the supernatant carefully removed using a pipette. Alternatively, small volumes (≤ 1.5 ml) were centrifuged in a bench-top microfuge (MSE Microcentaur) at full-speed (17,000 *g*) for 2 min.

2.9 *Assay of enzyme activities.*

2.9.1 *Lipase.*

Lipase activity was assayed at 37°C using a pH-Stat method in which the rate of fatty acid production due to the hydrolysis of olive oil (> 80 % w/w triolein) was measured from the rate at which a standard solution of NaOH needed to be continuously added to the reaction mix in order to maintain a constant pH. The optimum conditions were initially determined using a commercial solution of *Pseudomonas* lipase (Sigma). The reaction was carried out at pH 9.00, since preliminary experiments had indicated that this was the pH at which the fastest rate of hydrolysis was obtained (Davidson, 1987) and which allowed the oleic acid produced by the reaction to be largely ionised (approximate pKa 6.7 to 9.5; Benzonanna & Desnuelle, 1968). The NaOH (0.025 M) was made up from a standard Convol solution (BDH) and stored at 4°C in a stoppered bottle to minimise absorption of carbon dioxide from the atmosphere. The assay was carried out using a Titralab (Radiometer) comprising a VIT90 video titrator, an ABU93 titraburette and a SAM90 sample station (containing a glass reaction vessel surrounded by a water jacket linked to a circulating water bath), coupled to a Thinkjet printer (Hewlett-Packard). The olive oil substrate was a stable commercial emulsion (Sigma), prepared without the use of any potentially-detrimental emulsificants. This minimised the variation in droplet size between assays and therefore ensured reproducibility. It was diluted to 10 % (v/v) in 0.25 M NaCl plus 0.05 M $CaCl_2$ (optimal concentrations were determined from the range suggested by Brockerhoff and Jensen, 1974), and 5 ml was placed in the reaction vessel and allowed to equilibrate at 37°C. The pH of the reaction mix was adjusted to 9.00, and a base-line rate was obtained before starting the reaction by adding a sample (0.1 ml) of culture, culture supernatant or purified lipase. Automatic titration with alkali was allowed to continue for at

least 5 min. Lipase activity was calculated from the rate of addition of alkali, corrected for the rate in the absence of enzyme, and was expressed as LU ml^{-1}, LU (mg cells)$^{-1}$, or LU (mg protein)$^{-1}$. 1 LU (lipase unit) was defined as the release of 1 μmol titratable fatty acid per min under the assay conditions used. This method could not measure lipase activities of < 0.5 LU ml^{-1}.

It should be noted that for an insoluble substrate such as olive oil, the observed lipase activity reflected both the physicochemical properties of the substrate (*eg.* surface area and charge) as well as the concentration and activity of the enzyme.

2.9.2 *Esterase.*

(i) *Hydrolysis of* p-*nitrophenyl acetate.* Esterase activity was measured from the rate of formation of the chromogenic *p*-nitrophenyl anion from *p*-nitrophenyl acetate at pH 7.0, 37°C. It was necessary to carry out this assay at pH 7.0, rather than at pH 9.0 at which lipase activity was measured, because *p*-nitrophenyl acetate undergoes rapid non-enzymatic hydrolysis at alkaline pH. The assay mixture (1 ml final volume) contained 20 mM HEPES buffer, pH 7.0, plus 10 mM *p*-nitrophenyl acetate (final concentration, added from a freshly-prepared 200 mM stock solution in methanol). A base-line rate was determined which reflected the rate of spontaneous hydrolysis and the reaction started by the addition of an appropriate amount of culture, culture supernatant or purified lipase. The formation of the *p*-nitrophenyl anion (ε = 8.25 mM^{-1} cm^{-1}) was followed by the increase in absorbance of the solution at 410 nm using a SP1800 Pye-Unicam spectrophotometer. Esterase activity was expressed as μmol min^{-1} $(ml)^{-1}$, μmol min^{-1} (mg cells)$^{-1}$, or μmol min^{-1} (mg protein)$^{-1}$. This method could not detect esterase activities of < 0.05

μmol min^{-1} $(ml)^{-1}$, but was far more sensitive than the pH-stat-based lipase and esterase (Tween 80 hydrolysis; see below) assays.

(ii) *Hydrolysis of Tween 80.* Esterase activity was measured titrimetrically at pH 9.00, as described for lipase, except that the olive oil emulsion was replaced by 20% (v/v) Tween 80 and 0.2 ml of sample was added; NaCl and $CaCl_2$ were not present. Esterase activities were expressed as LU ml^{-1}, LU $(mg\ cells)^{-1}$, or LU mg $(protein)^{-1}$. This method could not detect activities of < 0.5 LU ml^{-1} and was less sensitive than the lipase and *p*-nitrophenyl acetate-based esterase assays.

2.9.3 *Protease.*

(i) *Azo-casein hydrolysis.* Protease activity of culture supernatants harvested from exponential and stationary phase cultures (0.4 ml) were assayed using a modification of the method of Jones *et al.* (1988) by measuring the release of azo-polypeptides and amino acids from azo-casein. The assay mixture (1 ml final volume) contained 0.6 % (w/v) azo-casein, 120 mM HEPES buffer, pH 8.0, and 0.019 % (w/v) $CaCl_2$ (final concentrations), and was incubated for 1 h at 37°C. The reaction was terminated by the addition of TCA (5 % w/v; final concentration) on ice. The resultant precipitate was removed by centrifugation in an MSE Microcentaur at full speed for 5 min, and the absorbance at 400 nm (A_{400}) relative to a blank (containing enzyme added after the TCA) was determined using a Perkin-Elmer λ5 UV-visible spectrophotometer. Absorbance readings were corrected against controls containing minimal-salts medium and buffer only. Protease activity was expressed as the change in absorbance at 400 nm at 37°C over 1 h.

(ii) *Casein hydrolysis.* Culture supernatants (0.1 ml) were assayed using a modification of the method described by Cowan and Daniel (1982).

The assay was essentially the same as the azo-casein assay described above, except that samples were incubated at 37°C for 1 h in a reaction mixture (2 ml final volume) containing 0.5 % (w/v) casein solution dissolved in 100 mM Tris-HCl buffer, pH 8.5. The reaction was terminated by the addition of TCA (1 % w/v; final concentration) on ice. The absorbance of the supernatant at 280 nm was determined against a blank solution (TCA added to casein only), and protease activity was expressed as the increase in absorbance at 280 nm at 37°C over 1 h.

2.9.4 *Glucose 6-phosphate dehydrogenase.*

Samples of exponential and stationary phase cultures were assayed at 37°C for glucose 6-phosphate dehydrogenase activity ($NADP^+$-linked; to determine the extent of cell lysis) by following the reduction of $NADP^+$ at 340 nm using a Pye-Unicam SP1800 spectrophotometer (3 ml reaction volume, 1 cm path length). Culture samples (0.1 to 0.5 ml) were mixed with 100 mM Tris-HCl buffer, pH 8.0 (2.29 to 2.69 ml) containing 0.1 ml of 100 mM $MgCl_2$ and 0.1 ml of 10 mM $NADP^+$. The rate of NADPH formation was measured against a reagent blank containing the above mixture (3 ml) with the exception of $NADP^+$. The reaction was started by the addition of 50 µl of 100 mM glucose 6-phosphate and followed over approximately 10 min.

2.10 *Determination of total organic carbon concentration.*

Samples of culture supernatants were diluted ten-fold in distilled water and total organic carbon contents (g C l^{-1}) were determined using a Shimadzu AS1-502 analyser calibrated with 0.5 mg ml^{-1} potassium phthalate.

2.11 *Determination of oleic acid concentration.*

A sample of culture supernatant (5 ml) was acidified with 2 drops of concentrated HCl, and fatty acids were extracted with four 5 ml volumes of dry diethyl ether using a solvent-cleaned separating funnel (any emulsion formed was broken by the addition of a small amount of NaCl). The organic phases were then pooled, the ether removed by rotary evaporation and the residue redissolved in 1 ml dry diethyl ether. The resultant solution was cooled on ice and mixed with a few drops of diazomethane (prepared according to the manufacturer's instructions [Aldrich]; Black, 1983) until a constant yellow colour was observed. The mixture was allowed to stand for approximately 5 min, the solvent was then removed by evaporation under nitrogen, and the residue redissolved in 0.5 ml methanol. The methylated products (1 to 5 μl) were applied to a CPSil 5 column (Chrompack) using a Hewlett-Packard 5890 gas chromatograph linked to a 3396A integrator with helium (at a flow rate of 1.5 ml min^{-1}) as the carrier gas. The products were separated by subjecting the column to a temperature gradient of 32°C min^{-1} from 100°C to 180°C and subsequently holding at this latter temperature for 20 min. Methyl-oleate was identified and quantified by reference to a standard mixture of C_{18} methyl esters; the residence time was approximately 14.45 ± 0.05 min.

2.12 *Determination of protein concentration.*

Protein concentration was determined by the method of Bradford (1976) using the Bio-Rad protein assay reagent according to the manufacturer's instructions. BSA (0 to 1.5 mg ml^{-1}) was used as a standard.

The methods of Warburg & Christian (see Dawson *et al.*, 1986) and Lowry *et al.* (1951) modified by Dulley & Grieve (1975) were shown to be ineffective either due to interference by Tween 80 (present in many culture samples) or their failure to detect low protein concentrations.

2.13 *Polyacrylamide gel electrophoresis.*

2.13.1 *SDS-PAGE.*

Discontinuous SDS-PAGE was carried out using a vertical slab-gel system (Hames, 1981) composed of a stacking gel containing 3.75 % (w/v) acrylamide, 0.1 % (w/v) SDS and 125 mM Tris-HCl buffer, pH 8.5, and a resolving gel containing 12.5 % (w/v) acrylamide, 0.1 % (w/v) SDS and 375 mM Tris-HCl buffer, pH 8.8. The solutions were degassed under reduced pressure and polymerised using 0.05 % (v/v) TEMED and 0.075 % (w/v) ammonium persulphate, added from a freshly-prepared solution. The reservoir buffer contained 25 mM Tris-HCl buffer, pH 8.3, 192 mM glycine and 0.1 % (w/v) SDS.

Samples (whole cultures, culture supernatants or purified lipase) were boiled for 1 to 5 min in dissociating sample buffer (60 mM Tris-HCl buffer, pH 6.8, 1 % [w/v] SDS, 5 % [v/v] β-mercaptoethanol, 5 % [v/v] glycerol, 1 % [w/v] bromophenol blue; Laemmli, 1970) and a volume corresponding to < 20 μg protein was added to each track. Proteins were electrophoresed at 40 mA constant current per gel until the bromophenol blue marker dye had reached the bottom. The gels were washed in distilled water and stained for protein overnight with Kenacid blue R (2.5 g dissolved in 454 ml of 50 % [v/v] methanol and 46 ml glacial acetic acid, filtered through a Whatman No. 1 filter) then destained by repeated washings in destain solution (7.5 % [v/v]

glacial acetic acid, 5 % [v/v] methanol) until an optimum contrast between stained protein and background was obtained. The M_r of proteins was estimated from a plot of the logarithm of the M_r of known standards against their relative mobility (R_f value). The following proteins were used as M_r standards (Dalton Mark VII-L prepared according to the manufacturer's instructions; Sigma): α-lactalbumin, (M_r 14,200); trypsin inhibitor (20,100); trypsinogen (24,000); carbonic anhydrase (29,000); glyceraldehyde 3-phosphate dehydrogenase (36,000); egg albumin (45,000); and bovine albumin (66,000).

When protein concentrations were low, such as in supernatant samples, a mini-gel system was used (Bio-Rad) and either stained by Kenacid blue R (as above) or by silver as follows: The gel was fixed in 50 % methanol (v/v) and 10 % acetic acid (v/v) for 1 h, rinsed in 5 % methanol (v/v) and 7 % acetic acid (v/v) for 30 min, then fixed in 10 % (v/v) glutaraldehyde for 30 min. The gel was then rinsed repeatedly in distilled water for 2 h, shaken gently in ammoniacal silver solution (21 ml of a 0.36 % [w/v] solution of NaOH, 4 ml of a 19.4 % [w/v] solution of $AgNO_3$ and 2 ml of concentrated ammonia solution, made up to 100 ml with distilled water) for 10 min, then washed again in distilled water for 2 min. Protein was developed by agitating the gel in a citric acid/formaldehyde solution (2 ml of a 0.5 % [w/v] citric acid solution, 100 µl of 38 % stock formaldehyde solution, made up to 200 ml with distilled water). Once bands became visible, the gel was washed in distilled water: Care was taken to not allow the gel to over-stain. Gels were photographed immediately, as they darkened rapidly upon standing.

The analysis of lipase purification fractions by SDS-PAGE was performed as described above. Samples (20 µg protein) of concentrated supernatant, pooled Mono-Q and pooled Superose fractions were applied to the gel. Due to the low protein concentration of the latter, 1.2 ml was

concentrated by dialysis against PEG_{600} to approximately 100 µl prior to the addition of sample buffer and application to the gel. The destained gel was scanned at 633 nm using an LKB laser densitometer linked to a recording integrator.

2.13.2 *Dissociating 6 M urea PAGE.*

Concentrated continuous culture supernatant (approximately 50 µg protein) was applied to three tracks of a 6 M urea polyacrylamide gel in a native sample buffer (Section 2.13.3) without boiling, and electrophoresed at room temperature at 40 mA constant current, until the bromophenol blue marker had migrated towards the bottom of the gel (concentrated continuous culture supernatant was shown to retain 79 % of its initial lipase activity when incubated in 6 M urea at room temperature for 7 h). The gel consisted of: A 4 % (w/v) acrylamide stacking gel made up in 740 mM Tris-HCl buffer, pH 8.45; a 7.5 % acrylamide spacer gel made up in 1 M Tris-HCl, pH 8.45 buffer; a 7.5 % acrylamide resolving gel made up in 1 M Tris-HCl, pH 8.45 containing 6 M urea. Following electrophoresis, the gel was washed in 20 mM Tris-HCl buffer, pH 8.5, at room temperature for 3 h, with three 500 ml changes of buffer to remove the urea. The tracks were then separated and stained for either protein or activity (Section 2.13.4).

2.13.3 *Non-dissociating (native) PAGE.*

Discontinuous native-PAGE was carried out using 7.5 % (w/v) acrylamide, high pH vertical slab gels (Hames, 1981). Gels were prepared as described above for SDS-polyacrylamide gels (Section 2.13.1), except higher pH stacking (pH 8.3) and resolving (pH 9.5) buffers were employed, the final acrylamide concentration was lower and SDS was omitted from all buffers. Likewise, both SDS and β-mercaptoethanol were omitted from the sample buffer, which was not boiled with the sample prior to application to the gel.

Duplicate samples of both purified lipase and concentrated culture supernatants were electrophoresed for over 10 h at a constant current of 40 mA per gel; one was stained for protein using Kenacid blue R or silver (Section 2.13.1), the other for lipase and/or esterase activity (Section 2.13.4).

A similar gel was run in which 0.1 M NaCl was incorporated into the stacking, resolving and running buffers.

2.13.4 *Activity staining.*

Staining for lipase activity was carried out by incubating the gel at 37°C for 24 h in contact with a 1 % (w/v) agarose overlay attached to a 1850-101 Gel Bond Film (LKB). The overlay contained either:

(i) a 5 % (v/v) olive oil emulsion (Sigma) with or without 0.01 % (w/v) Victoria Blue B. Lipase activity was detected as either a blue zone on a white background due to the release of oleic acid and its interaction with the pH indicator, or as a zone of clearing in the olive oil emulsion; or

(ii) a 2 % (v/v) Tween 80 plus 3mM $CaCl_2$ in 50 mM Tris-HCl buffer, pH 8.5. Esterase activity was detected as a white zone on a clear background due to the precipitation of calcium oleate

2.14 *Purification of lipase.*

Continuous culture supernatant (697 ml) was reduced in volume to approximately 10 ml by passage under nitrogen at 4°C through a 202 Ultrafiltration unit (Amicon) containing a YM10 (M_r 10,000) cut-off filter. Excess salts were removed by passage through a PD10 desalting column (pre-equilibrated with 20 mM Tris-HCl buffer, pH 7.5; Pharmacia) to give a final volume of 14 ml. The concentrated, desalted supernatant was passed

through an acrodisc filter (0.2 μm pore size; Gelman), then loaded on to an FPLC 10/10 Mono-Q anion-exchange column (Pharmacia) equilibrated with 20 mM Tris-HCl buffer, pH 7.5, and eluted with the same buffer containing a linear gradient of NaCl (0 to 1 M over 40 min) at a flow rate of 3 ml min^{-1}. The seven fractions (each 3 ml) containing the highest lipase activities were pooled, then concentrated by passage under nitrogen at 4°C through an 8010 Ultrafiltration unit (Amicon) containing a PM10 (M_r 10,000) cut-off filter and centrifuged at full speed for 10 min in an MSE Microcentaur centrifuge. The supernatant was divided into two 0.75 ml portions, each of which was loaded on to an FPLC Superose 6 gel-filtration column (Pharmacia) and eluted at a flow rate of 0.25 ml min^{-1} with 20 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl. The fractions (1 ml) containing the highest lipase activities were analysed by SDS-PAGE and those which contained pure lipase were pooled and stored at -20°C.

All buffers were prepared using double distilled water, filtered through a 0.2 μm pore size filter (Whatman or Sartorius) and degassed under reduced pressure prior to use. Tris-HCl (20 mM, pH 7.5) was shown not to interfere with protein estimation or the lipase assay, and to only interfere minimally with the esterase (*p*-nitrophenyl acetate) assay. Culture supernatant diluted in the buffer and left overnight at room temperature retained both lipase and esterase activities. No detectable loss of activity occurred throughout the purification procedure.

2.15 *Physico-chemical properties of lipase.*

2.15.1 *Determination of native M_r*

The native M_r of purified lipase was determined by gel-filtration FPLC through a Superose 6 column, as described previously (Section 2.14). This was pre-calibrated with molecular weight standards, applied as a mixture (approximately 1 mg ml^{-1} of each) and a standard curve was constructed from a plot of the logarithm of the M_r of each protein against the corresponding elution volume (ml), from which the M_r of the lipase was determined. The standard proteins were: cytochrome *c* (M_r 12,400); carbonic anhydrase (29,000); ovalbumin (45,000); bovine serum albumin (66,000 [monomer], 132,000 [dimer]); alcohol dehydrogenase (150,000); thyroglobulin (669,000). The procedure was carried out twice using Tris-HCl buffer, pH 7.5, as the equilibration and elution buffer, containing either 25 mM or 100 mM NaCl. Fractions were analysed by SDS-PAGE (Mini-Gel system) to confirm the presence of the lipase.

2.15.2 *Determination of isoelectric point.*

The isoelectric point (pI) of purified lipase was determined by isoelectric focussing through a 1 mm thick pre-cast 5 % (w/v) polyacrylamide gel (Pharmacia LKB). The gel was pre-focussed for 20 min at 15 mA, 15 W (maximum voltage setting) with pH 4.0 (0.1 M glutamic acid/ 0.5 M phosphoric acid) and pH 6.5 (0.1 M β-alanine) ampholytes applied to paper wicks at the anode and cathode respectively, using an LKB Multiphor II flat bed system. Purified lipase (80 µl, approximately 25 µg protein) and pI standards (approximately 35 µg each of soy bean trypsin inhibitor [pI 4.55], β-lactoglobulin A [5.20], bovine carbonic anhydrase B [5.85]; Pharmacia LKB) were applied at the cathode on paper sample wicks and focussed for 1 h at 25 mA, 15 W (maximum voltage setting), after which the sample wicks were

then removed and the gel was focussed under identical conditions for a further hour. The gel was then fixed for 1 h in a solution of methanol (350 ml), trichloroacetic acid (130 g) and sulphosalycylic acid (35 g) made up to 1 l with distilled water, then washed under running water overnight (to remove the ampholite). Proteins were then stained using Kenacid blue R, and the gel destained in methanol/acetic acid solution (Section 2.13.1).

2.15.3 *Determination of thermostability.*

Samples of purified lipase were diluted (to approximately 20 μg ml^{-1}) in 20 mM Tris-HCl buffer, pH 7.5, and incubated in a plastic Eppendorf tube at various temperatures (20°C to 100°C) in a circulating water bath. The determination at 100°C was performed in small glass vessels (made from melted Pasteur pipettes sealed with Nescofilm, to reduce the initial lag observed due to poor heat conductivity of the retaining vessels) in a boiling water bath. In all cases, samples were removed at time intervals (ranging from 2 s to 24 h, depending on the temperature) and assayed for lipase activity (Section 2.9.1). Activities were expressed as a percentage of the initial activity. The $t_{1/2}$ and D values (see Section 4.5.4) were calculated from a plot of the logarithm of residual activity against incubation time. The Z value was calculated from a plot of the logarithm of the calculated D value against the appropriate incubation temperature (Section 4.5.4).

2.15.4 *Determination of temperature and pH optima.*

Samples of purified lipase were diluted (to approximately 20 μg ml^{-1}) in 20 mM Tris-HCl buffer, pH 7.5, and the lipase and esterase (Tween 80 hydrolysis) activities of 0.1 ml were determined. This was essentially the same as the method described in Sections 2.9.1 and 2.9.2.ii, except that either the reaction temperature or the end-point titration pH was altered. These were attained either by altering the temperature of the circulating water

around the reaction vessel (using the connected water bath) over the range 20°C to 70°C, or by altering the amount of NaOH added to give end-point pH values of 7.0 to 10.5 or 11.0. In both cases the base-line rate in the absence of enzyme at these altered values was subtracted from the recorded activity in the presence of enzyme. It should be noted that the activity observed reflected not only the catalytic properties of the enzyme, but also the change in the physico-chemical nature of the substrates. The term optimum was therefore used with caution.

2.15.5 *Inhibition of lipase and esterase activities by DCI.*

The effect of the serine-active reagent 3,4-dichloroisocoumarin (DCI) on the activity of purified lipase was determined by incubating the enzyme (approximately 20 μg ml^{-1}) at 30°C in 20 mM Tris-HCl buffer, pH 7.5, containing various concentrations of DCI (up to 100 μM). This was supplied as a stock solution dissolved in DMSO (H. Djaballah, personal communication). However, as the lipase was shown to be sensitive to DMSO, this was removed under vacuum and the solid redissolved to 10 mM in methanol. The stock solution was then stored in the dark at -20°C for a limited period prior to use. Small samples of the reaction mixture were removed over a 3.5 h period and assayed for lipase and esterase (Tween 80) activity. Control samples were assayed for the effect of methanol alone. Part of this work was carried out by M. Podlusiuk (Undergraduate Student, Department of Biochemistry, University of Leicester).

2.15.6 *Inhibition of esterase activities by EDTA.*

Samples of purified lipase were diluted (to approximately 20 μg ml^{-1}) in 20 mM Tris-HCl buffer, pH 7.5, containing various concentrations of the divalent cation chelator EDTA (final concentrations 1 μM to 100 mM) and incubated at 30°C for 30 min. Aliquots were then removed and assayed for

esterase activity, measured as both *p*-nitrophenyl acetate and Tween 80 hydrolysis. Control samples containing only buffer plus EDTA were performed.

2.15.7 *Determination of regiospecificity.*

This was carried out at 37°C using a modification of the method of Okumura *et al.* (1976). For qualitative analysis the reaction mix (final volume 2.5 ml) contained 2.15 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 250 mM NaCl and 50 mM $CaCl_2$, 0.25 ml triolein (99 % pure) or diolein (85 % 1,3-diolein, 15 % 1,2-diolein) and 0.1 ml purified lipase. Identical mixes were placed in several 10 ml glass scintillation vials and agitated at 37°C. Each reaction was started by the addition of enzyme to a final concentration of approximately 20 μg ml^{-1} and terminated at intervals for up to 48 h by three successive extractions into 2.5 ml dichloromethane. The solvent was removed by rotary evaporation, and the residue was resuspended in 2.5 ml dichloromethane. Samples (1 μl) were applied to a 0.25 mm thick silica gel 60 F-254 chromatography plate, and were resolved in a 70:30:1 (by volume) mix of ligroin (mineral spirit)/diethyl ether/acetic acid. The spots were visualised by spraying the plate with 20 % (v/v) H_2SO_4 in ethanol and then heating in an oven at 100°C until charring occurred. They were then identified by comparing their R_f values with those of known standards.

For quantitative analysis, the procedure used was as described above except that the substrate consisted of 0.25 ml ^{14}C-labelled triolein (glycerol tri [1-^{14}C] oleate; 12 mCi $mmol^{-1}$, 0.44 GBq $mmol^{-1}$; Amersham) and 2 μl samples were applied to the chromatography plate. After separation, the spots were scraped off the plate and placed in plastic vials containing 4 ml

Optiphase T scintillation fluid prior to measuring the radioactivity by scintillation counting.

2.15.8 *Determination of N-terminal amino acid sequence.*

Semi-purified lipase (approximately 20 μg protein, consisting predominantly of lipase) was electrophoresed in a 12.5 % polyacrylamide SDS gel (Section 2.13.1). The proteins were then transferred on to a polyvinylidene difluoride (PVDF) membrane by electroblotting for 2.5 h, and visualised by staining briefly in Kenacid blue R (Section 2.13.1). The portion of the membrane containing the lipase (M_r 29,000) was removed and loaded on to an Applied Biosystems 470A gas-phase sequencer. The N-terminal amino acids were removed by sequential Edman degradation, analysed by high performance liquid chromatography and the N-terminal sequence deduced. This was performed by K. Lilley, Department of Biochemistry, University of Leicester.

2.16 *Bacteria and plasmids used in DNA manipulations.*

2.16.1 *Bacterial strains.*

(i) *Pseudomonas aeruginosa* EF2: a novel lipase-positive strain isolated from soil, as described previously (Section 2.1).

(ii) *Pseudomonas putida* P2440: a gift from M. O'Callahan, Department of Microbiology, University of Leicester, UK. No further details were supplied.

(iii) *Escherichia coli* JM109: F' *traD36 lacI^q Δ(lac Z) M15 proAB/recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* (r_k-m_k+) *supE44 relA1 Δ(lac-proAB)*

(iv) *Escherichia coli* NM522: F' *lacI^q Δ(lac Z) M15 proAB/supE thi Δ(lac-proAB) Δ(hsdMS-mcrB)5* (r_k-m_k-McrB-)

Ps. putida P2440 and *E. coli* JM109 and NM522 were deemed to be lipase-negative, as random colonies incubated on M9 minimal-salts agar medium supplemented with Tween 80 (Section 2.19.1) failed to grow.

2.16.2 *Plasmid vectors.*

(i) *pUC18;* a small (2,686 kbp), high copy number *E. coli* plasmid vector containing portions of pBR322 and M13mp19 (Yanisch-Perron *et al.*, 1985), which conferred ampicillin resistance to ampicillin-sensitive bacteria. Strains harbouring the plasmid were therefore selected for their ability to grow on media supplemented with ampicillin at 50 μg ml^{-1} (added from a 50 mg ml^{-1} stock solution, stored at -20°C). Recombinant plasmids contained a DNA insert at the unique *Eco*R I restriction site (Section 2.17.11) within the polylinker. This was located within the *lacZ'* gene which encoded the enzymatically inactive N-terminal α-peptide of β-galactosidase. In the absence of any insert, this was synthesized (following induction of the gene with IPTG) and complemented the C-terminal peptide encoded by the *Δlac Z M15* gene present in the *E. coli* strains to form an active enzyme; cells were therefore able to hydrolyse the artificial substrate X-Gal and produce blue colonies. In the presence of an insert, a functional α-peptide was not synthesized; cells were therefore unable to hydrolyse X-Gal following induction with IPTG and produced white colonies. This was known as blue-white selection.

(ii) *pKT230*; a *Pseudomonas/Escherichia* broad host range, RSF 1010-derived plasmid (Bagdasarian *et al.*, 1981), which conferred kanamycin and streptomycin resistance to sensitive bacteria. Resistant strains were therefore selected using media supplemented with either kanamycin or streptomycin, both at 50 μg ml^{-1} (added from 50 mg ml^{-1} stock solutions, stored at -20°C). Insertion of foreign DNA at the unique *Eco*R I site resulted

in inactivation of the streptomycin-resistance gene, hence colonies grew on media supplemented with kanamycin but not streptomycin.

2.17 *Manipulation of bacterial DNA.*

2.17.1 *Preparation of genomic DNA.*

Genomic DNA was prepared from exponentially-growing cells of *Ps. aeruginosa* EF2 by the method of Wilson (1990), which involved the selective precipitation of cell wall debris, polysaccharides and proteins with CTAB. Mid-exponential growth phase cultures (1.5 ml; OD_{600} approximately 0.4 to 0.5) were harvested by centrifugation in an MSE microcentaur for 2 min. Twelve samples were prepared and the contents pooled at a later stage. Pelleted cells were resuspended in 567 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), 30 µl of 10 % (w/v) SDS, and 3 µl of 20 mg ml^{-1} proteinase K (previously self-digested at 37°C for 30 min to remove any DNAase and stored at -20°C prior to use; NBL), mixed and incubated at 37°C for 1 h. This was then mixed thoroughly with 100 µl of 5 M NaCl, followed by 80 µl of pre-warmed CTAB-NaCl solution (10 % [w/v] CTAB in 0.7 M NaCl) and incubated at 65°C for 10 min. An equal volume (approximately 0.7 to 0.8 ml) of chloroform/isoamyl alcohol (24:1 v/v ratio) was added, mixed thoroughly and the solution spun at full speed in a microfuge for 5 min. The aqueous phase was transferred to a fresh tube and shaken gently with phenol (containing 8-hydroxyquinoline and equilibrated with Tris-HCl buffers, pH 8.0, as described by Maniatis *et al.*, 1982)/chloroform/isoamyl alcohol (25:24:1 v/v ratio), then spun for 5 min in a microcentrifuge and the aqueous phase transferred to a fresh tube. Samples were pooled at this stage to give volumes of approximately 1 ml. Isopropanol (0.6 vol) was added, and the tubes gently agitated for about 10 min to precipitate the DNA. The

aqueous solution was removed following centrifugation in a MSE Microcentaur at full speed for 2 min, and the DNA pellet washed in 70% (v/v) ethanol. This was then removed and the remaining pellet dried under vacuum in a desiccator for 10 min. The pellet was resuspended in 10 µl TE buffer.

Isolation of chromosomal DNA from stationary phase cells, or by using the method of Chow *et al.* (1977) which was specific for *E. coli* chromosomal DNA, were unsuitable. Pseudomonads are prolific producers of extracellular proteins and polysaccharides and these were shown to adversely interfere with the isolation procedure, resulting in highly viscous solutions that could not be completely removed by repeated phenol extractions.

2.17.2 *Preparation of plasmid DNA.*

Plasmid DNA was isolated from cells by one of two methods, depending on the subsequent use of the plasmid.

(i) *Triton lysis method.* This was used for the large-scale preparation of plasmid DNA which was subsequently purified to a very high degree by ultra-centrifugation in a CsCl gradient. The plasmids pUC18 and pKT230 were prepared in this way, prior to restriction and ligation with chromosomal DNA.

E. coli harbouring the appropriate plasmid was grown overnight in 1 l of nutrient broth supplemented with the appropriate selection antibiotic (50 µg ml^{-1}) on an orbital shaker at 37°C. The cells were harvested by centrifugation at 10,000 *g* for 10 min at 4°C in an MSE high-speed centrifuge, washed in an equal volume of 20 mM HEPES buffer, pH 7.0 at 4°C and re-spun. Cells were then re-suspended in 6.5 ml of 25 % (w/v) sucrose,

50 mM Tris-HCl buffer, pH 8.0, containing 1 mg RNAase A (100 µl of a 10 mg ml^{-1} stock stored at -20°C) and 33 mg lysozyme. The RNAase A stock was made up in 10 mM Tris-HCl buffer, pH 7.5, containing 15 mM NaCl and heated to 100°C for 15 min to denature any endogenous DNAase. This was incubated at room temperature for 10 min, then gently mixed with 1 ml of 0.25 M EDTA, pH 8.0, and incubated for a further 10 min at room temperature. A 20 % (v/v) Triton X-100, 50 mM Tris-HCl, pH 8.0, solution (8 ml) was then added, mixed by gently inverting the centrifuge tubes, then immediately centrifuged at full-speed in an MSE high-speed centrifuge for 20 min at 4°C. The resultant supernatant was collected, the volume measured and 2/3 of the volume of a 1.25 M NaCl, 25 % (w/v) PEG_{6000} solution was added, then left on ice for 2 to 3 h. The solution was then centrifuged at 10,000 *g* for 10 min at 4°C, and the resultant pellet (which contained predominantly plasmid DNA) was re-suspended in 0.7 ml of TNE buffer (50 mM Tris-HCl, pH 8.0, 5 mM NaCl, 5 mM EDTA).

The dissolved plasmid solution was mixed with 4.2 g CsCl, 3 ml of TNE buffer and 200 µl of a 10 mg ml^{-1} stock of ethidium bromide and heat-sealed in an ultracentrifuge tube. This was then centrifuged overnight in a Beckman L5-65 ultra-centrifuge at 48,000 rpm (approximately 298,000 *g*) under vacuum at room temperature. DNA was visualised under a low energy UV light and closed circular plasmid DNA was removed using a sterile hypodermic needle, as described by Maniatis *et al.* (1982). The solution was then mixed with an equal volume of butan-1-ol and the upper organic layer removed. The procedure was repeated until all of the ethidium bromide had been extracted; seen as a colourless solution and the absence of any pink colouration. The solution was then dialysed in Visking tubing 32/34 (previously boiled in the presence of a small amount of EDTA for 5 min and stored at 4°C) for 2 to 3 h against aliquots of TE buffer (1 l). The dialysate was

then removed, the plasmid DNA precipitated with ethanol, resuspended in 100 µl TE buffer and the concentration and purity of the solution determined (as described elsewhere).

(ii) *Alkaline lysis method.* This was performed as described by Maniatis *et al.* (1982) and was used to recover recombinant plasmid DNA from cells of *E. coli.*

E. coli harbouring the appropriate plasmid was grown overnight in either 5 or 20 ml of nutrient broth containing the appropriate selection antibiotic on an orbital shaker at 37°C (the volume depended on the quantity of DNA required). Cells were removed from 1.5 ml of culture by centrifugation in a Microfuge for 1 min, and residual media removed using a fine-tipped aspirator. The pellet was resuspended in 100 µl of GTE buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) and left at room temperature for 5 min. To this, 200 µl of a a freshly-prepared solution of 0.2 M NaOH and 1 % (w/v) SDS was added, mixed by inverting the Eppendorf tube rapidly and stored on ice for 5 min. An ice-cold solution of 3 M potassium 5 M acetate, pH 4.8 (150 µl) was then added and mixed by vortexing the inverted tube gently for 10 s. The tube was again placed on ice for 5 min, centrifuged for 5 min at 4°C, and the supernatant transferred to a fresh tube. Protein was then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) at least once, the DNA then precipitated from solution with ethanol (Section 2.17.3) and redissolved in an appropriate volume of TE buffer containing 20 µg ml^{-1} of RNAase A (typically 10 µl for pUC18-based plasmids; less for the lower copy number pKT230-based plasmids).

2.17.3 *Ethanol precipitation of DNA.*

A DNA solution was mixed with 1/10 volume of 3M sodium acetate, pH 5.2 (to give a final concentration of 0.3 M). To this, exactly two volumes of ice-cold ethanol was added, mixed thoroughly and then stored on either dry ice for at least 15 min, or at -20°C for 1 to 2 h. The resultant precipitate was removed by centrifugation in an MSE Microfuge at full speed for 3 to 5 min and the supernatant discarded by inverting the Eppendorf tube. The pellet was washed in 1 ml of ice-cold 70 % ethanol and re-spun. The supernatant was removed and the pellet dried under vacuum in a desiccator (approximately 5 to 10 min). The DNA was then resuspended in appropriate volume of TE buffer.

2.17.4 *Determination of the purity of a DNA solution.*

The absorbance of a DNA solution (generally diluted 100-fold in distilled water) was determined at 260 nm and 280 nm using a Pye-Unicam SP1800 spectrophotometer (quartz cuvettes, 1cm pathlength). A solution was deemed pure if the ratio of A_{260}/A_{280} was 1.8.

2.17.5 *Purification of DNA.*

Residual protein was removed by re-extracting the DNA solution with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The upper aqueous layer was removed, the DNA precipitated with ethanol, then redissolved in an appropriate volume of TE buffer.

Alternatively, both protein and RNA were removed using the commercial kit Geneclean II (Bio 101 Inc., California, USA). This resulted in highly purified solutions of DNA and was also employed to recover electrophoresed DNA from TAE agarose gels as follows: Three volumes of NaI stock solution (6 M) were mixed with the sample (any agarose was then

dissolved by incubating at 45°C to 55°C for 5 min) then approximately 5 µl of Glass milk (a suspension of silicon glass beads in water, to which DNA binds under high salt conditions) was added, mixed thoroughly, then left at room temperature for 5 min. The Glass milk/DNA complex was removed by centrifugation in an MSE Microcentaur at full speed for 5 s, then washed three times in approximately 500 µl of New Wash (a solution of Tris-HCl buffer, pH 7.0 to 8.5, NaCl, EDTA and ethanol; prepared from a concentrate according to the manufacturer's instructions). The DNA was eluted in an appropriate volume of either TE buffer or distilled water at 55°C for 5 min, and the silicon matrix removed by centrifugation.

2.17.6 *Determination of the concentration of nucleic acid solutions.*

The absorbance of a double-stranded DNA (chromosomal or plasmid) or oligonucleotide solution was determined at 260 nm using a Pye-Unicam SP1800 spectrophotometer (quartz cuvettes; pathlength 1 cm). Solutions were generally diluted 100-fold in distilled water and an absorbance of 1 was assumed to reflect concentrations of 50 µg ml^{-1} and 20 µl ml^{-1} for double stranded DNA and oligonucleotide solutions, respectively.

Alternatively, when concentrations and/or volumes were small, an aliquot was run on an agarose gel (Section 2.17.8) with 1 µl of standard M13 DNA (Pharmacia LKB) at 100 ng $µl^{-1}$. A rough estimate of the concentration was made by comparing the intensity of the bands when viewed under UV light.

2.17.7 *Restriction of chromosomal and plasmid DNA.*

Aliquots of DNA (1 to 20 µl; up to 10 µg) were restricted for 1 to 18 h at 37°C with various restriction endonucleases (*Sal* I, *Bam*H I, *Pst* I, *Eco*R I and *Hind* III; Gibco BRL or Pharmacia LKB) and the appropriate 10 x REact

or 10 x One-Phor-All buffer, respectively. These were both added at concentrations of 1/10 the final reaction volume (*ie.* generally 3 µl of each to a final volume of 30 µl), which was adjusted appropriately with TE buffer.

2.17.8 *Horizontal agarose slab gel electrophoresis.*

DNA fragments were separated by horizontal slab electrophoresis in a TAE agarose gel (0.6 % w/v). This was prepared by heating 0.6 g Miles agarose in 2 ml of 50 x TAE buffer (l^{-1}: tris base, 242 g; glacial acetic acid, 57.1 ml; 0.5 M EDTA [pH 8.0], 100 ml) made up to 100 ml with distilled water (final concentration 0.04 M Tris-acetate, 1 mM EDTA, pH 8.0) until fully dissolved. The solution was allowed to cool slightly, ethidium bromide added to a final concentration of 2 µg ml^{-1} (5 µl of a 10 mg ml^{-1} stock stored at 4°C), poured and allowed to set.

Samples were mixed immediately prior to application to the gel in 1/6 volume of 6 x sample buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol, 30 % glycerol) and electrophoresed in TAE buffer containing ethidium bromide at approximately 100 V at room temperature (although potential differences of between 5 and 140 V were employed depending on the time required for resolution). DNA on the gel was analysed by visualisation under UV light and the size of the fragments estimated by comparing their relative mobility with those of λ *Bst*E II molecular weight standards (Section 2.17.9). Where necessary gels were illuminated by UV light on a trans-illuminator, photographed on to Kodak T_{max} 100 (4052; f = 5.6, 15 s exposure) and developed using standard procedures.

2.17.9 *Preparation of λ Bst*E II molecular weight standards.

λ DNA (100 µl of 250 to 600 µg ml^{-1}; Gibco-BRL) was mixed with 30 µl *Bst*E II, 20 µl REact 2 and 50 µl distilled water, overlayed with sterile

mineral oil and incubated at 65°C for 2 h. The oil was then removed and the reaction stopped by the addition of 200 µl TE buffer and 100 µl 6 x sample buffer. The stock was split into 50 µl aliquots and stored at -20°C; 3 µl of this was applied to each track.

The fragment sizes were as follows (in kbp): 8.45, 7.24, 6.37, 5.69, 4.82, 4.32, 3.68, 2.32, 1.93, 1.37, 1.26, 0.70 (a 14.14 kbp fragment formed due to association of the 8.45 and 5.69 kbp fragments, although this could have been prevented by heating the standards at 65°C for 2 to 3 min prior to use).

A plot of the logarithm of molecular weight against relative mobility (R_f) of the standards resulted in a straight line relationship, from which the sizes of unknown DNA fragments were determined.

2.17.10 *Southern analysis of DNA.*

(i) *Transfer of DNA.* DNA was restricted with various endonucleases and the fragments separated by agarose gel electrophoresis (Section 2.17.8). The gel was viewed and photographed under UV light, rinsed briefly in distilled water and washed in 250 ml of 0.25 M HCl for 7 min with gentle shaking. The DNA was denatured for 30 min with 250 ml of 1.5 M NaCl and 0.5 M NaOH, then neutralised for 30 min in 250 ml of 3 M NaCl and 0.5 M Tris-HCl, pH 7.4. The DNA was transferred by capillary blotting, as described by Southern (1979). The gel was placed on Whatman 3 MM paper soaked with 20 x SSC (3 M NaCl and 0.3 M Na citrate, pH 7.4) and a piece of pre-soaked (in 3 x SSC) nylon Hybond N (Amersham) membrane the same size as the gel placed carefully on top. This was surrounded by waterproof Saran Wrap to prevent the transfer buffer by-passing the gel and membrane. Two further layers of 3 MM paper, a stack of absorbant paper towels and a weight were then placed on top of the membrane and the DNA allowed to transfer overnight at room temperature.

Following transfer, the well origin and the gel orientation were marked and the membrane rinsed briefly in 3 x SSC, blotted and allowed to air dry at room temperature. The membrane was enclosed in Saran Wrap and the DNA cross-linked to the nylon by a 15 s exposure on a UV transilluminator.

(ii) *Synthesis and labelling of oligonucleotide probe.* Two 20-mer oligonucleotide probes, based on the N-terminal amino acid sequence of purified *Ps. aeruginosa* EF2 lipase, were synthesized using an Applied Biosystems 380B synthesizer by J. Kyte and K. Lilley, (Department of Biochemistry, University of Leicester). The first contained 256 nucleotide degeneracies, which included all possible codon combinations encoding the seven amino acid sequence. The second contained inosyl residues at two sites where the use of all four nucleotides was possible, thus reducing the number of degeneracies to 16 (Section 5.3). Both probes were radiolabelled and used in an identical manner.

The oligonucleotides (200 µl) were precipitated with ethanol (Section 2.17.3), redissolved in 100 µl of TE buffer and the concentrations determined from their absorbances at 260 nm (Section 2.17.6). Aliquots were diluted in TE buffer to give 20 pmol of 5' ends in 5 µl. The diluted probe (5 µl) was mixed with 5 µl TE buffer, 2.5 µl 10 x kinase buffer (500 mM Tris-HCl, pH 7.6, 100 mM $MgCl_2$), 1.25 µl of 0.1 M dithiothreitol and 3 µl polynucleotide kinase (Gibco-BRL). To this, 10 µl of γ^{32}P-ATP was added and the solution was incubated at 37°C for 1 h in a lead container, followed by 5 min at 70°C to inactivate the kinase. Incorporation of the ^{32}P into the probe was tested by spotting a small aliquot (approximately 0.4 µl) on to a strip of Whatman DE81 paper. This was chromatographed in 0.3 M ammonium formate, air-dried, wrapped in Saran Wrap and autoradiographed for 10 min. If labelling was

complete, the ^{32}P remained at the origin where it was applied and did not migrate.

(iii) *Southern hybridization.* The DNA-containing Hybond N membranes were pre-hybridized at the appropriate hybridization temperature (generally 37°C; Section 5.3) for 1 h in a pre-warmed solution containing 0.1 g Marvel dried milk powder, 1.2 g PEG_{6000}, 2 ml 15 x SSPE (2.25 M NaOH, 150 mM NaH_2PO_4, 15 mM EDTA, pH 7.4), 2 ml 10 % (w/v) SDS and 15 ml distilled water in an air-tight hybridization chamber. The labelled probe was then added and hybridization was allowed to proceed overnight at the same temperature.

The probes were then removed (and stored for further use, if required within the next seven days) and the membranes rinsed briefly in 3 x SSC at the hybridization temperature (unless otherwise stated). This was followed by three washes in 3 x SSC containing 0.1 % (w/v) SDS, then three washes in 0.5 x SSC containing 0.1 % SDS at the appropriate temperature. The membranes were blotted and allowed to dry thoroughly in air at room temperature, then wrapped in Saran Wrap. They were then exposed next to Fuji RX X-ray film (autoradiography) in between intensifying screens at -70°C for a variable period (24 to >50 h) depending on the amount of hybridized ^{32}P-labelled probe. The autoradiographs were developed using standard procedures.

2.17.11 *DNA ligations.*

(i) *Chromosomal DNA.* Genomic DNA was isolated, restricted with *Eco*R I and the fragments separated by agarose gel electrophoresis. A portion of the gel containing fragments ranging from approximately 5 to 9 kbp in size was cut out using a clean scalpel blade (this was thought to contain the lipase gene, as it encompassed the region to which the

oligonucleotide probe hybridized during Southern analysis). The DNA was eluted using Geneclean (Section 2.17.5), resuspended in 50 µl TE buffer and the concentration determined.

Plasmids (pUC18 and pKT230) were isolated by the triton lysis method, purified in a caesium chloride gradient, and aliquots restricted with *Eco*R I. The endonuclease was removed by extracting with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and the DNA was cleaned by precipitating with ethanol. To prevent re-circularisation of the restricted plasmid, the 5' phosphate groups were removed by incubating 8 µl with 1 µl of calf intestine phosphatase (Gibco-BRL or Pharmacia LKB) and 1 µl of the supplied buffer at 37°C for 30 min. The enzyme was then removed by extraction with the phenol mix, the DNA precipitated with ethanol, redissolved in 8 µl of TE buffer and the concentration determined. Unfortunately, this procedure apparently degraded the pKT230 DNA.

Ligation of the restricted chromosomal and plasmid DNA was performed overnight at 15°C in 10 µl total volume containing 1 µl of T4 DNA ligase (Gibco-BRL), 2 µl of the supplied 5 x reaction buffer, and volumes of chromosomal and plasmid DNA calculated (from their concentrations and approximate molecular weights) to give various molar ratios (optimal ratios were not known) made up with TE buffer. Ratios of 1:1, 1:2, 1:3 and 1:4 (plasmid to insert) were employed with the pKT230 vector, whilst ratios of 0.1:1, 0.5:1, 1:1 and 3:1 (plasmid to insert) were employed with the pUC18 vector.

(ii) *Sub-cloning.* The pUC18-based construct harbouring an 8.6 kbp *Eco*R I chromosomal DNA fragment (pJG5; Section 5.6) was restricted with an appropriate endonuclease and the generated fragments separated by agarose gel electrophoresis. The required fragment was removed from the

gel using a scalpel and the DNA isolated using Geneclean (dissolved in 5 µl TE buffer). This was then ligated into either pUC18 or pKT230, as described previously. Equal ratios of plasmid to insert were generally employed.

2.18 *Transformation of bacterial strains.*

2.18.1 *E. coli* JM109 *and* NM522.

Ligated DNA was used to transform strains of *E. coli* using the rubidium chloride method of Kushrer (1978). Cultures were grown aerobically at 37°C in nutrient broth to the mid-exponential growth phase (OD_{600} approximately 0.4 to 0.5) and 1.5 ml samples were harvested by centrifugation in a MSE Microcentaur for 30 s at full speed. Cells were resuspended in 500 µl of a pH 7.0 MOPS solution containing 50 µl of each of 100 mM MOPS, pH 7.0, 100 mM RbCl, 500 mM $CaCl_2$ and 350 µl distilled water, then removed again by centrifugation for 15 s, and resuspended in 500 µl of a pH 6.5 MOPS solution containing 50 µl each of 1.0 M MOPS, pH 6.5, 100 mM RbCl, 500 mM $CaCl_2$ and 350 µl distilled water. They were then held on ice for at least 1 h, removed by centrifugation and resuspended in 150 µl of the above pH 6.5 MOPS solution. To this, 3 µl of DMSO and the contents of the ligation mix (10 µl) were mixed and held on ice for a further hour. The cells were then heat-shocked at 55°C for 30 s, cooled in an ice-water bath for 1 to 2 min, then diluted to 1 ml final volume with pre-warmed nutrient broth. This was incubated at 37°C for 1 h to enable the cells to synthesize the associated proteins required to confer antibiotic resistance. Aliquots (generally 50 to 100 µl) were spread using a sterile glass rod on to nutrient agar media containing the appropriate selection antibiotic (50 µg ml^{-1}) and, with the pUC18-derived plasmids, IPTG (0.3 mM; added from a 0.1 M stock solution) and the chromogenic X-Gal (50 µl of a 3 % w/v

solution dissolved in DMF spread onto the surface of each plate). Plates were incubated at 37°C overnight and the number of colonies noted. Colonies containing pKT230-derived plasmids (kanamycin resistant) were patched on to a nutrient agar-streptomycin medium using a sterile tooth pick to test for insertional inactivation of the streptomycin resistance gene. Colonies with pUC18 plasmids containing DNA inserts, were determined by blue-white selection (Section 2.16.2.i).

2.18.2 *Ps. putida* P2440.

Ps. putida P2440 was transformed with the pKT230-based construct pJG3 (Section 5.7) from *E. coli* NM522 using the method described by Olsen *et al.* (1982). A culture was grown aerobically at 37°C in nutrient broth until the mid-exponential phase (OD_{600} approximately 0.4). Cells were removed from 2 ml of culture by centrifugation at 4°C in an MSE Microcentaur at full speed, resuspended in 1 ml of ice-cold 0.1 M $MgCl_2$ and held on ice for 5 min. This was then repeated, except the cells were held on ice for 20 min. The cells were then removed, resuspended in 200 µl of 0.1 M $MgCl_2$, mixed with 9 µl of plasmid and held on ice for 1 h. This was followed by a heat-shock treatment at 37°C for 3 min with gentle swirling, cooling in an ice-water bath for 5 min, then the addition of 0.5 ml of nutrient broth which was incubated at 37°C for 1 h. Aliquots (100 µl) were spread on to nutrient agar-antibiotic media and incubated at 37°C overnight.

2.19 *Selection of strains harbouring recombinant plasmids containing the* Ps. aeruginosa *EF2 lipase gene.*

2.19.1 *Selection on Tween 80 media.*

Colonies containing recombinant plasmids were selected on the basis of their ability/failure to grow on nutrient agar-antibiotic media as described above. The presence of the functional lipase gene was determined from the ability of the plasmid to confer the lipase-positive phenotype to the lipase-negative host. This was determined by patching selected colonies on to M9 minimal-salts agar medium supplemented with Tween 80 and the appropriate selection antibiotic, using a sterile tooth pick. The plates were incubated overnight at 37°C, followed by incubation at room temperature for a further few days until growth was visible. The medium contained (l^{-1}): Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g; Oxoid technical agar, 15 g and the pH adjusted to 7.4 with concentrated KOH. Following sterilisation by autoclaving, 2 ml of 1 M $MgSO_4$, 1 ml of 1 M $CaCl_2$, 10 ml Tween 80, 1 ml of thiamine (10 mg ml^{-1}) and the appropriate antibiotic (to 50 μg ml^{-1}) were added once the medium had cooled, just before the agar set. This medium was the M9 minimal-salts medium described by Maniatis *et al.* (1982), except that Tween 80 replaced glucose as the carbon source and the $CaCl_2$ concentration was increased ten-fold to precipitate any free oleic acid in the Tween 80.

2.19.2 *Western analysis.*

Samples of purified lipases from *Ps. aeruginosa* EF2 and *Ps. aeruginosa* PAC1R, and cellular proteins from *Ps. aeruginosa* EF2 and *E. coli* JM109 were separated by SDS-PAGE using a mini-gel system (Section 2.13.1) in duplicate. One gel was stained for protein using Kenacid blue R. Proteins present in the other gel were transferred to a nitrocellulose

membrane (0.2 μm pore size; Schleider & Schuell, Dassel, Germany) using a LKB Novablot system as follows: 18 Whatman No. 1 filters and the nitrocellulose membrane (all cut to exactly the same size as the polyacrylamide gel) were soaked in blot buffer (48 mM Tris-base, 39 mM glycine, 10 % [v/v] methanol and 0.0375 % [w/v] SDS) until fully saturated. Half (9) of the filters were carefully placed on the bottom graphite electrode (anode), ensuring that no air bubbles were trapped. The nitrocellulose membrane, then the polyacrylamide gel (the orientation noted), followed by the remaining 9 filters were then layered on top. Any excess buffer was wiped away around the stack, then the top graphite electrode (cathode) was carefully applied to this. Proteins were transferred for 1.5 h at a constant current of 0.8 mA per cm^2 of filter (typically 45 mA), the electrodes were then removed, and the gel was stained in Kenacid blue R to determine residual protein. The nitrocellulose filter was stained for transferred protein by agitating it briefly in Poncea's Red solution (5 % [w/v] in TCA). The position of the molecular weight standards and other major proteins were marked lightly, then the remaining stain was removed by washing in distilled water.

The membrane was then blocked at 37°C for 1 h in TBS (Tris-buffered saline; 10 mM Tris-HCl, pH 7.4, 0.9 % [w/v] NaCl) containing 0.1 % (v/v) Tween 20 and 3 % (w/v) Marvel dried milk, then washed for three 10 min periods in TBS until the solution became clear. Rabbit serum containing polyclonal antibodies raised against purified *Ps. aeruginosa* PAC1R lipase (gift of K.-E. Jaeger, Ruhr-Universität Bochum, Bochum, Germany) was diluted to 0.1 % (v/v) in TBS containing 0.1 % [w/v] BSA (total volume 50 ml) and incubated with the nitrocellulose membrane at 37°C for 1.5 h, which was then washed in TBS/Tween 20 for three 10 min periods. Detection of the bound rabbit polyclonal antibodies was performed using one of two methods:

(i) The membrane was incubated at room temperature for 1 h with 0.15 % (v/v) swine anti-rabbit immunoglobulin (diluted in the TBS/BSA; total volume 10 ml; Dakopattz, Denmark) and washed in TBS/Tween 20. The membrane was over-layed with a 0.15 % (v/v) mixture of horse radish peroxidase and rabbit anti-horse radish peroxidase (Dakopattz), diluted in TBS/BSA for 30 min at room temperature and washed in TBS/Tween 20. Bands were developed with 30 µl of a β-chloronaphthol solution (30 mg dissolved in 10 ml methanol, mixed with 50 ml TBS) and 30 µl of H_2O_2 (100 volume).

(ii) The membrane was incubated at room temperature for 1 h with goat anti-rabbit antibodies conjugated to alkaline phosphatase (0.1 % [v/v] in 10 ml block buffer), washed in TBS, then washed again in 10 ml of 100 mM Tris-HCl buffer, pH 9.5, containing 1 mM $MgCl_2$. Bands were visualised by incubating in the latter buffer containing 0.1 % (v/v) of each of 5-bromo-4-chloro indolyl phosphate (50 mg ml^{-1} in DMF) and nitro-blue tetrazolium (50 mg ml^{-1} in 70 % [v/v] DMF; gifts of S. Sweeney).

2.20 *Growth of strains containing the recombinant lipase gene in liquid media.*

Batch cultures were grown aerobically at 37°C as described in Section 2.5.1, except that the minimal-salts medium was the M9 medium pH 7.5 described above (Section 2.19.1; agar omitted) supplemented with either Tween 80 or glucose (3.83 mg ml^{-1}) and the appropriate antibiotic where necessary. Culture samples were removed at various time intervals (5 to 48 h, depending on the strain) and cell density and esterase activities (*p*-nitrophenyl acetate) determined. Aliquots (400 µl) were centrifuged at full speed in an MSE Microcentaur for 1 min, and the cell and supernatant

fractions separated. The cells were resuspended in the same volume of ice-cold 20 mM HEPES buffer, pH 7.0, and a 200 µl aliquot was sonicated on ice (2 to 3 15 s bursts at low energy until the cloudy suspension became clear) to disrupt the cells. All fractions were then assayed for esterase activity (*p*-nitrophenyl acetate; Section 2.9.2.i).

2.21 *DNA sequencing.*

The pUC18-based constructs pJG5.1 and pJG5.2 (containing 4.3 kbp *Bam*H I and 2.5 kbp *Sal* I fragments respectively) were sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using a Sequenase kit (Version 2.0; United States Biochemical) according to the manufacturer's instructions. The oligonucleotide probe containing 256 degeneracies (Section 2.17.10.ii) was used as a primer at a primer:template ratio of approximately 50:1, as suggested by Konrad (1990).

2.22 *Chemicals and reagents.*

All chemicals were of the highest grade available and were obtained from either Sigma, BDH or Fisons, unless stated otherwise. Solutions were prepared using distilled water; those used in DNA manipulations were prepared using sterile double-distilled water and were then sterilised either by autoclaving at 15 psi for 15 min, or by filtration through an Acrodisc filter (0.2 µm pore size; Gelman) prior to use. These solutions and reagents were prepared and stored as described by Maniatis *et al.* (1982).

CHAPTER 3.

THE PHYSIOLOGICAL REGULATION AND OPTIMISATION OF LIPASE PRODUCTION BY *PSEUDOMONAS AERUGINOSA* EF2.

3.1 *Abstract.*

The physiological regulation of lipase production by a newly-isolated strain of *Pseudomonas aeruginosa* (strain EF2) was investigated during growth in batch, fed-batch and continuous cultures. Growth on various carbon sources in batch culture suggested that lipase activity (measured as the rate of hydrolysis of olive oil) by *Ps. aeruginosa* EF2 was strongly induced by growth on long chain fatty acyl esters (especially Tween 80) and repressed by long-chain fatty acids, including oleic acid. Maximum activities were observed towards the end of the exponential and the onset of the stationary growth phases, when carbon and energy became limiting. The highest activities were obtained in Tween 80-limited fed-batch and continuous cultures grown at low specific growth rates. From this it was also concluded that lipase production by *Ps. aeruginosa* EF2 was also weakly induced by carbon and/or energy limitation. Lipase production by *Ps. aeruginosa* EF2 was optimised with respect to temperature and pH in a Tween 80-limited continuous culture using statistical response surface analysis at a fixed dilution rate of 0.05 h^{-1} (optimum values 35.5°C, pH 6.5). Maximum activities were obtained under these conditions at a dilution rate of 0.04 h^{-1} (39 LU [mg cells]$^{-1}$; where 1 LU equalled 1 μmol titratable fatty acid released min^{-1}). These were over eight-times greater than the maximum activities detected following growth on Tween 80 (8.3 LU mg $cells^{-1}$), and over forty-times greater than growth on glucose (< 1 LU mg $cells^{-1}$) in batch cultures. Esterase activities (measured as both the rate of hydrolysis of both *p*-nitrophenyl acetate and Tween 80) varied approximately in parallel with lipase activities under nearly all growth conditions, suggesting that a single enzyme catalysed both activities.

3.2 *Introduction.*

3.2.1 *The regulation of enzyme synthesis in bacteria.*

Bacteria respond to changes in the environment by differentially expressing genes encoding specific proteins necessary for survival. The expression of these genes are regulated at the molecular level by negative and/or positive feed-back mechanisms. Negative control involves the binding of a specific repressor protein to the gene which blocks transcription; expression can only take place once this has been effectively removed. In contrast, positive control requires the interaction of an activator protein with the gene to initiate transcription (Busby, 1986). The activity of these repressor and activator proteins are rigorously controlled by their interaction with defined molecules which act as signals.

The production of extracellular enzymes is generally controlled by a combination of induction, end-product repression and catabolite repression mechanisms (Harder, 1979). Induction and end-product repression are an example of a negative control system, where a product of an enzymic reaction combines with a unique repressor protein (co-repressor) within the cell. This enables it to bind to the operator region of the gene encoding the enzyme, thus preventing messenger RNA (mRNA) synthesis. Inactivation of the co-repressor, by the binding of a specific inducer molecule (commonly the substrate or a substrate analogue) allows transcriptional re-activation.

These mechanisms are often superseded by a form of positive control, known as carbon catabolite repression or catabolite repression control (CRC; Postma, 1986). This occurs when a more readily catabolisable substrate, such as glucose, is present. A pre-requisite for the transcription of these repressible genes is the binding of a catabolite activator protein to the DNA, prior to binding of the RNA polymerase. This has been well characterised in

Escherichia coli and other enteric bacteria, and shown to be cyclic AMP-mediated. Glucose metabolism reduces the intracellular cAMP concentration, thus lowering the frequency of transcriptional activation of such catabolite-repressible genes. Exhaustion of glucose subsequently leads to increased cAMP levels and hence transcription of the genes; this phenomenon is observed in diauxic growth.

CRC is not thought to be cAMP-mediated in *Pseudomonas* species (Phillips & Mulfinger, 1981). Recently, mutants of *Ps. aeruginosa* PAO defective in CRC were isolated and mapped to the 11 min region of the chromosome (Wolff *et al.*, 1991). Complementation studies using these CRC-defective mutants, indicated that the gene responsible for repression encoded a protein of M_r 30,000, although its precise role was not defined (MacGregor *et al.*, 1991).

These strict control mechanisms enable microorganisms to regulate their physiological state in response to varying nutritional changes within their environment, without the wasteful extravagance of concomitant enzyme synthesis. Consequently, when grown under carbon substrate limitation microorganisms respond either by producing increased amounts of the enzyme(s) involved in the initial metabolism of the substrate, or by metabolising the substrate *via* an alternative route (Harder and Dijkhuizen, 1983). Such responses are commonly studied during growth in batch, fed-batch and continuous culture systems.

3.2.2 *Bacterial cultivation techniques.*

In a batch culture all essential nutrients are initially present in excess, and growth typically occurs in three distinct phases. These are

(i) An initial lag phase following inoculation, in which catabolic enzymes required for growth on a particular substrate are synthesized;

(ii) an exponential growth phase, which is a consequence of cell division; and

(iii) a stationary phase, where the growth rate decreases and eventually ceases due to the depletion of one or more essential nutrients.

During the exponential phase, the rate of growth can be expressed by the differential equation:

$$\frac{dX}{dt} = \mu X \qquad (1)$$

where X is the cell number, t is time (h), and μ is the specific growth rate (h^{-1}). By integrating, it can be expressed as:

$$\ln X = \ln X_0 + \mu(t) \qquad (2)$$

where X_0 is the cell number at time 0, and X is the cell number at time t. Taking the antilogarithm of both sides gives:

$$X = X_0 e^{\mu t} \qquad (3)$$

As the population is doubling, $\frac{X}{X_0} = 2$, hence the equation can be substituted and rearranged to give:

$$2 = e^{\mu td} \qquad (4)$$

where t_d is the doubling time. By taking the natural log and rearranging, this can be expressed in a practical form:

$$\mu = \frac{\ln 2}{t_d} \qquad (5)$$

During logarithmic growth, the specific growth rate μ is equal to the maximum specific growth rate, μ_{max}. Hence:

$$\mu_{max} = \frac{0.693}{t_d} \qquad (6)$$

As the maximum specific growth rate of an organism in batch culture is generally dependent on the culture medium, an insight into the ability of the organism to utilise a variety of different substrates can be made by comparing growth rates under a set of defined conditions. In addition, the final cell density attained upon nutrient exhaustion in the stationary phase enables the cellular yield [Y; g cell biomass (g substrate)$^{-1}$] to be calculated.

A batch culture which is fed continuously with a single growth-limiting substrate, where all the other nutrients are present in excess, is known as a fed-batch culture. Here the growth rate μ is regulated by the rate at which the growth-limiting substrate is supplied, and is less than μ_{max}. As the culture volume increases with increasing substrate input, μ therefore decreases with time. A quasi-steady state exists in which the biomass increases in a linear fashion (cf. exponentially during batch growth) with the linear input of substrate. This fed-batch culture system has been employed successfully to reduce the effects of catabolite or product repression on microbial product formation.

As described above, the specific growth rate of an organism is dependent upon the nutrient concentration, and can be expressed by the following mathematical relationship first defined by Monod (1942) (see Pirt, 1975):

$$\mu = \frac{\mu_{max}.S}{S + K_s} \qquad (7)$$

where μ is the specific growth rate, μ_{max} is the maximum specific growth rate, S is the substrate concentration and K_s is the saturation

constant for the growth limiting nutrient, which is similar to a Michaelis-Menten constant.

A continuous culture system enables growth to occur under precisely defined conditions, unlike that in batch and fed-batch cultures. This often takes the form of a chemostat culture, where the growth rate (which is usually less than μ_{max}) is dependent upon the rate at which the medium is fed into the constant volume fermentation vessel. Its major advantage is that both growth rate, μ, and cell density, $\tilde{x}$, can be controlled independently of each other. At steady-state a self-regulating system exists in which μ equals the dilution rate, D, which is defined as:

$$D = \frac{F}{V} \qquad (8)$$

where F is the flow rate of medium (ml h^{-1}), and V is the culture volume, V (ml). D, like μ, therefore has units of h^{-1}.

The cell density depends on the concentration of the growth-limiting substrate, when all other nutrients are present in excess. The cellular yield, Y, is defined as:

$$Y = \frac{\text{cell biomass (g)}}{\text{mass of substrate consumed (g)}} \qquad (9)$$

This can be re-expressed as:

$$Y = \frac{\text{cell density (g } l^{-1})}{[S - \tilde{s}] \quad (\text{g } l^{-1})} \qquad (10)$$

where S is the input growth-limiting substrate concentration (g l^{-1}) and $\tilde{s}$ is the residual growth-limiting nutrient concentration (sometimes called the standing concentration; g l^{-1}).

In a steady-state culture $\tilde{s}$ is very low, and can be expressed mathematically by substituting and rearranging the Monod equation:

$$\tilde{s} = \frac{K_s . D}{\mu_{max} - D} \qquad (11)$$

As D tends towards μ_{max}, then $\tilde{s}$ increases exponentially, and the effect of substrate concentration on enzyme regulation can therefore be investigated at various dilution rates (see for example Harder & Dijkhuizen, 1982, and Melling, 1977).

This chapter describes the use of batch, fed-batch and continuous cultures to study the regulation of lipase production by a newly-isolated strain of *Pseudomonas aeruginosa*, together with the optimisation of lipase activity during growth in continuous culture using statistical response-surface analysis. The results are discussed with reference to lipase production by other bacteria.

3.3 *The isolation of* Pseudomonas aeruginosa *EF2.*

The isolation strategy employed was intended to select for microorganisms producing lipases suitable for incorporation into a detergent formulation. Samples from various environmental sites rich in grease and oil, were subjected to conditions similar to that in a typical detergent wash (that is an alkaline pH, moderate temperature and the presence of surfactants and sequestrants), where olive oil was the sole source of both carbon and energy. This ensured the positive selection of desirable lipolytic microorganisms. The use of a continuous cultivation system to isolate lipolytic microorganisms has recently been described by Lie *et al.* (1991) and is probably superior to conventional batch isolation procedures.

Several pure bacterial cultures were isolated. One of these, *Pseudomonas aeruginosa* EF2, was chosen for further characterisation, as initial studies had indicated that it produced an alkaline, thermotolerant lipase (Davidson, 1987). This organism was used in all subsequent work.

3.4 *Lipase production by* Pseudomonas aeruginosa *EF2 during growth in batch culture.*

3.4.1 *Effect of the nitrogen source.*

Pseudomonas aeruginosa EF2 was grown in batch culture at 37°C in a glucose-minimal salts medium supplemented with either trypticase peptone or casein hydrolysate (0.1% w/v). As growth could not be directly related to the utilisation of specific components of the complex nitrogen sources, and as lipase activities were below the limit of detection, it was decided to use only defined minimal media.

Instead, *Ps. aeruginosa* EF2 was grown in a glucose-minimal salts medium containing ammonium sulphate, potassium nitrate or urea (30mM) as the nitrogen source. The cultures exhibited high specific growth rates (μ_{max} 0.48-0.56 h^{-1}) under all three conditions, but the final cell density was approximately seven times higher following growth on nitrate than on the other two nitrogen sources, and would have been even higher had the culture not run out of glucose. This difference probably reflected the relatively rapid formation of extracellular ammonia from ammonium sulphate and urea compared with the rate from potassium nitrate, followed by the partial loss of this volatile product from the growth medium at alkaline pH. Acidification of the minimal salts medium occurred following growth on ammonium sulphate, whilst it remained alkaline when grown on nitrate and urea. Potassium nitrate was therefore used as the nitrogen source in all subsequent experiments. The lipase activity of nitrate-grown batch cultures was below the limit of detection [<1 LU $(mg\ cells)^{-1}$] during both the exponential and stationary phases of growth; similarly, no esterase activity was detected during the exponential growth phase, but a low activity was detected during the stationary phase. The extent of esterase production on glucose was independent of the nitrogen source.

3.4.2 *Effect of the carbon source.*

When *Pseudomonas aeruginosa* EF2 was grown in an olive oil-minimal salts medium, optical density determinations were not possible (preventing the calculation of specific growth rates and specific enzyme activities), due to extensive emulsification and saponification of the lipid substrate. Growth on a range of different carbon sources was therefore carried out to identify a suitable alternative. Low esterase activities were detected at the onset of the stationary phase of growth using glucose, glycerol, oleic acid and the soluble triglyceride triacetin as the carbon source;

no growth was observed on the semi-soluble tributyrin. Lipase activities of all cultures were below the limit of detection.

Ps. aeruginosa EF2 grew at intermediate specific growth rates on the non-ionic surfactants tested, *ie.* Tweens (polyoxyethylene sorbitan fatty acyl esters) and Spans (sorbitan fatty acyl esters) (Fig. 3.1). The chain length and degree of saturation of the esterified fatty acyl residue determined the solubility of the various Tweens and Spans in aqueous solution. Optical density determinations were possible at all stages of growth on Tweens 20 and 80, but only during the late exponential phase of growth on Tweens 40, 60, 85 and Span 85 (*ie.* after a significant proportion had been utilised).

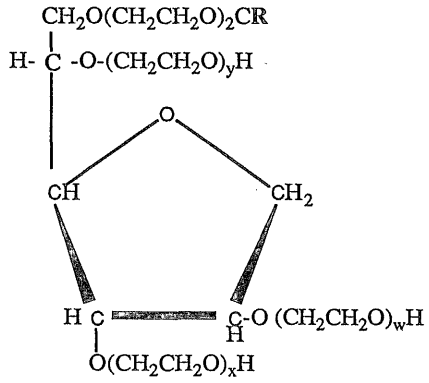
Lipase activities varied depending on the nature of the fatty acyl ester, with maximum activities being observed on Tweens 40 (monopalmitate), 80 (monooleate) and 85 (trioleate). These activities were substantially higher than activities during growth on glucose (Table 3.1). As minimal salts medium supplemented with Tweens 40 and 85 were fairly turbid, whereas Tween 80 was completely soluble, the latter was used as the carbon source in all subsequent experiments.

3.4.3 *Effect of Tween 80.*

Lipase and esterase activities were barely detectable during the exponential phase of growth, but increased several-fold to a maximum during the onset of the stationary phase (Fig. 3.2). This was thought to correspond to depletion of the carbon source, as a linear relationship between the final cell density and initial Tween 80 concentration was observed. The pH of the minimal salts medium fell from an initial pH 8.5 to 7.5 during growth. Lipase activities of *Ps. aeruginosa* EF2 grown Tween 80-limited in continuous culture were shown to be strongly influenced by the pH of the medium (Section 3.6.1). However, it is doubtful whether the difference in pH

Figure 3.1

a. *Structure of Tweens (polyoxyethylene sorbitol esters).*

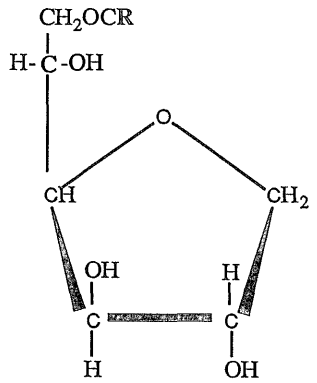


w+x+y+2=20

R, fatty acyl chain:

Tween 20, mono laurate;
Tween 40, mono palmitate;
Tween 60, mono stearate;
Tween 80, mono oleate;
Tween 85, tri oleate.

b. *Structure of Spans (sorbitol esters).*



R, fatty acyl chain:

Span 80, mono oleate;
Span 85, tri oleate.

Table 3.1. *Effect of carbon source on the lipase and esterase activities of* Pseudomonas aeruginosa *EF2 grown in batch culture.*

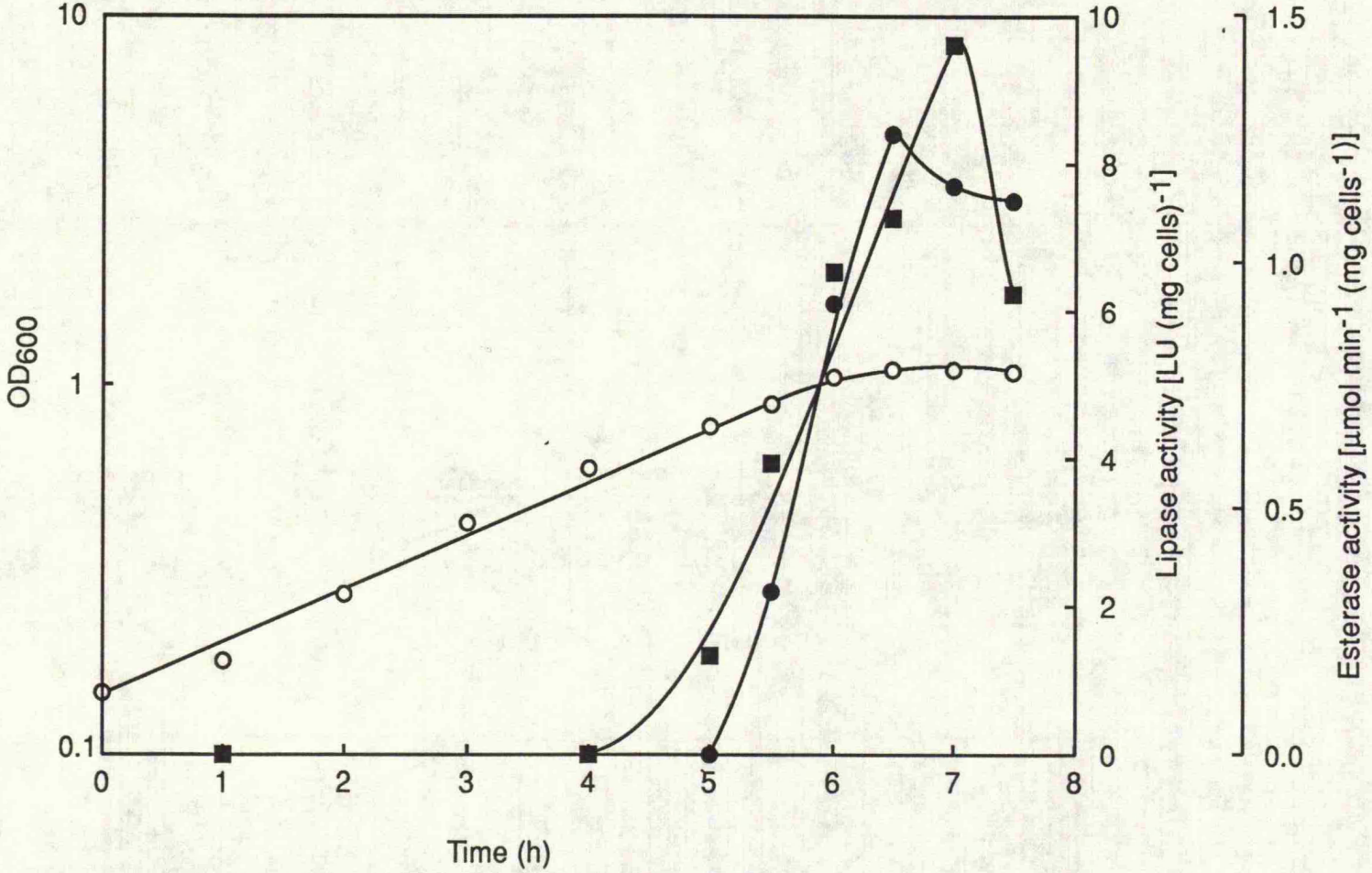
Ps. aeruginosa EF2 was grown in batch culture (37°C, initial pH 8.5) on a minimal-salts medium, with KNO_3 as the nitrogen source and supplemented with various carbon sources (3.85 mg ml^{-1}). Specific growth rates were measured during the exponential growth phase, which generally lasted about 6h; in some cases these measurements were only possible during the later stages when the turbidity due to the initial concentration of carbon substrate had disappeared. Lipase and esterase activities were measured on untreated culture samples at 30 min intervals between the mid-exponential phase and the later stages of the stationary phase, as described in Methods. The highest activity acheived was recorded. The results are the mean of up to six independent determinations.

Carbon source	Final cell density	μmax	Enzyme activities		Optical state of culture
	(mg cells ml^{-1})$_{max}$	$(h)^{-1}$	Esterase max [μmol min^{-1} (mg cells)$^{-1}$]	Lipase max [LU mg $cells^{-1}$]	
Glucose	1.79	0.48	0.19	<1	Clear
Oleic acid	ND	ND	0.35	<1	Very turbid
PEG (600)	0.0	ND	ND	ND	Clear
Triacetin	0.78	0.06	0.30	<1	Clear
Tributyrin	0.0	ND	ND	ND	Slightly turbid
Olive Oil (>80% w/v triolein)	ND	ND	0.20*	0.2*	Very turbid
Tween 20	0.69	0.37	0.48	3.6	Clear
Tween 40	0.85	0.37	1.31	7.2	Slightly turbid
Tween 60	0.81	0.36	1.46	6.3	Turbid
Tween 80	1.03	0.36	1.36	8.3	Clear
Tween 85	1.53	0.33	1.89	7.7	Turbid
Span 80	ND	ND	1.53*	2.7*	Very turbid
Span 85	2.06	0.27	0.88	1.0	Turbid

LU, Lipase Units; μmol titratable fatty acid released min^{-1}.
ND, Not determined due to high turbidity.
*, Activities expressed on a volumetric basis [LU (ml culture)$^{-1}$ or μmol min^{-1} (ml culture)$^{-1}$].

Figure 3.2. *Lipase and esterase activities of* Ps. aeruginosa *EF2 grown in batch culture.*

A minimal salts medium supplemented with Tween 80 (3.85 g l-1) was inoculated to give an initial density of approximately 0.1 mg dry mass ml-1. Culture samples were taken at regular intervals over an 8h period and assayed for growth and enzyme activities as described in Methods. o , Cell density; ● , lipase activity; ■ , esterase activity.



observed during batch growth would be enough to account for the large increase in activities detected at the onset of the stationary phase of growth.

Ps. aeruginosa EF2 failed to grow on polyethylene glycol (PEG_{600}), indicating it was unable to utilise this component of the Tween molecule as a carbon source. There is evidence from work with continuous cultures (Section 3.6.3) that *Ps. aeruginosa* EF2 only uses the oleyl residue as a source of carbon for growth, and that it exhibits a growth yield (Y) on Tween 80 equivalent to 0.7 g cells (g oleic acid)$^{-1}$ [197 g cells (mol oleic acid)$^{-1}$]. The lipase activities detected during exponential growth, although very low, were nevertheless at least three-fold greater than the *in vivo* rate of oleic acid utilisation (q_{OA}) of a Tween 80 batch culture growing at μ_{max} (0.37 h^{-1}). This was calculated according to the following relationship:

$$q_{OA} = \frac{\mu_{max}}{Y} = \frac{0.37}{197}$$

$$= 1.88 \text{ mmol } h^{-1} \text{ (g cells)}^{-1}$$

$$= 31 \text{ nmol } min^{-1} \text{ (mg cells)}^{-1}$$

The concentration of free oleic acid in the culture supernatant remained approximately constant during the exponential growth phase (average concentration 60 mg l^{-1}, equivalent to 7% of the oleic acid potentially available in Tween 80) but this rapidly decreased to a non-detectable level at the end of the exponential growth phase (ie. concomitant with the large increase in lipase and esterase activities).

Measurement of lipase and esterase activities in culture supernatants and whole cells prepared by centrifugation of early stationary phase batch cultures grown on Tween 80 showed that over 90% of the total activity in each case was present in the culture supernatant, indicating that both activities were predominantly extracellular.

Upon attaining their maximal values during the early stationary phase of growth, both lipase and esterase activities rapidly decreased. As *Ps. aeruginosa* is a prolific producer of the extracellular proteases elastase and alkaline protease (Kessler & Safrin, 1988) it was possible that proteolytic cleavage might account for this. However protease activity could not be detected using the azo-casein- (Jones *et al.*, 1988) and casein-hydrolysis (Cowan & Daniel, 1982) assays.

Cell lysis could not be detected in late exponential and early stationary phase cultures, when they were assayed for the exclusively intracellular enzyme glucose 6-phosphate dehydrogenase.

When culture supernatants taken from exponential and stationary phase culures were analysed by SDS-PAGE (silver stained), no single protein could be detected whose concentration varied in parallel to lipase activities.

The possibility that lipase production was subject to carbon catabolite repression was investigated by growing *Ps. aeruginosa* EF2 in a minimal salts medium supplemented with 1.93g l^{-1} of both glucose and Tween 80. Diauxic growth was not observed, indicating that lipase activity was not strongly repressed by glucose.

It was therefore concluded from this work with batch cultures that lipase and esterase activities were weakly induced by carbon and/or energy limitation, strongly induced by long-chain fatty acyl esters (most strongly by Tween 80 and 85) and repressed by oleic acid. Furthermore, as the variation in lipase and esterase activities during batch growth was very similar irrespective of which Tween was used as the carbon source, it seemed likely that other long-chain fatty acids (e.g. lauric, palmitic and stearic acids, released by the hydrolysis of Tweens 20, 40 and 60, respectively) could also repress these activities.

3.5 *Lipase production by* Pseudomonas aeruginosa *EF2 during growth in fed-batch culture.*

Lipase and esterase activities of carbon-limited fed-batch cultures of *Ps. aeruginosa* EF2 grown at low specific growth rates were substantially higher during growth on Tween 80 than on glucose, glycerol or oleic acid (Table 3.2). An inverse relationship between lipase and esterase activities and the instantaneous growth rate in Tween 80-limited cultures was noted (Fig. 3.3), supporting the conclusions drawn from growth on Tween 80 in batch culture (Section 3.4.3). Furthermore lipase and esterase activities of the Tween 80-limited cultures were several-fold higher than those of Tween 80-excess cultures grown at similar rates under oxygen or nitrate limitation.

3.6 *Lipase production by* Pseudomonas aeruginosa *EF2 during growth in continuous culture.*

3.6.1 *Growth of* Ps. aeruginosa *EF2 under Tween 80 limitation.*

Pseudomonas aeruginosa EF2 was originally grown in a Tween 80-limited continuous culture at a dilution rate, D, of 0.05 h^{-1} (pH 8.5, 37°C); a growth rate at which reasonable lipase activities were detected in a Tween 80-limited fed-batch culture. The minimal salts medium was identical to that used during during batch and fed-batch determinations. Following growth in batch cultures, an input Tween 80 concentration of 3.38 g l^{-1} was calculated to yield a cell density of approximately 1 mg ml^{-1}. The steady-state cell density of the continuous culture was, however, only half this value, and the subsequent doubling of the minimal salts components and replacement of the phosphate source (Na_2HPO_4) with KH_2PO_4 (to reduce maintenance energy expenditure), did not change this. Doubling of the Tween 80

Table 3.2. *Effect of various nutrient limitations on the lipase and esterase activities of* Ps. aeruginosa *EF2 grown in fed-batch culture.*

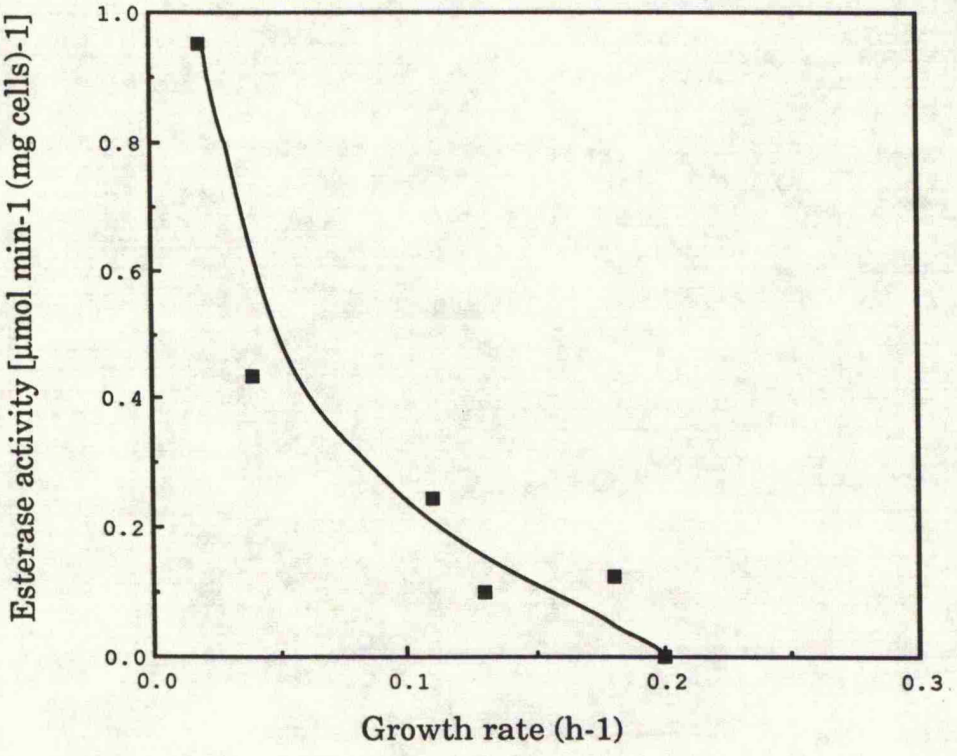
Ps. aeruginosa EF2 was grown in fed-batch culture (μ 0.09 ± 0.06 h^{-1} at the point of harvesting) on a minimal-salts medium supplemented with various carbon sources. The cell density in the oleic acid-limited culture was ≥ 1 mg dry wt ml^{-1}.

		Enzyme activities	
Carbon source	Limiting nutrient	Esterase	Lipase
		μmol min^{-1} $(mg\ cells)^{-1}$	LU $(mg\ cells)^{-1}$
Glucose	Glucose	0.37	< 1
Glycerol	Glycerol	0.22	< 1
Oleic acid	Oleic acid	0.28*	0.1*
Tween 80	Tween 80	0.95	3.5
	Oxygen	< 0.05	< 1
	Nitrate	0.08	< 1

*, Activities expressed on a volumetric basis [μmol min^{-1} $(ml\ culture)^{-1}$ or LU $(ml\ culture)^{-1}$.

Figure 3.3. *The effect of growth rate on the production of esterase activity by* Ps. aeruginosa *EF2 grown under Tween 80-limitation in fed-batch culture.*

Ps. aeruginosa EF2 was grown in fed-batch culture on a minimal-salts medium supplemented with Tween 80 at variable rates. The growth rate at the point of harvesting was calculated and esterase activities determined, as described in Methods.



concentration to 6.76 g l^{-1} resulted in a doubling of the cell density (to approximately 1 mg ml^{-1}), indicating that the culture was genuinely carbon limited. This concentration was used in all subsequent experiments.

The direct addition of iron into the culture medium led to extensive precipitation, as a result of this the culture became iron-limited. This was accompanied by the production of a fluorescent green pigment (probably the iron chelator pyoverdine; Palleroni, 1984). Acidified ferric citrate (to prevent auto-oxidation) was therefore supplied *via* an independent input line at a rate calculated to give a concentration similar to that in the original minimal-salts medium.

Growth on Tween 80 (even under limiting conditions) caused extensive foam formation due to its surfactant properties, and this was controlled by the periodic input of a silicon-based antifoam. An antifoam concentration of 200 ppm (the calculated concentration within the fermentation vessel) was shown to have very little effect on the growth rate or on the lipase and esterase activities of *Ps. aeruginosa* EF2 in batch culture.

Difficulties were also experienced when trying to establish a steady-state culture of *Ps. aeruginosa* EF2 grown under Tween 80 limitation at a dilution rate of 0.05 h^{-1}, at 37°C and pH 8.5. These were overcome by reducing the pH of the medium, whilst keeping the dilution rate and temperature constant. Lipase and esterase activities of the culture (the latter measured as both *p*-nitrophenyl acetate and Tween 80 hydrolysis) were shown to increase as the pH was decreased, although not in parallel (hinting at the possibility of a second esterolytic enzyme; Figs. 3.4 & 3.5). SDS-PAGE (silver stained) analysis of culture supernatants indicated that the concentration of a protein of M_r 29,000, thought to be the lipase (Section 4.3) was proportional to esterase (Tween 80 hydrolysis) activity, and possibly reflected the activity of the

Figure 3.4. *The effect of pH on the production of lipase activity by* Ps. aeruginosa *EF2 grown Tween 80-limited in continuous culture at a constant dilution rate (0.05 h^{-1}) and temperature (37°C).*

Each point was the mean of three independent determinations assayed in duplicate. The standard error was generally < 0.7.

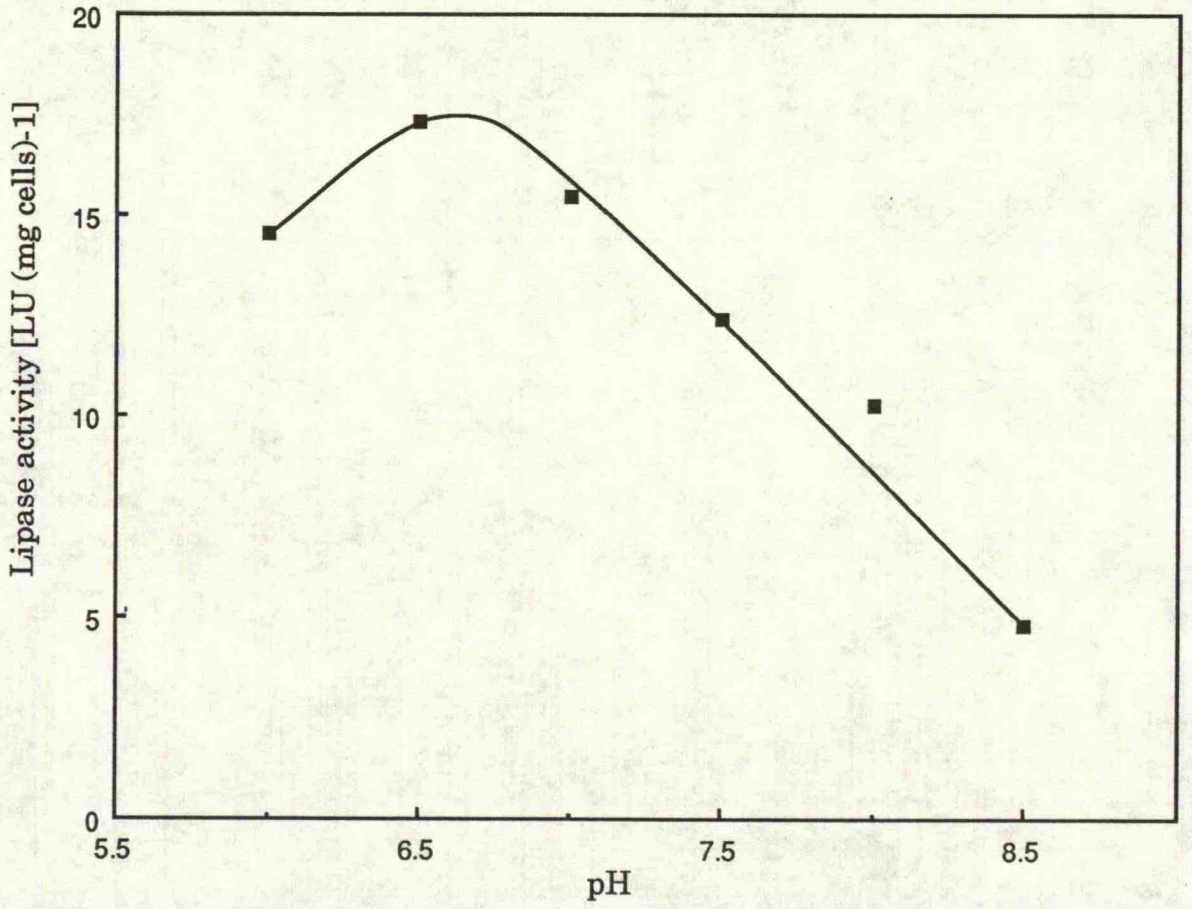
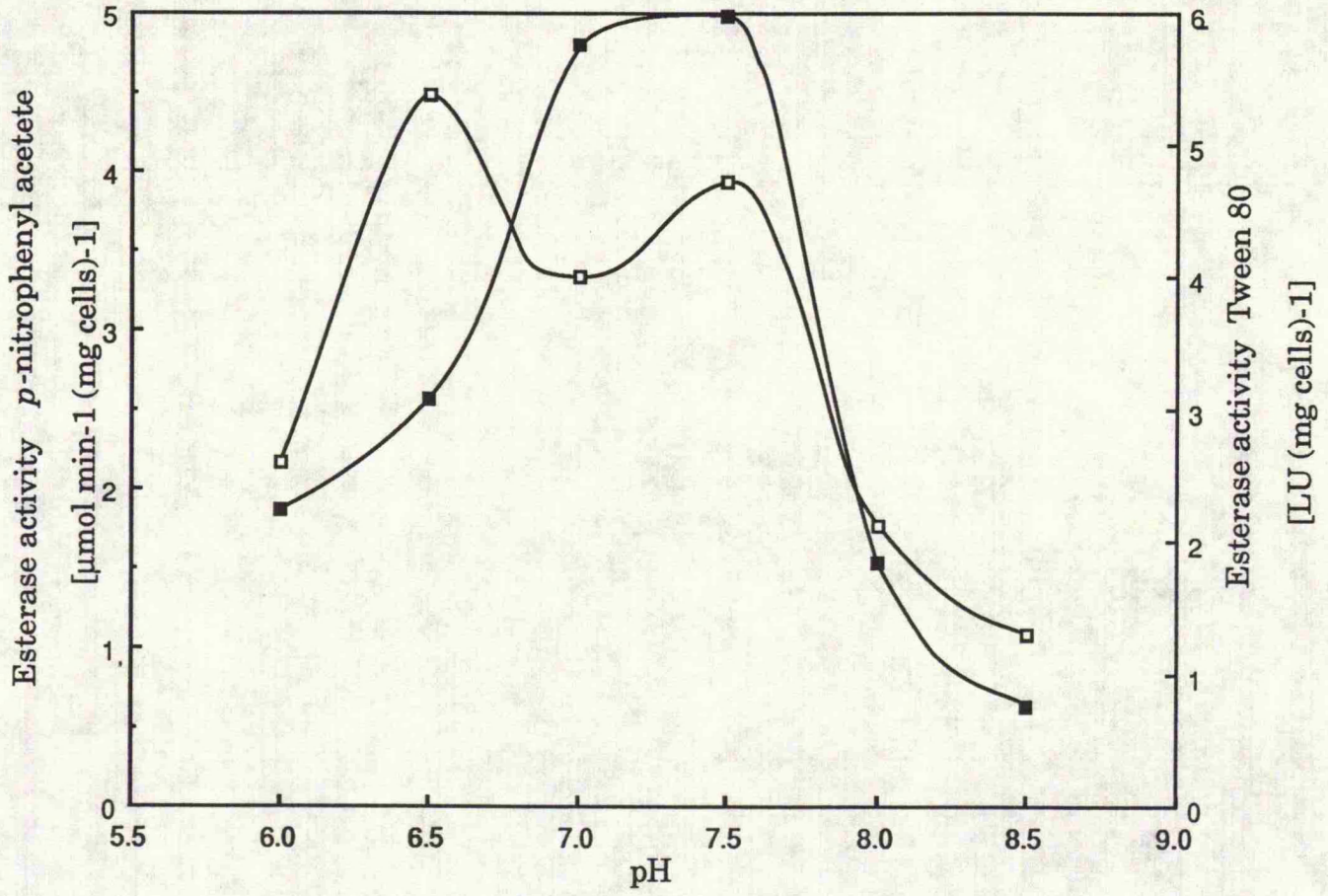


Figure 3.5. *The effect of pH on the production of esterase activities by* Ps. aeruginosa *EF2 grown Tween 80-limited in continuous culture at a fixed dilution rate (0.05 h^{-1}) and temperature (37°C).*

Each point was the mean of three independent determinations assayed in duplicate. The standard error was generally <0.25. □ , *p*-nitrophenyl acetate; ■ Tween 80.



enzyme towards the growth substrate. However, in view of the majority of the results obtained, where lipase and esterase activities of *Ps. aeruginosa* EF2 grown in batch, fed-batch and continuous cultures generally varied in parallel, this strongly suggested catalysis by a single enzyme. Experimental error in this particular experiment was probably the most likely explanation.

3.6.2 *The optimisation of lipase production.*

The effect of growth temperature and pH (D = 0.05 h^{-1}) on lipase activity was also determined to enable optimum conditions for lipase production to be calculated by statistical contour surface analysis (see Farrand *et al.*, 1983). Experimental data obtained at various values of pH and temperature (Table 3.3) indicated a significant interaction between the two variables, and allowed the formulation of a quadratic model for each. This was then incorporated into a single model, which was expressed algebraically as:

$$\text{Lipase activity} = M + A.pH + B.temp + pH^2D.temp^2 + E.pH.temp$$

where M, A, B, C, D and E are constants estimated from computer analysis of the data. The data was shown to fit the model well (R^2=0.98) and optimal values of pH 6.5 and 35.7 °C were calculated by resolving the equation. This is expressed graphically in Fig. 3.6. In practice a temperature of 35.5°C was employed due to limitations imposed by the apparatus used.

The calculated optimum values were confirmed by growing *Ps. aeruginosa* EF2 at various temperatures at pH 6.5 and dilution rate 0.05 h^{-1} (Fig. 3.7). Attempts to incorporate the dilution rate into the model were prevented by the observation that extensive interactions occurred between the three variables (pH, temperature and dilution rate), which could

Table 3.3. *Response surface variables and lipase activities for the optimisation of lipase production by* Ps. aeruginosa *EF2.*

Ps. aeruginosa EF2 was grown in Tween 80-limited continuous culture (D 0.05 h^{-1}) at different combinations of pH and temperature. The results are expressed as the mean ± SE (three independent determinations).

pH	Temperature (°C)	Lipase activity [LU (mg cells)$^{-1}$]
6.0	37	14.5 ± 1.9
6.5	37	17.3 ± 0.4
7.0	37	15.5 ± 0.3
7.5	37	12.4 ± 0.4
8.0	37	10.3 ± 0.1
8.5	37	4.8 ± 0.7
6.5	30	5.9 ± 0.2
6.5	42	1.6 ± 0.0
7.0	42	5.1 ± 0.2
8.0	42	1.9 ± 0.1

Figure 3.6. *Response surface analysis of the effect of pH and temperature on the lipase activity of* Ps. aeruginosa *EF2 during growth in Tween 80-limited continuous culture (D 0.05 h^{-1}).*

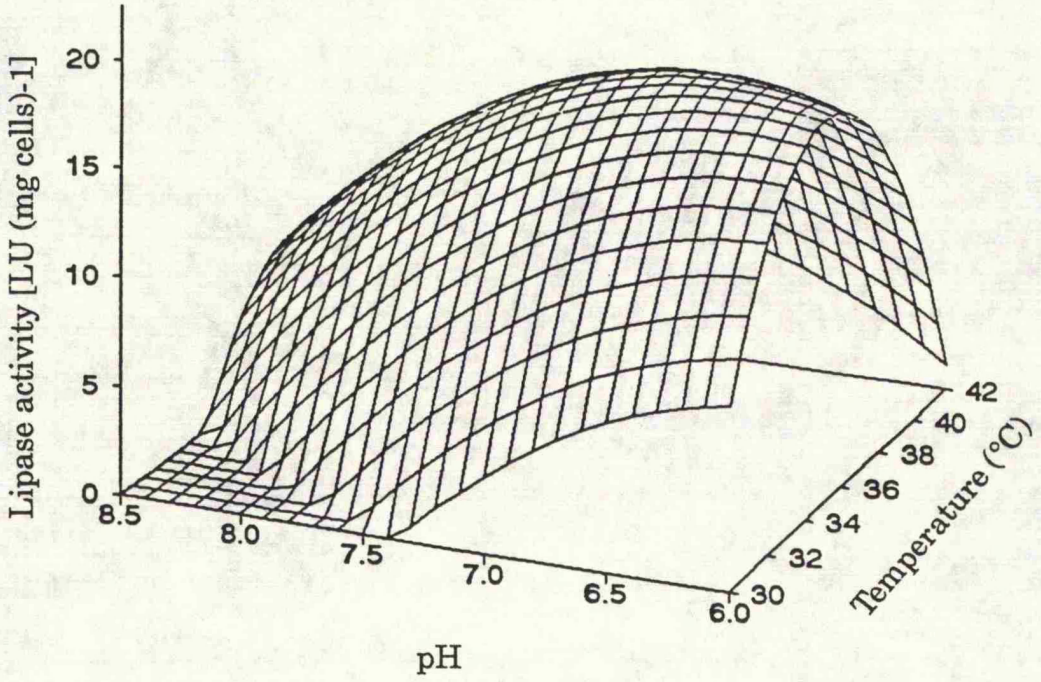
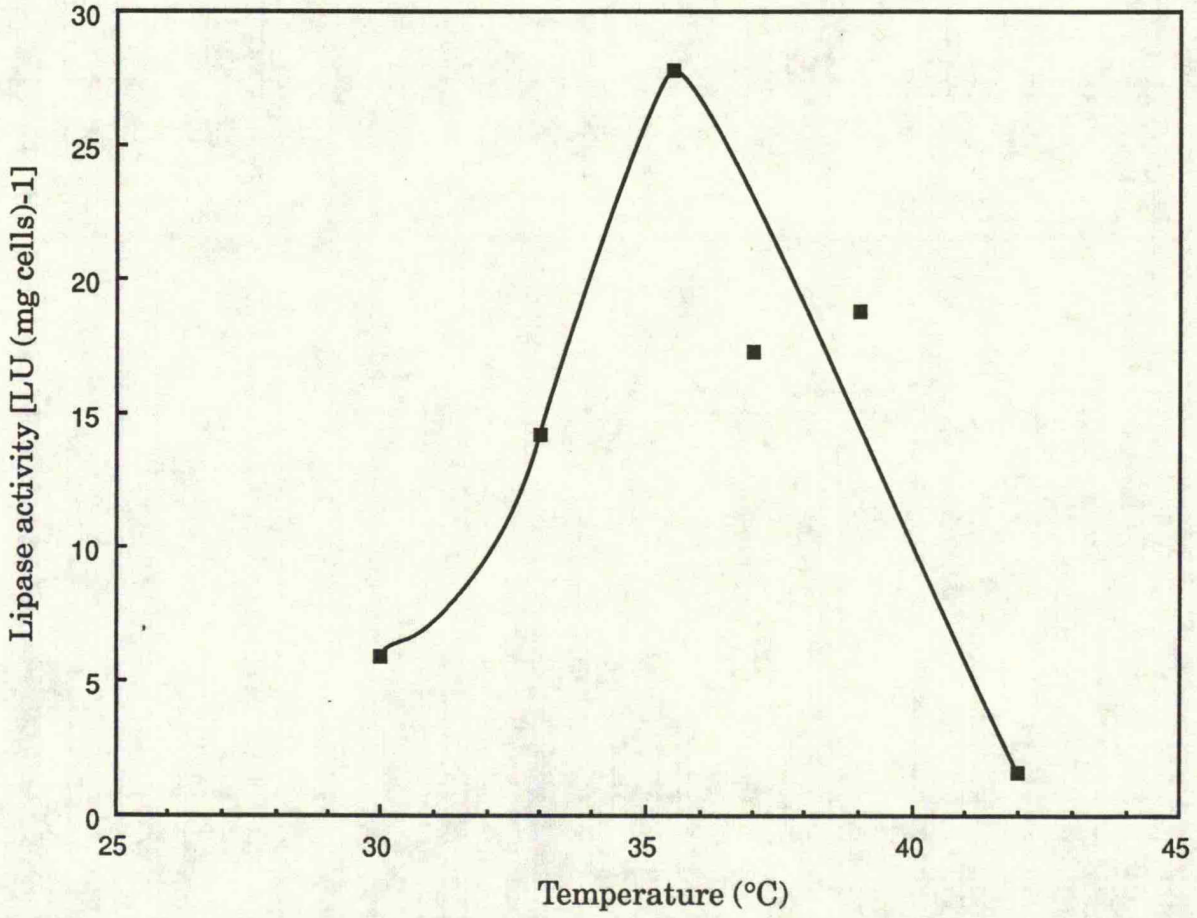


Figure 3.7. *The effect of temperature on the production of lipase activity by* Ps. aeruginosa *EF2 grown Tween 80-limited in continuous culture at a fixed dilution rate (0.05 h^{-1}) and pH (6.5).*

Each point was the mean of three independent determinations assayed in duplicate. The standard error was generally <0.4.



only have been overcome by carrying out a very large number of additional experiments.

3.6.3 *The effect of dilution rate on lipase production.*

The effect of dilution rate at the optimum values of pH (6.5) and temperature (35.5°C) on the production of lipase by *Ps. aeruginosa* EF2 was determined (Fig. 3.8). The inverse relationship between lipase activity and dilution rate confirmed the earlier observations made in Tween 80-limited fed-batch cultures. Lipase and esterase activities (the latter measured as both *p*-nitro phenyl acetate and Tween 80 hydrolysis) varied approximately in parallel (Fig. 3.9).

The concentration of oleic acid increased with dilution rate (from 0.4 to 32.9 mg l^{-1}; Fig.3.8), in accordance with the Monod equation (Eq. 11). Growth of *Ps. aeruginosa* EF2 on Tween 80 in batch and fed-batch cultures had earlier indicated that lipase production was regulated by end-product, that is oleic acid, repression. At first, this also appeared to hold true for the Tween 80-limited continuous culture. At a low dilution rate where the oleic acid concentration was low (hence repression was low), lipase activities were high; at a high dilution rate, the oleic acid concentration was greater (thus lipase production was more repressed) and activities were low. Furthermore, when *Ps. aeruginosa* EF2 was grown in both Tween 80- and nitrate-limited continuous cultures (D = 0.05 h^{-1}, 35.5°C, pH 6.5), lipase activities were over 100-fold higher in the Tween 80-limited culture (37 LU mg $cells^{-1}$ cf. 0.2 LU mg $cells^{-1}$), whereas the oleic acid concentration was almost 100-fold lower (0.5 mg l^{-1} cf. 46.6 mg l^{-1}).

However, the validity of these conclusions was brought into question when the specific rate of lipase production [q_{Lipase}; LU $(mg\ cells)^{-1}\ h^{-1}$] was calculated according to the equation:

Figure 3.8. *The effect of dilution rate on lipase activity,the specific rate of lipase production (q_{Lipase}) and oleic acid concentration during the growth of* Ps. aeruginosa *EF2 in Tween 80-limited continuous culture under optimised conditions of pH and temperature (pH 6.5, 35.5°C).*

●, Lipase activity; ■, q_{Lipase}; ○, oleic acid concentration. Each point was the mean of three independent determinations assayed in duplicate. The standard error was generally < 5 % of the mean.

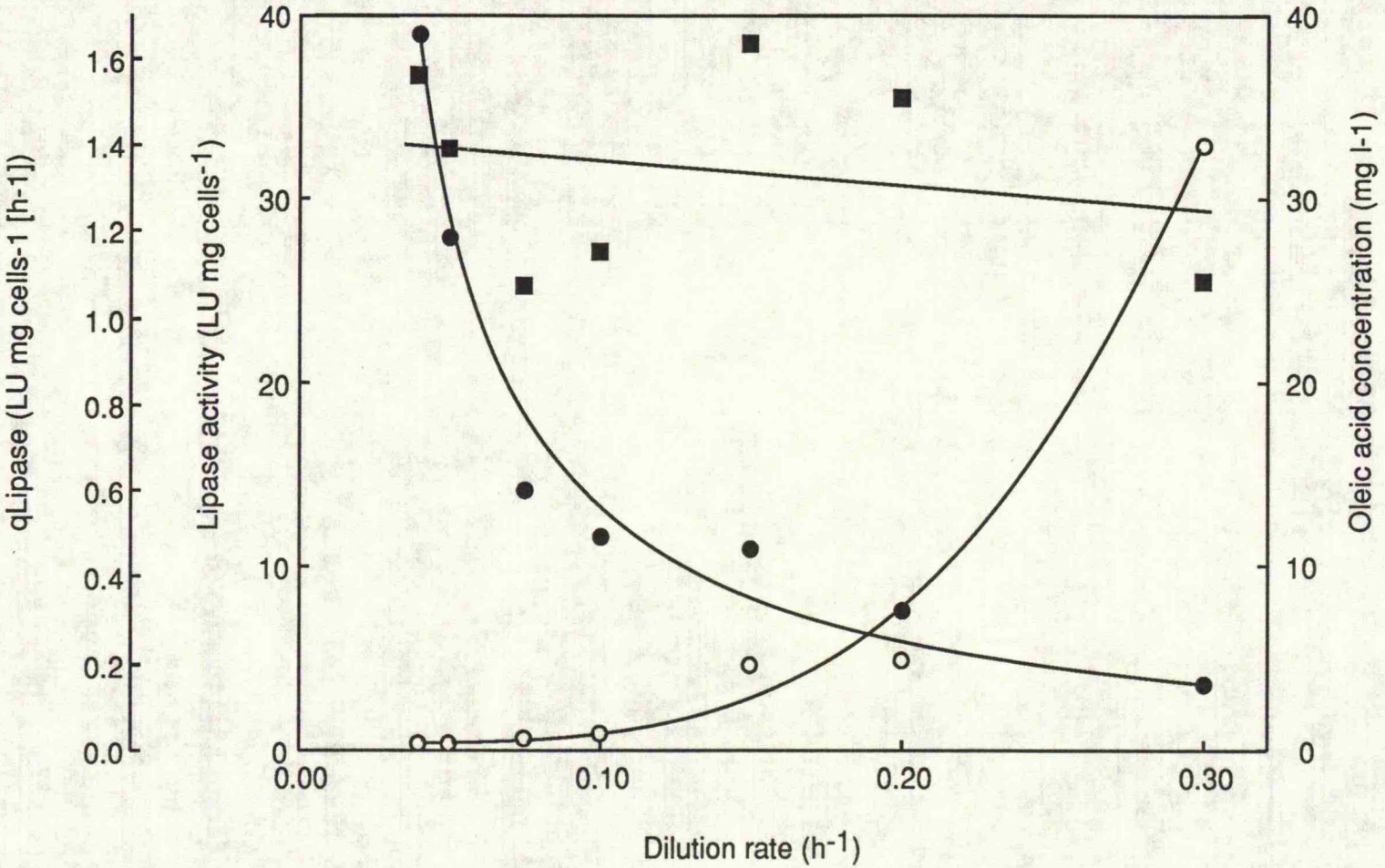
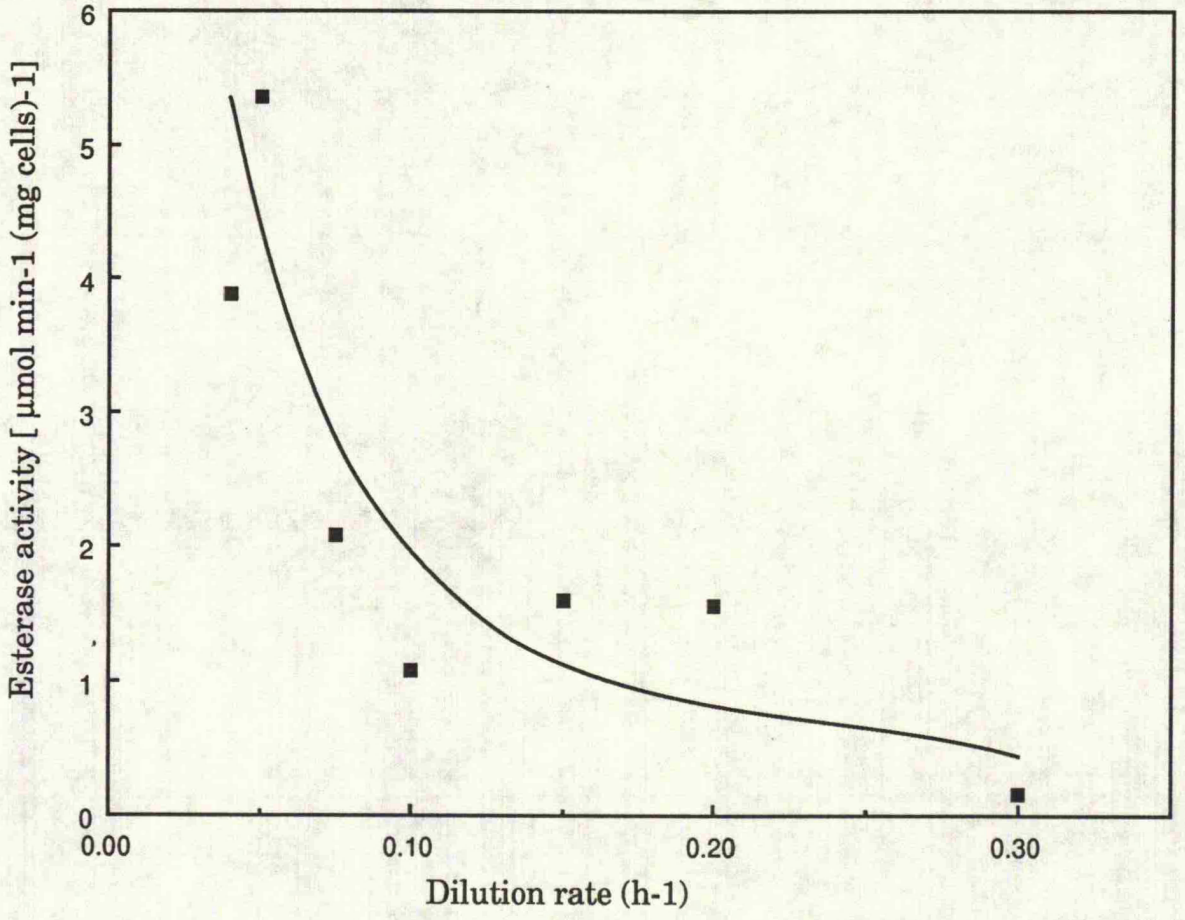


Figure 3.9. *The effect of dilution rate on esterase activity (p-nitrophenyl acetate) during the growth of* Ps. aeruginosa *EF2 in Tween 80-limited continuous culture under optimised conditions of pH and temperature (pH 6.5, 35.5°C).*

Each point was the mean of three independent determinations assayed in duplicate. The standard error was generally less than 0.2.



$$q_{Lipase} = \text{Lipase activity [LU (mg cells)}^{-1}] \times D\ (h^{-1})$$

This remained virtually constant [1.34 ± 0.22 LU (mg cells)$^{-1}$ h^{-1}] over the entire dilution rate range (Fig. 3.8), as did the specific rate of esterase production [0.19 ± 0.03 μmol min^{-1} (mg cells)$^{-1}$]. The increase in lipase activity with decreasing dilution rate may therefore be purely a function of the residence time of the culture within the fermentation vessel. However, it seems likely that repression of lipase production by oleic acid occurs above an undetermined threshold value (between 32.9 and 46.6 mg l^{-1}). Observations made throughout these experiments that small perturbations in the rate of medium input had profound effects on lipase and esterase activities, also indicated that their production was very tightly controlled.

The total organic carbon content of supernatant samples also increased slightly (from 3.01 to 3.27 g l^{-1}) as the dilution rate increased, probably reflecting the increase in the oleic acid concentration over a much larger background concentration of organic carbon. An estimation of the utilisation of Tween 80 by *Ps. aeruginosa* EF2 in continuous culture can be obtained from these measurements. By assuming that the fatty acyl content of Tween 80 is comprised only of oleate residues (it is in fact approximately 70% oleate; the balance comprising of linoleate, palmitate and stearate esters) and assuming a formula weight of $C_{64}H_{124}O_{26}$ (M_r 1308), the input organic carbon content of a 6.76 g l^{-1} Tween 80 solution can be calculated as:

$$\frac{64 \times 12}{1308} \times 6.76 = 3.97\ g\ l^{-1}$$

The input organic carbon due soley to the oleyl (C18) residues was therefore equal to:

$$\frac{18}{64} \times 3.97 = 1.12\ g\ l^{-1}$$

(*ie.* 28% of the carbon in each Tween 80 molecule), and the organic carbon due to the residual sorbitan and polyethylene glycol residues was equal to:

$$3.97 - 1.12 = 2.85\,g\,l^{-1}$$

(*ie.* 72% of the carbon in each Tween 80 molecule). The average total organic carbon content of the continuous culture supernatants at the various dilution rates was calculated to be 3.02 g l^{-1} (76% of the input carbon). Assuming that significant cell lysis or extracellular protein secretion had not occurred (this was supported by both protein concentration determinations and SDS-PAGE analysis of culture supernatants) relative to the standing substrate concentration, the actual residual organic carbon concentration was similar to that predicted to be present as residual sorbitan and polyethylene glycol residues. As growth was not observed on polyethylene glycol in batch culture, it therefore seems likely that *Ps. aeruginosa* EF2 was only able to utilise the oleic acid residues released by Tween 80 hydrolysis. Attempts to clarify this by identifying the components of a Tween 80 solution (which was a complex mixture of both free fatty acids and polyoxyethylene sorbitan to which esterified fatty acyl residues were attached) and Tween 80-limited continuous culture supernatants (at low and high dilution rates) by mass spectroscopy (both positive and negative fast atom bombardment and thermospray) were unsuccessful because of the presence of many molecular species.

The cell density of a Tween 80-limited continuous culture remained approximately constant throughout the range of dilution rates employed, and was calculated to be 1.04 g cells l^{-1}. Given the M_r values of Tween 80 and oleic acid were 1308 and 282 respectively, the growth yield (Y) on oleic acid was calculated to be:

$$1.04 \times \frac{1308}{282} \times \frac{1}{6.76} = 0.7 \text{ g cells (g oleic acid)}^{-1}$$

ie. 197 g cells (mol oleic acid)$^{-1}$

The possibility that growth of *Ps. aeruginosa* EF2 under Tween 80-limited conditions in continuous culture would select for mutant strains producing lipase/esterase with altered catalytic properties was highly unlikely. As the production of an altered extracellular enzyme would benefit all cells within the culture, no selective pressures would exist to directly select for a mutant strain; in addition, the passage of six culture volumes prior to assuming a steady state culture was far too short to select for mutants. A sample of Ps. aeruginosa EF2 grown in continuous culture for over 6 culture volumes (>120h) and subsequently transferred to a Tween 80 batch culture was shown to grow at a similar rate and produced similar lipase and esterase activities as the wild-type strain.

3.7 *Discussion.*

It is well known that the composition of the medium in which lipase-producing microorganisms are grown influences the lipase activity obtained (see for example reviews by Bloquel & Veillet-Poncet, 1984; Stead, 1986). The study of lipase production by various *Pseudomonas* species has generally been directed towards maximizing activities by media engineering, based on empirical observations made during growth in batch cultures, often on complex media. An understanding of the physiological regulation of lipase production was therefore somewhat limited. A more detailed analysis of lipase production by a newly-isolated strain of *Pseudomonas aeruginosa* (*Ps. aeruginosa* EF2) was obtained during growth in batch, fed-batch and continuous cultures.

The nitrogen source utilised during growth has been implicated in lipase production by various *Pseudomonas* species. These nitrogen sources were generally complex and included soy-bean meal, peptone, casein hydrolysate, yeast extract and amino acid mixtures (Alford & Pierce, 1963; Lawrence *et al.*, 1967 b; Nadkarni, 1971; Bloquel & Veillet-Poncet, 1984; Sztajer *et al.*, 1988; Sztajer & Maliszewska, 1988; Fernandez *et al.*, 1990; Minghua *et al.*, 1991). The study of lipase production by *Ps. aeruginosa* EF2 was therefore confined to the use of defined minimal media, where the effects of individual components could be determined. Preliminary work growing *Ps. aeruginosa* EF2 on a variety of complex media did not yield any detectable lipase activities. This was also reported by Breuil *et al.* (1978) and Fernandez *et al.* (1988), who noted that lipase secretion by *Ps. aeruginosa* NRCC 5005 and *Ps. fluorescens* B52, respectively, was significantly lower on complex, rather than minimal media.

The source of inorganic nitrogen used was shown not to affect lipase activity; the decision to use nitrate above ammonium or urea was based on the higher rate of loss of the volatile conversion product ammonia at alkaline pH, prior to assimilation.

Investigations of the effect of carbon source on *Pseudomonas* lipase production has also generally been confined to batch cultures. Lipids are generally thought to induce or stimulate lipase production, and many papers report the effects of various sources (see for example, Bloquel & Veillet-Poncet, 1984; Sztajer *et al.*, 1988; Minghua *et al.*, 1991). The mode of utilisation of these lipid substrates has not been well characterised (but see Tan & Gill, 1987; Suzuki *et al.*, 1988; Del Rio *et al.*, 1990); the insoluble nature of the natural substrates of lipases has undoubtedly restricted further detailed investigations.

The study of the effect of carbon source on lipase production by *Pseudomonas aeruginosa* EF2 initially centred around identifying a suitable soluble substrate for growth, as an alternative to the insoluble olive oil on which the organism had been isolated. The non-ionic surfactant Tween 80 (polyoxyethylene sorbitan monooleate) has been implicated as a suitable carbon source for lipase and esterase-producing microorganisms (Howe & Ward, 1976; Ohkawa *et al.*, 1979; Morinaga *et al.*, 1986; Vermeire *et al.*, 1987; Espinosa *et al.*, 1990). It was also reported to release bound enzyme from the surface of fungal cells (Vermeire *et al.*, 1987; Gomez-Alarcon *et al.*, 1989; Jacobsen *et al.*, 1989 a; Espinosa *et al.*, 1990), and enhance the activity of a purified *Pseudomonas* lipase (Yamamoto & Fujiwara, 1988), although Hegedus *et al.* (1988) suggested it inhibited extracellular lipase production by *Beauveria bassiana*. Lipase activities of *Ps. aeruginosa* EF2 were induced by growth on various Tweens (polyoxyethylene sorbitan fatty acyl esters) and Spans (sorbitan fatty acyl esters). This was similar to lipase production by a

thermophilic *Bacillus* species, which was induced by growth on Tween 80 (Gowland *et al.*, 1988). [It should be noted, that care must be taken to assay for genuine lipase activities, as Tween 80 hydrolysis does not always correlate to lipase activity (Ohkawa *et al.*, 1979, and Rollof *et al.*, 1988)].

The rapid decrease in lipase and esterase activities observed during the stationary growth phase could not be directly related to proteolytic digestion. Similar effects were also observed by Lawrence *et al.* (1967 b), and Finkelstein *et al.* (1970). Furthermore, Fox & Stepaniak (1983), demonstrated that the decreased lipase activity of *Pseudomonas fluorescens* AFT 36 during the stationary phase coincided with maximum protease activity. Therefore, this was still thought to be the most likely explanation for these observations.

Lipase production by *Ps. aeruginosa* EF2 appeared to be predominantly extracellular during both the exponential and stationary phases when grown on Tween 80 and glucose. The extracellular lipases of a variety of *Pseudomonas* species have been reported, including a postulated amino-terminal secretory signal sequence of *Ps. fragi* (Section 5.2; Table 5.1). However, complete secretion was not always observed, with a significant portion of the activity remaining cell-bound (Finkelstein *et al.*, 1970; Breuil *et al.*, 1978); a phenomenon that was shown to vary depending on the culture conditions (Watanabe *et al.*, 1977; Shabtai & Mishne-Daya, 1991), and which may be due to an association of the amphiphilic lipase molecule with outer membrane lipopolysaccharide (Jaegar *et al.*, 1991).

Growth under nutrient-limited conditions in fed-batch cultures supported the conclusions drawn during batch growth; since maximum lipase and esterase activities were produced under Tween 80-limitation. The inverse relationship between lipase and esterase activities and the instantaneous growth rate in Tween 80-limited cultures further implied that

production was induced at low substrate concentrations when carbon/energy became limiting, and the effects of product (oleic acid) repression were minimal.

The production of lipase by a strain of *Pseudomonas fluorescens* was also shown to be inducible (by growth on olive oil), with maximum activities obtained during olive oil-limited growth in fed-batch cultures (Suzuki *et al.*, 1988). This suggests, not unexpectedly, that similar mechanisms regulate lipase production in different *Pseudomonas* species.

As low lipase and esterase activities were detected during oleic acid and glycerol-limited growth, this suggests that these substrates probably did not act as inducers or signals to the cell to indicate the presence of an exogenous lipid substrate. A paradox therefore exists as to how these large hydrophobic molecules, and more specifically the large amphiphilic Tween 80 molecule, enters the cell to induce lipase production. However, as only one molecule per cell is theoretically required, simple diffusion may therefore suffice. It is interesting to note here that *Pseudomonas aeruginosa* NRCC 5005 produced lipase when grown on a variety of different alkanes (Breuil *et al.*, 1978), which, following terminal oxidation to the corresponding fatty acid, are metabolised *via* the β-oxidation pathway (Watkinson & Morgan, 1990). Thus, the possibility exists that an intermediate common to both alkane and fatty acid metabolism may induce lipase production in this isolate.

The growth and production of lipase by *Ps. aeruginosa* EF2 in a Tween 80-limited continuous culture using the same minimal-salts medium as used during batch and fed-batch determinations was complicated by a number of problems. Lipase production appeared to be unstable, with the culture exhibiting a temporal loss of activity; a problem also encountered by

Persson *et al.* (1990). By systematically determining the effects of the various media components, certain factors were eliminated as possible regulatory elements governing lipase production in *Ps. aeruginosa* EF2.

Iron has been implicated as an effector of extracellular product formation in *Ps. aeruginosa* PA-103 (Bjorn *et al.*, 1979), whilst lipase synthesis by *Pseudomonas fluorescens* B52 was thought to be regulated by a specific Fe^{3+} repressor, following studies using a pyoverdine-deficient mutant (Fernandez *et al.*, 1988). However, observations with iron-limited cultures of *Ps. aeruginosa* EF2 did not support these conclusions. Also, the silicon anti-foam used did not affect lipase production, unlike that of *Ps. nitroreducens* nov. var. *thermotolerans* (Watanabe *et al.*, 1977), which increased more than two-fold.

The extent of aeration has been suggested to affect fungal (see for example, Giuseppin, 1984; Vermeire *et al.*, 1987), but not bacterial (Lawrence *et al.*, 1967 b) lipase production (however shear forces at the gas/liquid interface have recently been reported to inactivate a *Staphylococcal* lipase [Falk *et al.*, 1991]). Cultures of *Ps. aeruginosa* EF2 grown under low or high aeration rates did not exhibit drastically different lipase activities, when the control of air into the chemostat was erratic, due to problems with the lead/silver galvanic oxygen electrode.

Lipase production by *Ps. aeruginosa* EF2 grown in a Tween 80-limited continuous culture was strongly dependent upon the pH of the growth medium, and to a lesser extent the temperature, at a constant dilution rate. These factors have previously been shown to affect lipase production in batch cultures of other organisms (see for example, Bloquel & Veillet-Poncet, 1984, and Stead, 1986), but their effects on continuous cultures have not previously been reported. As the pH of the growth medium was reduced (from 8.5 to

6.0), lipase activities increased. Initial experiments indicated a pH optimum for lipase activity to be 9.0 (Davidson, 1987). *Ps. aeruginosa* EF2 may therefore have compensated for this decrease in lipase activity in the more acidic growth media by producing more enzyme. Lipase activities, which were measured at a constant pH 9.00, thus appeared to increase. Alternatively, growth of *Ps. aeruginosa* EF2 at these low pH values might have reflected an overall increased ability to produce and secrete lipase.

The use of statistical contour surface analysis enabled the production of lipase by *Ps. aeruginosa* EF2 to be optimised with respect to pH and temperature at a fixed dilution rate. The calculated optima (pH 6.5 and 35.5°C) were very different from those reported by Harris *et al.* (1990) for *Ps. fluorescens* grown in batch cultures (pH 8.07, 25°C). The maximum lipase activity of *Ps. aeruginosa* EF2 grown at these optimum values dilution rate ($0.04h^{-1}$) was nearly five times the maximum activity attained following growth on Tween 80 in batch culture, and at least forty times higher than when grown on glucose (*ie.* 39, 8.3 and <1 LU $[mg\ cells]^{-1}$ respectively). This activity compares favourably with that expressed by *Ps. fluorescens* in fed-batch culture under olive oil limitation (Suzuki *et al.*, 1988).

The determination of the total organic carbon content of supernatant samples from continuous cultures provided an insight into the utilisation of Tween 80 by *Ps. aeruginosa* EF2, and enabled the growth yield on its hydrolysis product, oleic acid, to be calculated. The high lipase and esterase activities produced by *Ps. aeruginosa* EF2 grown under Tween 80 limitation in continuous culture, supported the conclusions drawn from growth in batch and fed-batch cultures, *ie.* that lipase activity was induced by a general carbon/energy limitation and strongly induced by Tween 80. Similar studies have not yet been reported for other organisms.

The repression of lipase production by oleic acid was also demonstrated. This was in direct contrast to lipase production by a low-water tolerant strain of *Ps. aeruginosa* YS7, where extracellular lipase production in a soybean oil fed-batch culture was greatly enhanced when the triglyceride was replaced by lauric acid (Shabtai & Mishne-Daya, 1991). The repression of lipase production by fatty acid remains unreported in the literature. However, Fernandez *et al.* (1990) reported the repression of *Ps. fluorescens* 32A lipase production by arginine and arginine analogues, which was thought to result from the accumulation of catabolic intermediates.

CHAPTER 4.

THE PURIFICATION AND BIOCHEMICAL PROPERTIES OF THE *PSEUDOMONAS AERUGINOSA* EF2 LIPASE.

4.1 *Abstract.*

Lipase was isolated from a continuous culture of *Pseudomonas aeruginosa* EF2 grown under near-optimal conditions for lipase production (Tween 80-limited, dilution rate 0.05 h^{-1}, 37°C, pH 6.5). The enzyme was purified to homogeneity (99.5 % pure from SDS-PAGE analysis) by ultrafiltration of the culture supernatant, followed by anion-exchange and gel-filtration FPLC. The lipase was composed of a single sub-unit protein (M_r 29,000, pI 4.9) which appeared to aggregate variably under non-dissociating conditions. The enzyme was a true lipase (6,606 LU mg $protein^{-1}$, k_{cat} 3191 s^{-1} for the hydrolysis of olive oil) that exhibited some esterase activity towards *p*-nitrophenyl acetate (421 μmol min^{-1} [mg protein]$^{-1}$, k_{cat} 204 s^{-1}) and Tween 80 (415 LU mg $protein^{-1}$, k_{cat} 201 s^{-1}). The enzyme preferentially hydrolysed the 1,3-oleyl residues of radiolabelled triolein, was relatively stable at moderate temperatures (exhibiting a biphasic loss of activity with an initial $t_{1/2}$ of 17.5 min at 60°C) and very stable to freezing and thawing. The presence of a catalytic serine at the active site could not be confirmed, as the enzyme was only weakly inhibited when incubated with the serine-active reagent 3,4-dichloroisocoumarin. Similarly, the chelating agent EDTA did not inhibit esterase activities. The N-terminal amino acid sequence of the *Ps. aeruginosa* EF2 lipase showed a significant homology with those of other *Pseudomonas* lipases.

4.2 *Introduction.*

Pseudomonas aeruginosa EF2 was originally isolated under strong selective pressures designed to obtain a bacterium capable of producing a detergent-stable lipase. The physiological regulation of lipase production by this organism was subsequently investigated, and growth conditions for optimum lipase production in a Tween 80-limited continuous culture were determined (Chapter 3).

The biochemical study of microbial and mammalian lipases has been of both academic and industrial interest. The unique problems associated with studying enzyme catalysis in heterogeneous systems have generated considerable interest in the kinetics of lipolysis. These have involved the successful development and use of mono-layer techniques to investigate substrate and product relationships (see, for example, Brockman, 1984), whilst substrate binding and the subsequent catalysis have been investigated using group-specific inhibitors (see, for example, Lóokene and Sikk, 1991).

Industry as a whole has maintained an interest in lipases for many years (Harwood, 1989). The food industry has initiated much research, aimed at either improving the production of various foods (*eg.* during maturation or flavour synthesis) or preventing the spoilage of stored foods. The latter has included the dairy industry, where the presence of psychrotrophic *Pseudomonas* species in refrigerated milk products has been especially problematic. The purification and subsequent characterisation of the associated lipases showed that many were thermostable and therefore not destroyed by existing pasteurisation techniques (Stead, 1986).

The incorporation of lipases into detergent formulations has resulted in the extensive characterisation of novel microbial strains, particularly with respect to the activity of the associated enzymes at different

temperatures and pH values, in the presence of various surfactants and metal ion chelators (see, for example, Andree *et al.*, 1980; Kawase *et al.*, 1985; Tatara *et al.*, 1985).

The ability of lipases to catalyse various transformation reactions under non-aqueous conditions is the subject of considerable current research. In particular, the stereo-specific nature of many lipases (see, for example, Kloosterman *et al.*, 1991; Ransac *et al.*, 1991) has far-reaching implications for the speciality chemical and drug industries.

The recent structural determinations of the human pancreatic, *Rhizormucor miehei* and *Geotrichum candidum* lipases (Section 1.3) have enabled the construction of precise active site models which have led to a much greater understanding of the processes involved during the lipolytic reaction (Blow, 1991; Brzozowski *et al.*, 1991). This has also coincided with the elucidation of the primary structures of many other lipases, made possible by nucleotide sequencing of the structural genes. This has shown that the sequence of amino acids surrounding the active site serine residue is highly conserved (see, for example, Kordel *et al.*, 1991 a), suggesting they play a crucial role. (It is interesting to note that the structure of human pancreatic lipase was initially determined in order to elucidate the binding properties of the irreversible inhibitor tetrahydrolipstatin, which has a potential commercial application within developed countries as an anti-obesity drug; Hadváry *et al.*, 1991).

The purification and biochemical properties of many microbial lipases have been reported (for a review see Antonian, 1988). Some procedures employed the use of novel techniques, such as synthetic polymers (Cernia *et al.*, 1991), reversed micelles (Aires-Barros & Cabral, 1991) and capillar membranes (Sztajer & Bryjak, 1989), which exploit the unique properties of

lipases. Many fungi have been shown to produce multiple forms of lipases, with slightly different characteristics, which are either encoded by separate genes or differ in the extent of post-translational modifications (commonly the extent of glycosylation; Section 5.2). In contrast, nearly all bacteria produce a single lipase which is often active over a wide range of pH and temperature values.

This chapter describes the purification of the *Ps. aeruginosa* EF2 lipase obtained under near-optimum conditions. An evaluation of the properties of any enzyme is imperative if it is to be considered for commercial application. Some of the biochemical properties of the purified lipase were therefore determined and are discussed in detail with reference to other *Pseudomonas* lipases, much of which stems from work carried out at the same time as the work described in this thesis.

4.3 *Purification of lipase.*

Lipase was recovered from a continuous culture supernatant of *Ps. aeruginosa* EF2 grown Tween 80-limited under near-optimum conditions for extracellular lipase production (37°C, pH 6.5, D = 0.05 h^{-1}). This culture was used as a source of lipase in preference to a stationary phase batch culture, where the effects of cell lysis would be greater. Lipase was purified 31-fold by ultra-filtration, followed by anion-exchange (Mono-Q; Fig. 4.1) and gel-filtration (Superose; Fig. 4.2) FPLC (Table 4.1). This indicated that the concentration of lipase in the culture supernatant was approximately 2 μg ml^{-1} (equivalent to approximately 3% of the total supernatant protein). This was, however, variable as a value as low as 5 (equivalent to 20% of the culture supernatant protein) was obtained for one purification and probably reflected variations in cell lysis between the cultures.

The lipase partly co-eluted off the Superose column with a slightly larger protein, thus reducing the yield of pure enzyme obtained (Fig. 4.2). To overcome this, the final purification step was attempted using hydrophobic-interaction (Phenyl Superose) FPLC, as lipases are generally thought to have a relatively hydrophobic surface. No lipase activity was recovered at all, even upon over-night elution in distilled water. The lipase may have bound irreversibly to the column, but evidence within the department suggested that the column was defective.

The analysis of purified lipase by SDS-PAGE (Fig. 4.3) was troublesome. Although plenty of purified lipase was available in terms of activity, the protein concentration was extremely low (towards the minimum limits of detection), and it was necessary to reduce the volume of the enzyme by dialysing against PEG before the sample could be loaded on to the gel. Analysis of elution fractions off both the Mono-Q and Superose columns by

Figure 4.1. *Lipase elution profile off the Mono Q anion-exchange column during FPLC.*

Concentrated supernatant from a culture of *Ps. aeruginosa* EF2 grown in Tween 80-limited continuous culture was applied to the column and eluted in 20 mM Tris-HCl in a gradient of NaCl (0-1 M) at a flow rate of 3 ml min^{-1}, as described in Methods. Fractions (3 ml) were collected and assayed for lipase activity.
Protein concentration (A_{280}), shown as a thin black line; NaCl gradient, shown as a thick black line; lipase activity, shown as a hatched line.

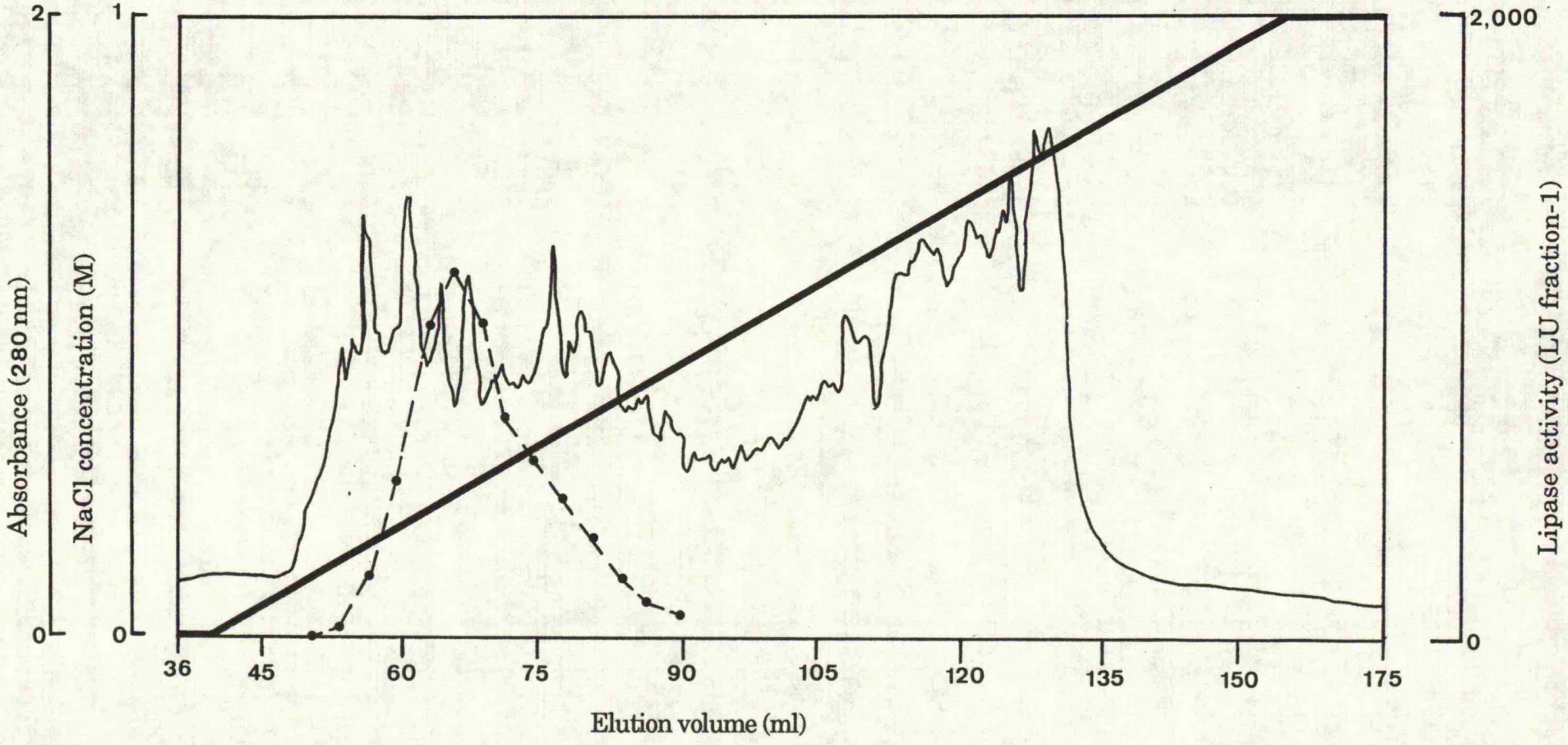


Figure 4.2. *Lipase elution profile off the Superose gel filtration column during FPLC.*

Pooled, concentrated Mono Q fractions were applied to the column and eluted in 20 mM Tris-HCl buffer at a flow rate of 0.25 ml min^{-1}, as described in Methods. Fractions (1 ml) were assayed for lipase activity.
Protein concentration (A_{280}), shown as a thin black line; lipase activity, shown as a hatched line.

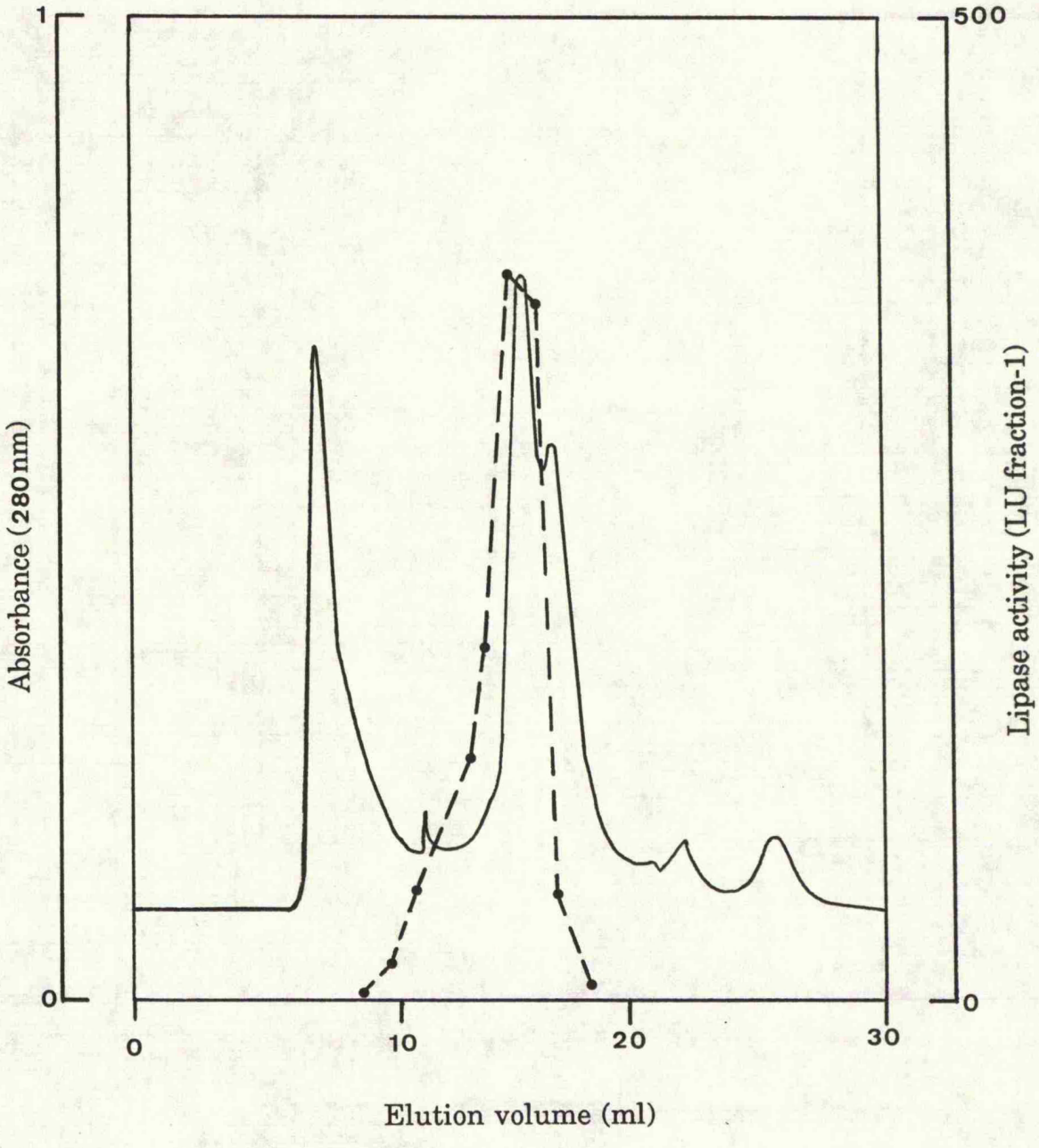


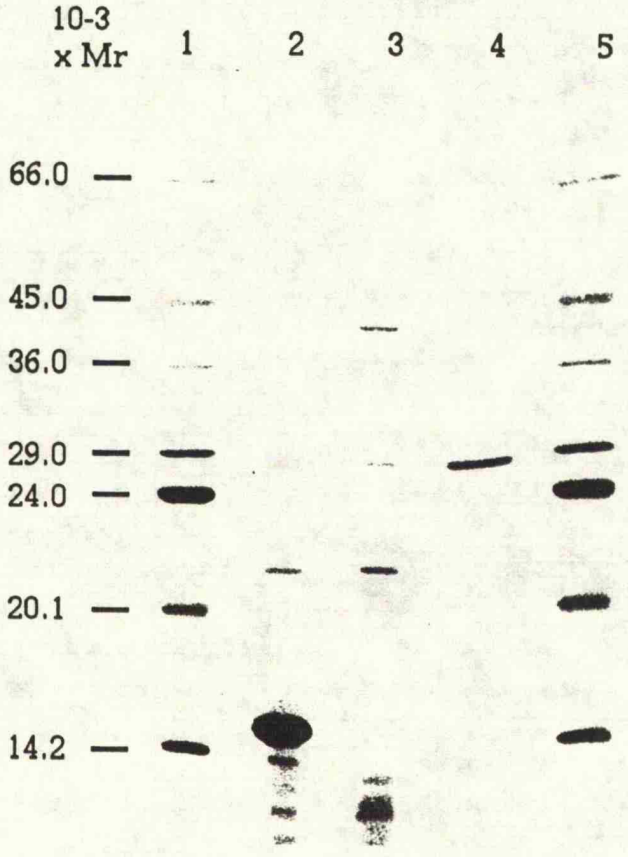
Table 4.1. *Purification of extracellular lipase from* Ps. aeruginosa *EF2.*

Lipase was purified from the culture supernatant of a Tween 80-limited continuous culture of *Ps. aeruginosa* EF2 (D 0.05 h^{-1}, pH 6.5, 37°C). The supernatant was concentrated by ultrafiltration and the lipase was subsequently purified using anion-exchange (Mono Q) and gel-filtration (Superose) FPLC, as described in Methods.

Fraction	Protein concentration	Total protein	Specific activity	Purification	Total activity	Yield
	(mg ml^{-1})	(mg)	(LU mg^{-1})		(LU)	(%)
Supernatant	0.07	48.8	213	1.0	10,406	100
Concentrated supernatant	1.43	20.0	468	2.2	9,377	90
Mono Q	1.75	5.4	1,226	5.7	6,650	64
Superose	0.02	0.28	6,606	31.0	1,853	18

Figure 4.3 *SDS-PAGE showing the purification of lipase from a culture supernatant of* Ps. aeruginosa *EF2 grown under Tween 80-limitation.*

Proteins were separated using SDS-PAGE and stained with Kenacid blue as described in Methods. Track 1, M_r standards; 2, concentrated culture supernatant; 3, bulked Mono-Q fractions; 4, bulked Superose fractions; 5, M_r standards.



SDS-PAGE indicated that the lipase was composed of a protein with a sub-unit M_r of approximately 29,000. Scanning laser densitometry showed that the lipase was 5.9, 12.7 and 99.5 % pure in the concentrated supernatant, Mono Q and Superose fractions respectively (Fig. 4.4).

The lipase activity of the purified enzyme from *Ps. aeruginosa* EF2 was 6,606 LU mg^{-1}, which was equivalent (assuming a minimum native M_r of 29,000) to a k_{cat} of 3191 s^{-1}. Similarly, the esterase activities were 421 μmol min^{-1}(mg protein)$^{-1}$ (*p*-nitrophenyl acetate) and 415 LU mg $protein^{-1}$ (Tween 80), which was equivalent to a k_{cat} of 204 s^{-1} and 201 s^{-1} respectively. Throughout the purification procedure, the ratio of lipase to esterase activities remained essentially constant. The average ratios were:

lipase	:	esterase (*p*-nitrophenyl acetate)	:	esterase (Tween 80)
8.2	:	0.9	:	1.0

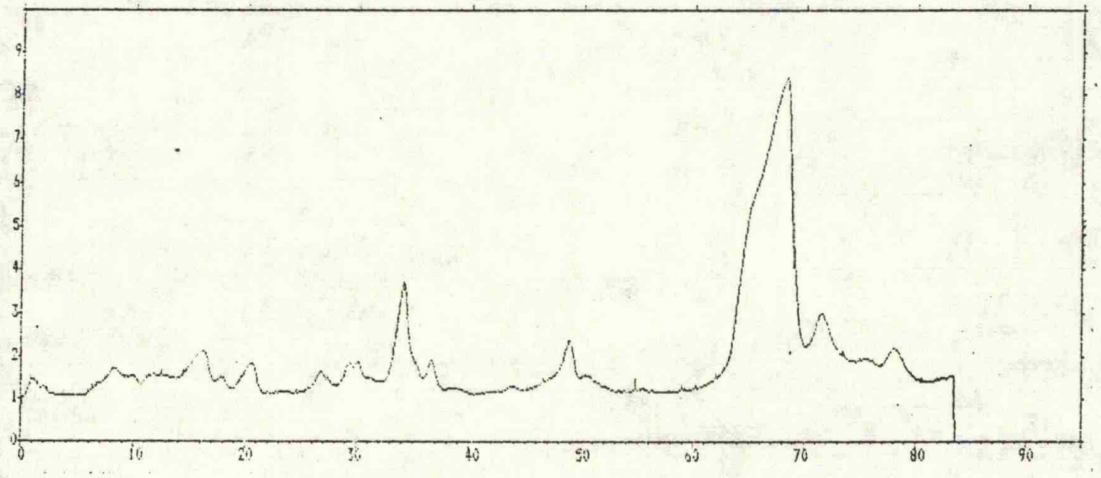
This was in accordance with observations made during the growth studies, and indicated catalysis by a single enzyme. As the lipase activity was approximately eight-times the esterase activity, this confirmed that the enzyme was a true lipase which also exhibited some esterase activity (*ie.* it showed a greater activity towards insoluble than soluble substrates).

4.4 *Kinetic properties and assay of lipase.*

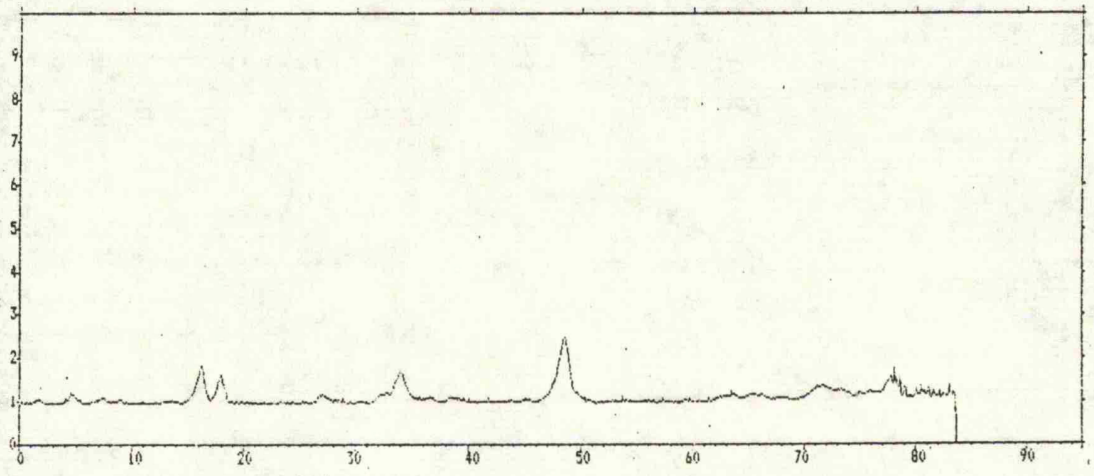
The optimum conditions for assaying lipase activity were initially determined using a commercial solution of *Pseudomonas* lipase (Sigma), as described previously (Section 2.9.1). Purified *Ps. aeruginosa* EF2 lipase was assayed over a range of substrate, NaCl and $CaCl_2$ concentrations, and the conditions previously determined were shown to be suitable (10% [v/v] olive oil emulsion, 0.25 M NaCl, 0.05 M $CaCl_2$). Lipase activities more than

Figure 4.4. *Scanning laser densitometry of lipase purification fractions folowing SDS-PAGE.*
Samples were electrophoresed in a dissociating SDS polyacrylamide gel and stained with Kenacid Blue (Figure 4.3). The purity of the lipase protein (M_r 29,000) was determined by scanning laser densitometry, as described in Methods.

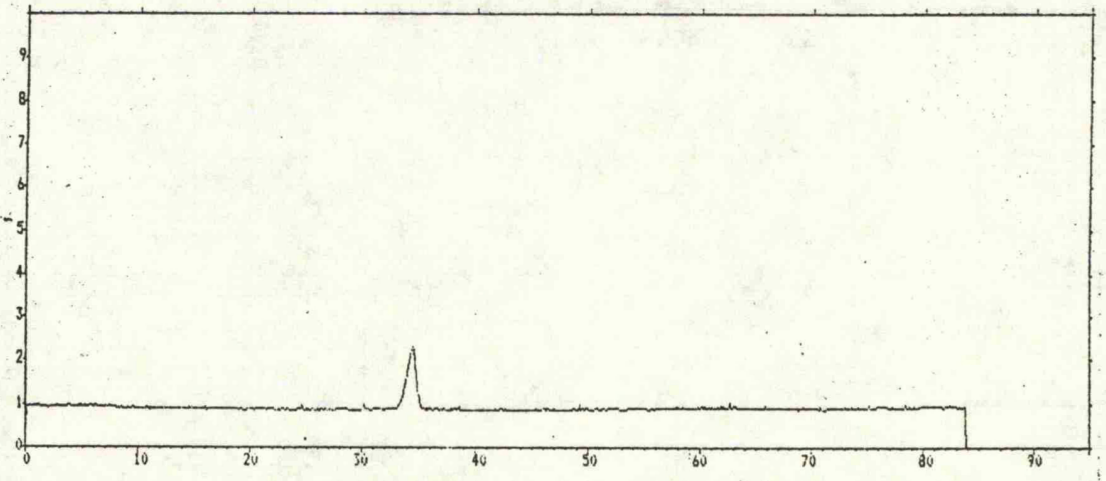
i. Concentrated culture supernatant (5.9 % pure).



ii. Pooled Mono Q fractions (12.7 % pure).



iii. Pooled Superose fractions (99.5 % pure).



Relative intensity

Relative distance from top of gel.

doubled in the presence of NaCl and $CaCl_2$ (0.25 M and 0.05 M), however, esterase activities (Tween 80 hydrolysis) were shown not to be dependent upon $CaCl_2$, as no increase in activity was detected upon the addition of up to 0.2 M $CaCl_2$. This therefore suggested that it was a soley interfacial effect on the insoluble olive oil substrate.

4.5 *Physico-chemical properties of purified lipase.*

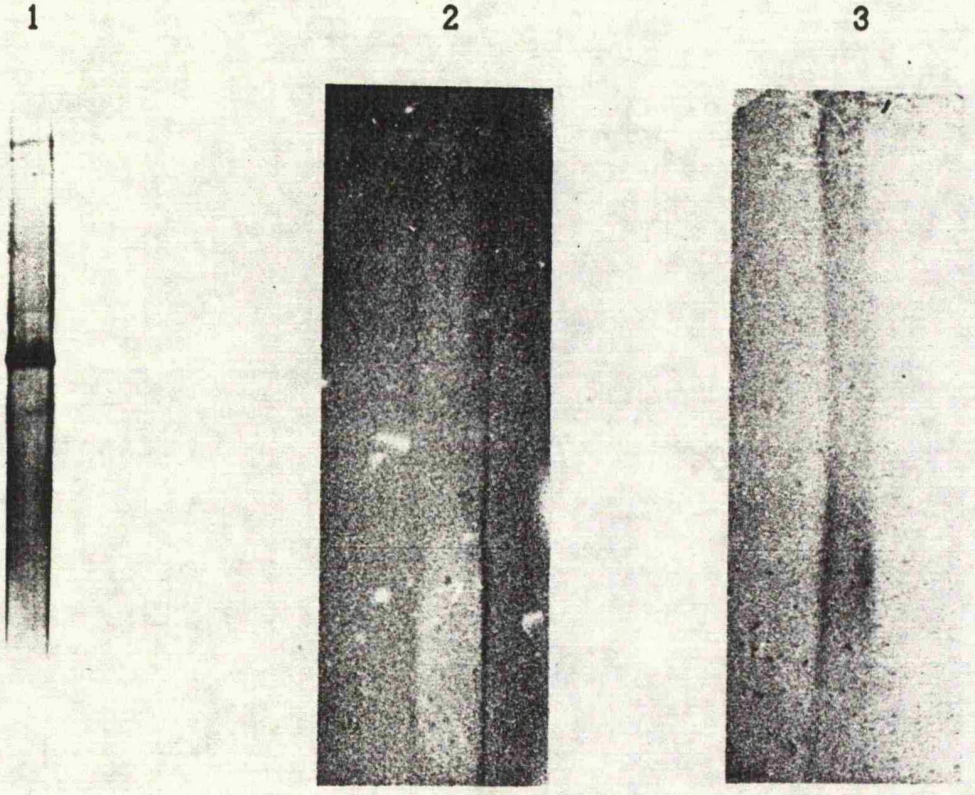
4.5.1 *Aggregation of the native enzyme.*

Non-dissociating (native) PAGE of concentrated supernatant samples was attempted using a high pH buffer system (pH 9.5), as this was near the expected pH optimum of the lipase. Also, the reported pI values of other lipases (*eg.* 5.8 for *Ps. aeruginosa* PAC1R, Stuer *et al.*, 1986; 7.0, for *Ps. fragi* 22,39B, Nishio *et al.*, 1986) suggested that the enzyme should be in its fully anionic form at this pH and hence migrate readily towards the anode. Following electrophoresis and subsequent silver staining, protein was observed as a streak through the gel, with the majority remaining in the stacking gel. These were both shown to correspond to the lipase when stained for activity using Tween 80 and olive oil agarose overlays (Fig. 4.5).

Native lipase thus appeared to be present in a high molecular weight form, possibly as hydrophobic aggregates. To overcome this, native PAGE was performed under mildly dissociating conditions. An SDS polyacrylamide gel (12 % acrylamide) was run according to the method of Von Tigerstrom & Stelmashuk (1989), where the supernatant was added to native sample buffer (without heating) containing 0.01 %, 0.1 % and 1 % SDS immediately prior to loading. Following electrophoresis, the gel was incubated in a Triton/Tris-HCl or EDTA/Tris-HCl buffer for at least 1 h at

Figure 4.5. *Native PAGE of lipase.*

Concentrated culture supernatant was electrophoresed in a discontinuous 7.5 % polyacrylamide native gel using a high pH buffer system. Tracks were stained for protein and activity using agarose overlays, as described in Methods. Track 1, silver stain; track 2, activity stain-olive oil hydrolysis; track 3, activity stain-Tween 80/CaCl-2.



37°C, and then stained for protein and activity. No protein remained in the stacking gel, but lipase and esterase activities could not be detected. This indicated that the *Ps. aeruginosa* EF2 lipase was extremely sensitive to the anionic detergent SDS. Attempts to electrophorese the lipase in a 6 M urea gel (to break any hydrogen bonds) and a 7.5 % acrylamide native gel containing 0.1 M NaCl (to break any hydrophobic bonds), also failed to remove the protein from the stacking gel. An attempt to determine whether retention within the stacking gel was due to association of the *Ps. aeruginosa* EF2 lipase with lipopolysaccharide, following the method described by Stuer *et al.* (1986), was unsuccessful.

Purified *Ps. aeruginosa* EF2 lipase was passed through a pre-calibrated Superose column to determine the M_r of the native enzyme. This was essentially the same as the final step performed in the purification procedure, except that duplicate samples were eluted in Tris-HCl buffer, pH 7.5 containing either 25 mM or 100 mM NaCl. Any variation in the elution patterns between the two samples would have indicated that the enzyme molecules associated by hydrophobic interactions. In both cases, the protein eluted at volumes corresponding to approximate native M_r values of both 49,000 and 200,000. Analysis of representative elution fractions by SDS-PAGE indicated the presence of a protein of M_r 29,000, thought to be the lipase sub-unit. These results were somewhat confusing, especially as the native M_r was not a multiple of the sub-unit M_r. It was therefore concluded, that under non-dissociating conditions, *Ps. aeruginosa* EF2 lipase aggregated to varying extents, although there was no direct evidence to suggest the nature of the interactions between enzyme molecules.

4.5.2 *Isoelectric point.*

Isoelectric focussing of purified *Ps. aeruginosa* EF2 lipase indicated a pI value of 4.95. Extensive smearing of the protein occurred from the site of application of the sample through the polyacrylamide matrix, presumably due to molecular sieving of the higher M_r aggregates (Fig. 4.6). The pI value also confirmed the earlier expectation, that the native enzyme would be in its anionic state at pH 9.5 and should have migrated readily in the native polyacrylamide gel described previously (Section 4.5.1).

4.5.3 *Temperature and pH optima.*

The effect of temperature and pH on the lipase and esterase (Tween 80 hydrolysis) activities of the purified enzyme were determined. Maximum activities were obtained at 50°C (Figs. 4.7 & 4.8), and at pH 8.5 and 9.0, respectively (Figs. 4.9 & 4.10).

4.5.4 *Thermostability.*

Purified lipase was stable at 20°C and 30°C, whilst 91% of the initial activity remained after 24 h at 37°C. At 45°C and above, a bi-phasic loss of activity occurred (Figs. 4.11 & 4.12). The time taken for the initial lipase activity to decrease by 50% ($t_{1/2}$) and 90% (the decimal reduction time, D; Brock *et al.*, 1984) measured by extrapolation of the initial inactivation phase, are summarised in Table 4.2 and Fig. 4.13, respectively. A Z-value (defined as the change in temperature required to decrease D by an order of magnitude; Brock *et al.*, 1984) was calculated to be 9.75°C.

The lipase was very stable to freezing and thawing, retaining 86% of its activity after six freeze-thaw cycles (Fig. 4.14).

Figure 4.6. *Isoelectric focussing of purified lipase.*

Purified lipase was applied to the cathode and focussed in a polyacrylamide gel between pH 4.0 and pH 6.5, as described in Methods. Tracks 1 & 3, pI markers; track 2, purified lipase.

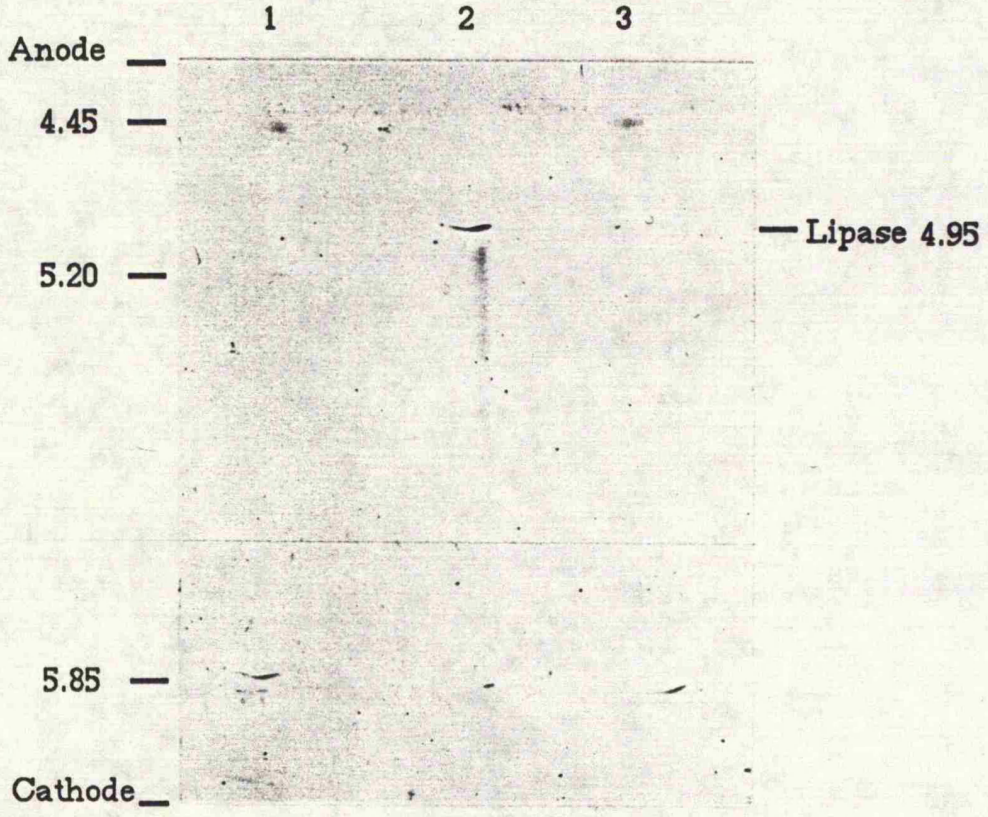


Figure 4.7. *Variation in lipase activity at different temperatures.*

Purified enzyme was assayed for lipase activity (pH 9.00) at various temperatures, as described in Methods. Results are expressed as a percentage of the maximum.

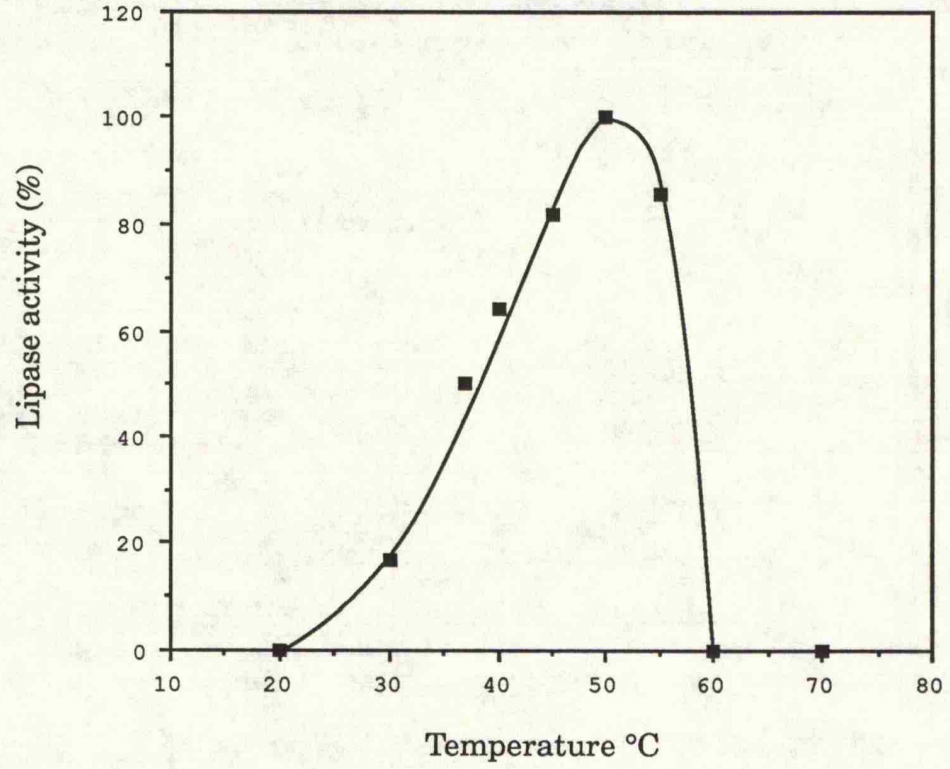


Figure 4.8. *Variation in esterase activity at different temperatures.*

Purified enzyme was assayed for esterase activity (Tween 80; pH 9.00) at various temperatures, as described in Methods. Results are expressed as a percentage of the maximum.

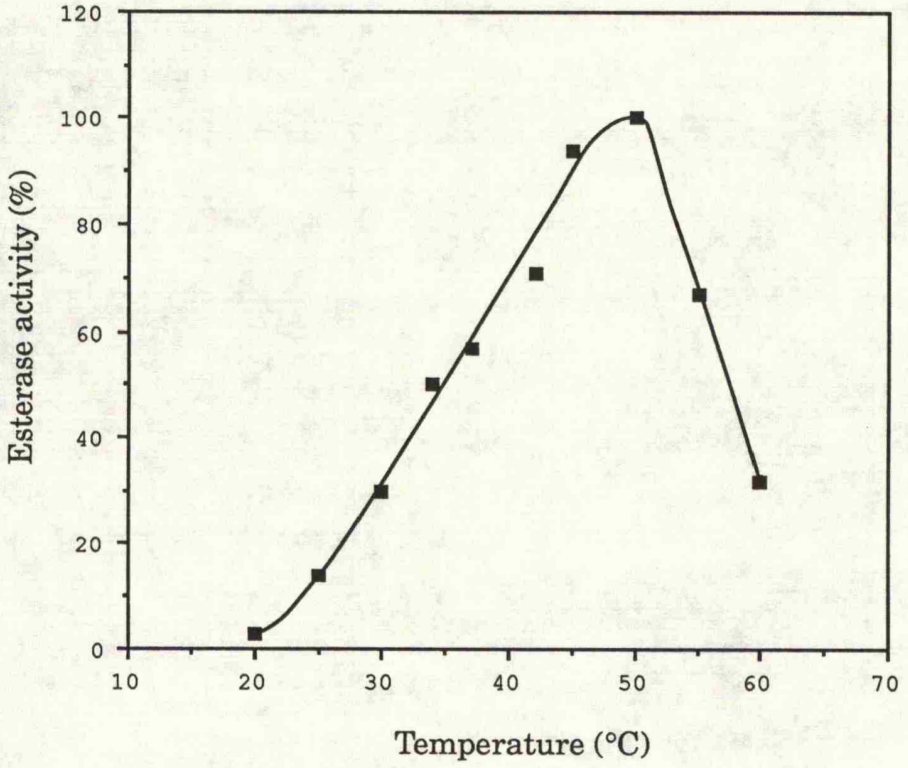


Figure 4.9. *Variation in lipase activity at different pH values.*

Purified enzyme was assayed for lipase activity (37°C) at various pH values, as described in Methods. Results are expresssed as a percentage of the maximum.

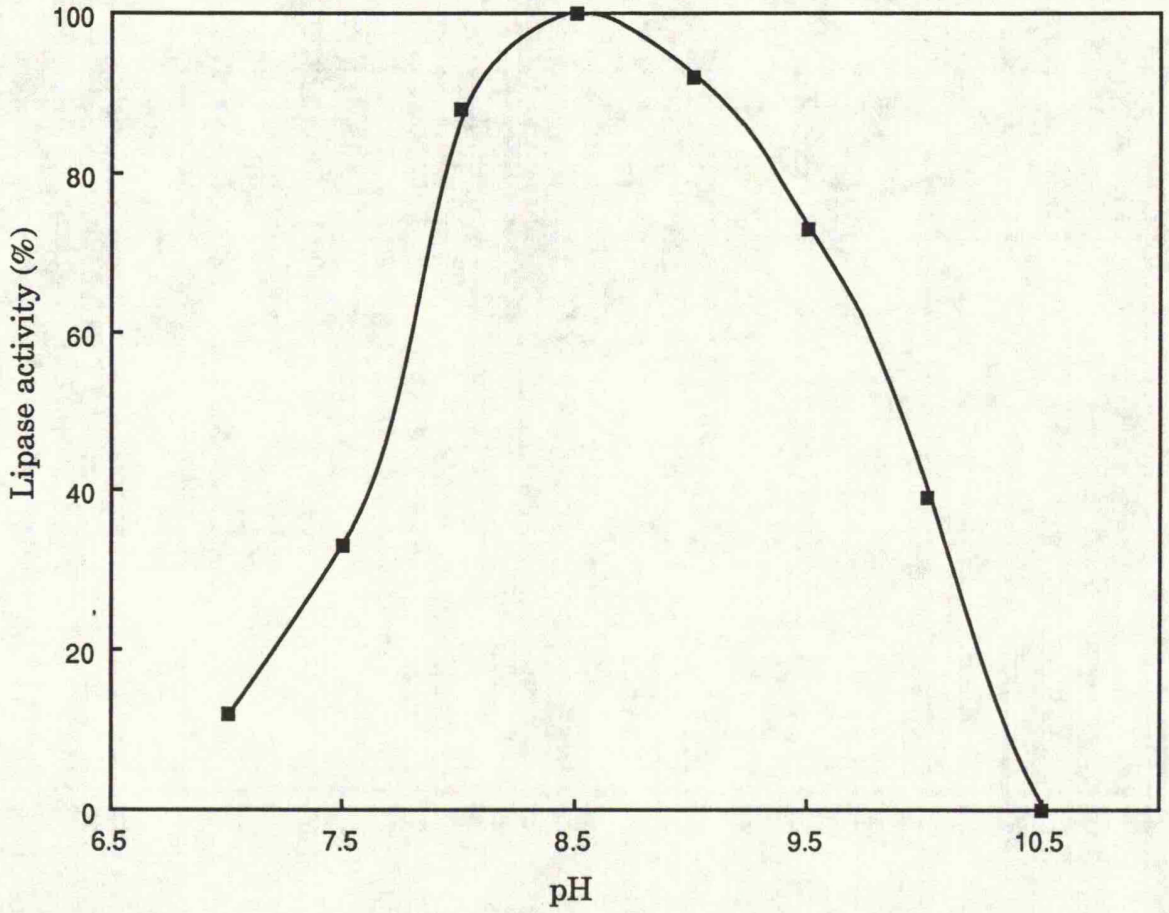


Figure 4.10. *Variation in esterase activity at different values of pH.*

Purified enzyme was assayed for esterase activity (Tween 80; 37°C), as described in Methods. Activities are expressed as a percentage of the maximum.

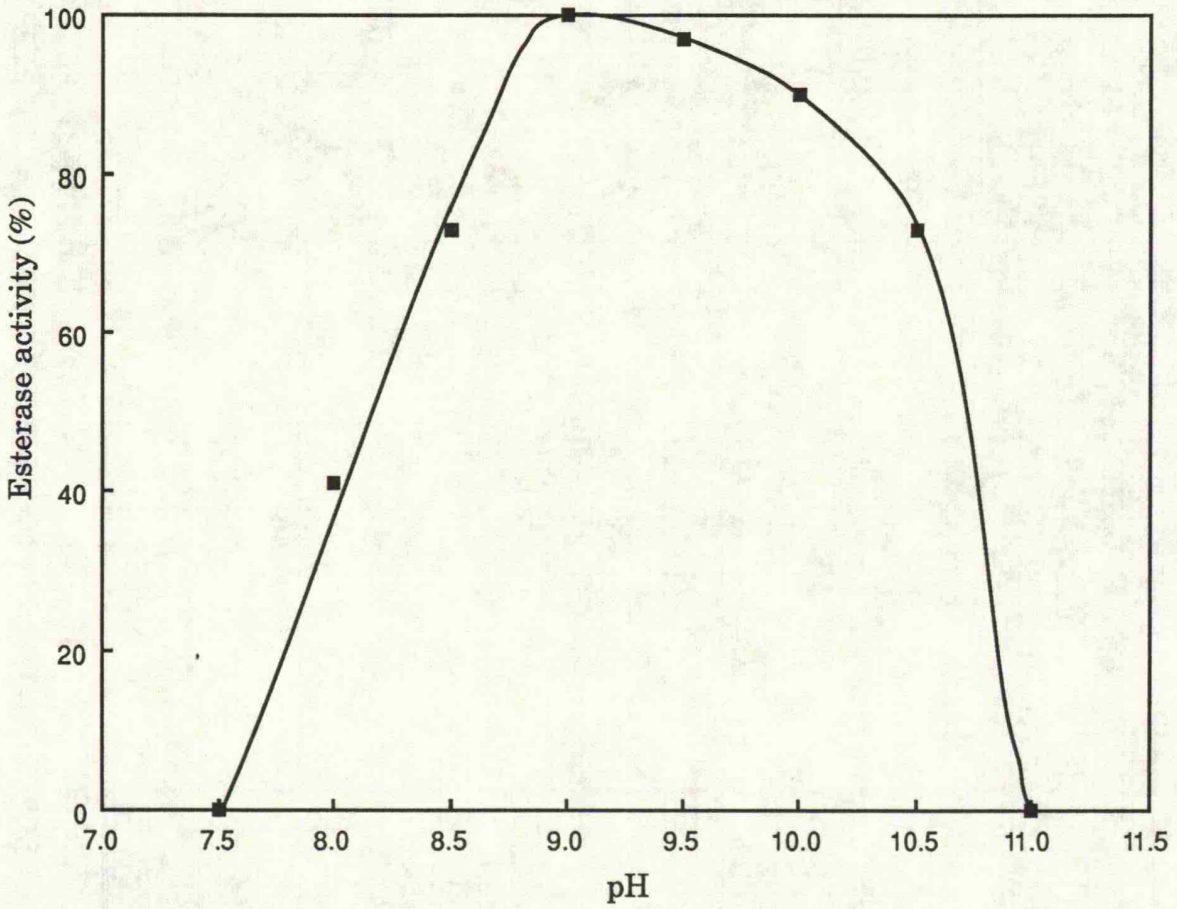


Figure 4.11. *Thermostability of lipase at 45°C and 55°C.*

Purified enzyme was incubated at various temperatures for up to 7h. Samples were removed and assayed for lipase activity, as described in Methods. Activities are expressed as a percentage of the maximum.
□, 45°C; ■, 55°C.

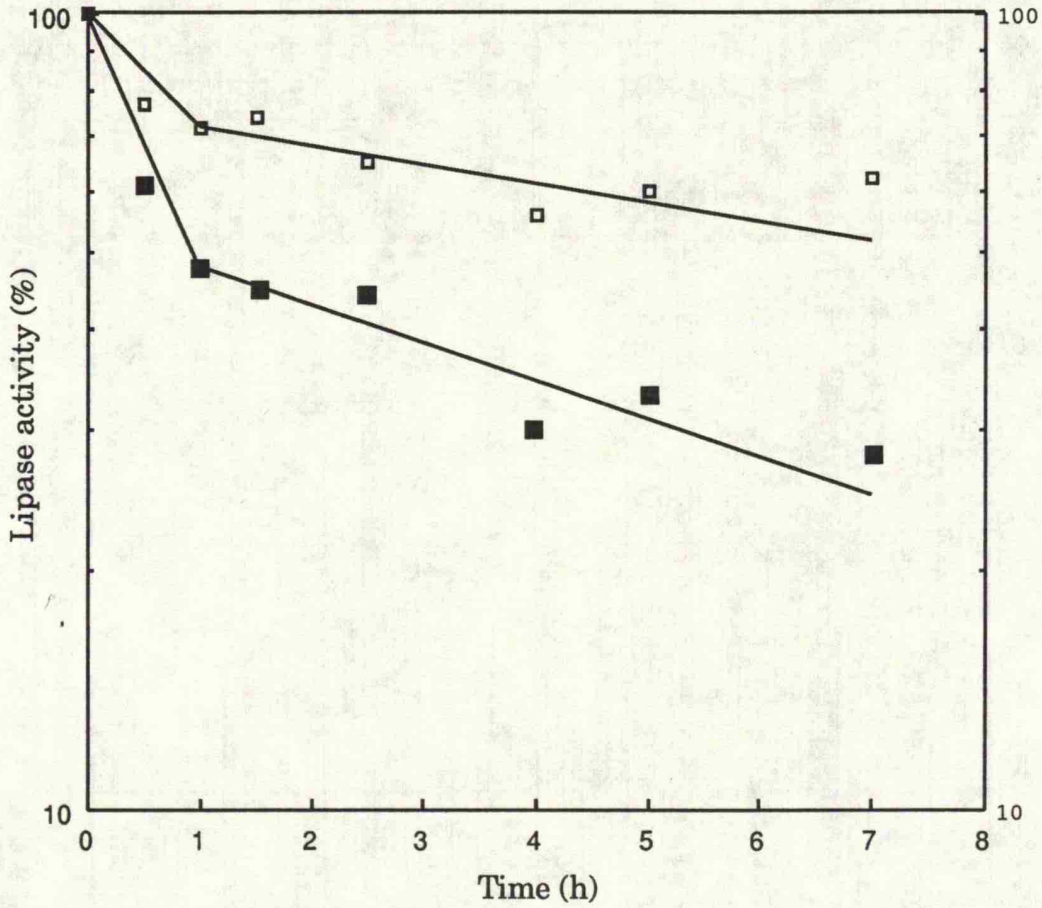


Figure 4.12. *Thermostability of lipase at 60°C.*

Purified enzyme was incubated for up to 1.5h. Samples were removed and assayed for lipase activity, as described in Methods. Activities are expressed as a percentage of the maximum.

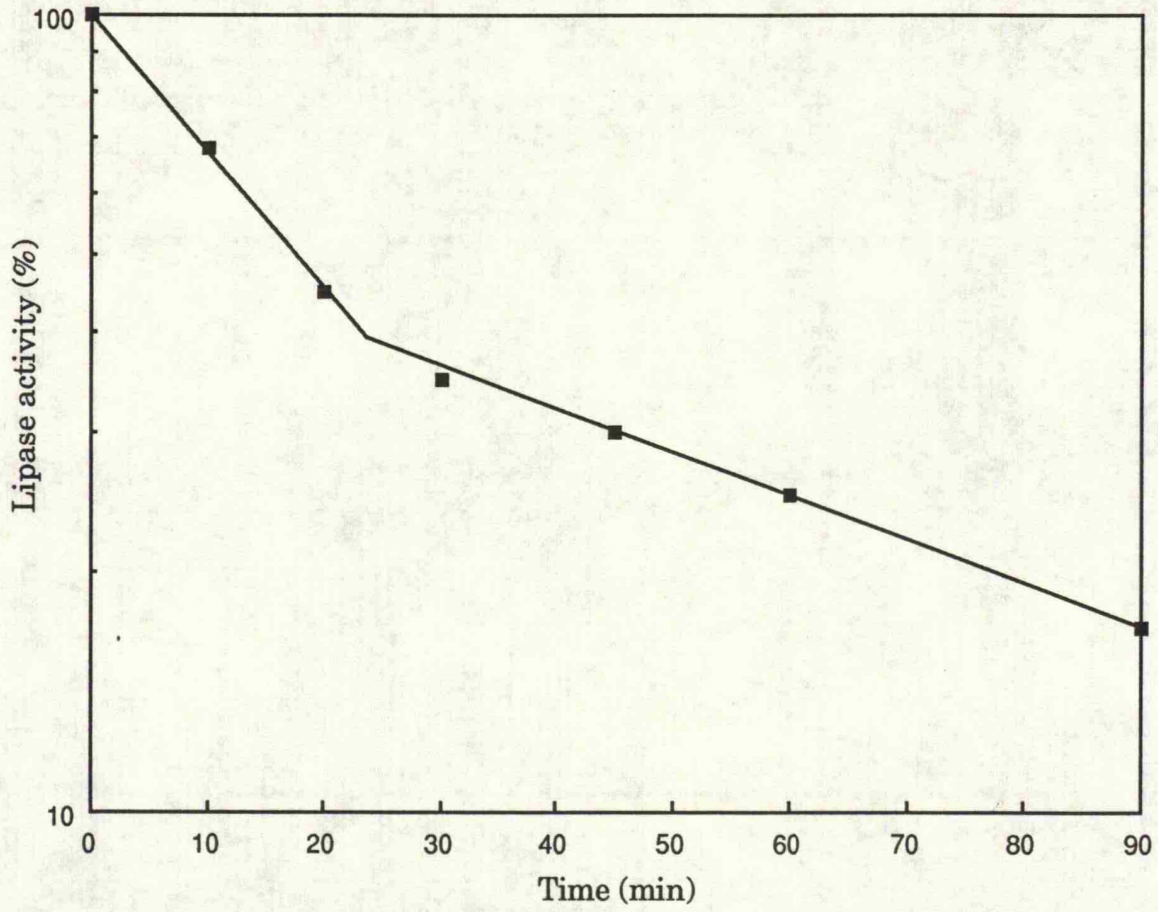


Table 4.2. *Thermostability of purified lipase.*

Purified lipase (approximately 20 μg ml^{-1} in 20 mM Tris-HCl buffer, pH 7.5) was incubated at different temperatures. Samples were removed at various time intervals and assayed for lipase activity, as described in Methods. The time taken for the initial activity to decrease by 50 % was calculated by extrapolation of the initial inactivation phase on a semi-log plot of activity versus time (see, for example, Figs. 4.11 and 4.12).

Temperature (°C)	$t_{1/2}$ (min)
45	108
55	54
60	17.5
65	5.4
70	2.1
80	0.17

Figure 4.13. *Variation in the decimal reduction time of purified lipase with temperature.*

The thermostability of purified lipase was determined at various temperatures and the decimal reduction time (D; the time taken for the enzyme to lose 90 % of its initial activity, measured by extrapolation of the initial rapid phase of inactivation) calculated. The slope of the graph enables the Z value to be calculated.

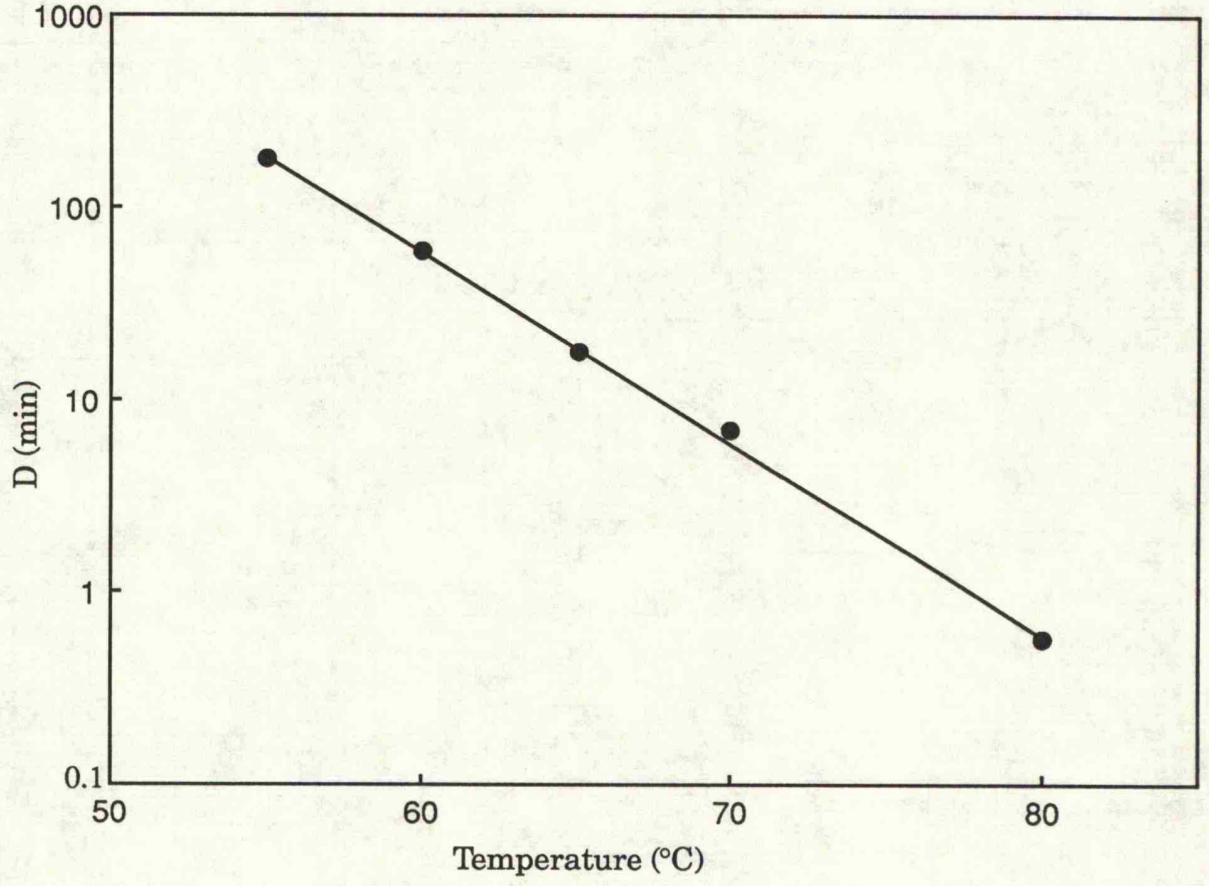
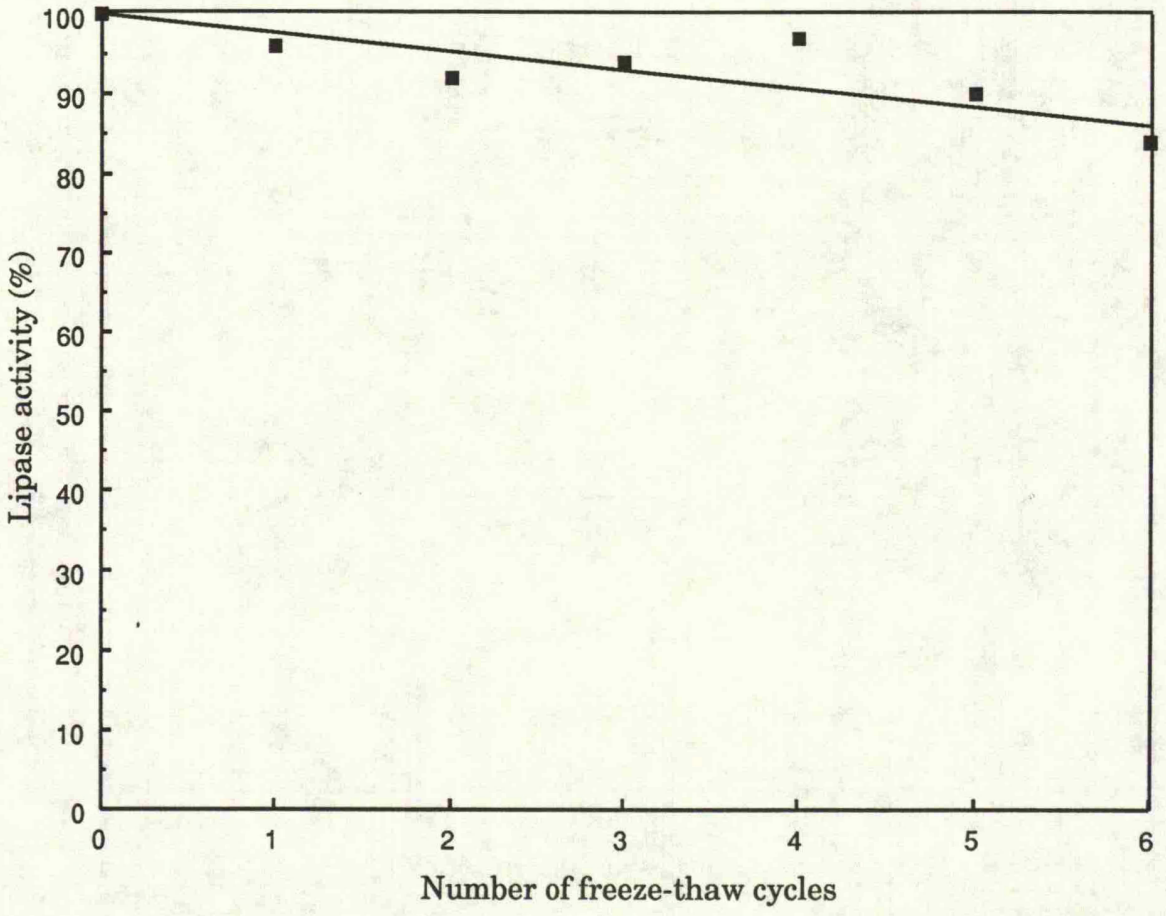


Figure 4.14. *Stability of lipase to freezing and thawing.*

Purified enzyme was assayed for lipase activity (37°C, pH 9.00), as described in Methods. The solution was placed at -20°C until frozen, then allowed to fully thaw at room temperature. Lipase activity was then measured again, and the process repeated.



4.5.5 *Sensitivity towards inhibitors.*

The divalent metal-chelating agent EDTA (1 mM) caused no significant inhibition of esterase (*p*-nitrophenyl acetate and Tween 80) activity following pre-incubation with the enzyme for 30 min at 30°C.

Lipase activity was weakly inactivated in a time-dependent, bi-phasic manner when pre-incubated with the serine-protease inhibitor, 3,4-dichloroisocoumarin (DCI; 100 μM). An apparent inhibition constant (k_{app}; [0.693/ time taken, in seconds, for lipase activity to decrease by 50 %] per molar concentration of inhibitor) of 10 $M^{-1}s^{-1}$ was calculated from the initial fast rate of inactivation (25% inhibition after 5 min incubation). Lipase activity was rarely inhibited by more than 50% during the subsequent slow inactivation phase. DCI also had a similar effect on esterase (Tween 80) activity.

4.5.6 *Regiospecificity.*

The positional specificity of the *Ps. aeruginosa* EF2 lipase towards triolein was determined by resolving the hydrolysis products using thin-layer chromatography. The amount of 1,2 (2,3)-diolein produced was always greater than the amount of the 1,3-isomer, indicating that the lipase showed a greater specificity towards the 1,3-oleyl residues of the triglyceride molecule (Fig. 4.15). The reaction was quantified by measuring the release of ^{14}C-labelled dioleins, monooleins and oleic acid from glycerol tri [1-^{14}C] oleate as a function of time (Fig. 4.16). Just over half the radioactivity in the triolein was released over the 7 h period of the experiment, the majority of which appeared in 1,2 (2,3)-diolein (19%) and oleic acid (23%), whilst only 6% was associated with 1,3-diolein, and 4% with monoolein. No detectable hydrolysis of triolein was observed in the absence of any lipase. The 1,3-specific nature of the *Ps. aeruginosa* EF2 lipase was confirmed by showing

Figure 4.15. *Hydrolysis of triolein by purified lipase.*

Lipase was incubated with triolein at 37°C, pH 7.5 for up to 48 h. Samples were removed, the products extracted and applied to a silica gel chromatography plate, as described in Methods.

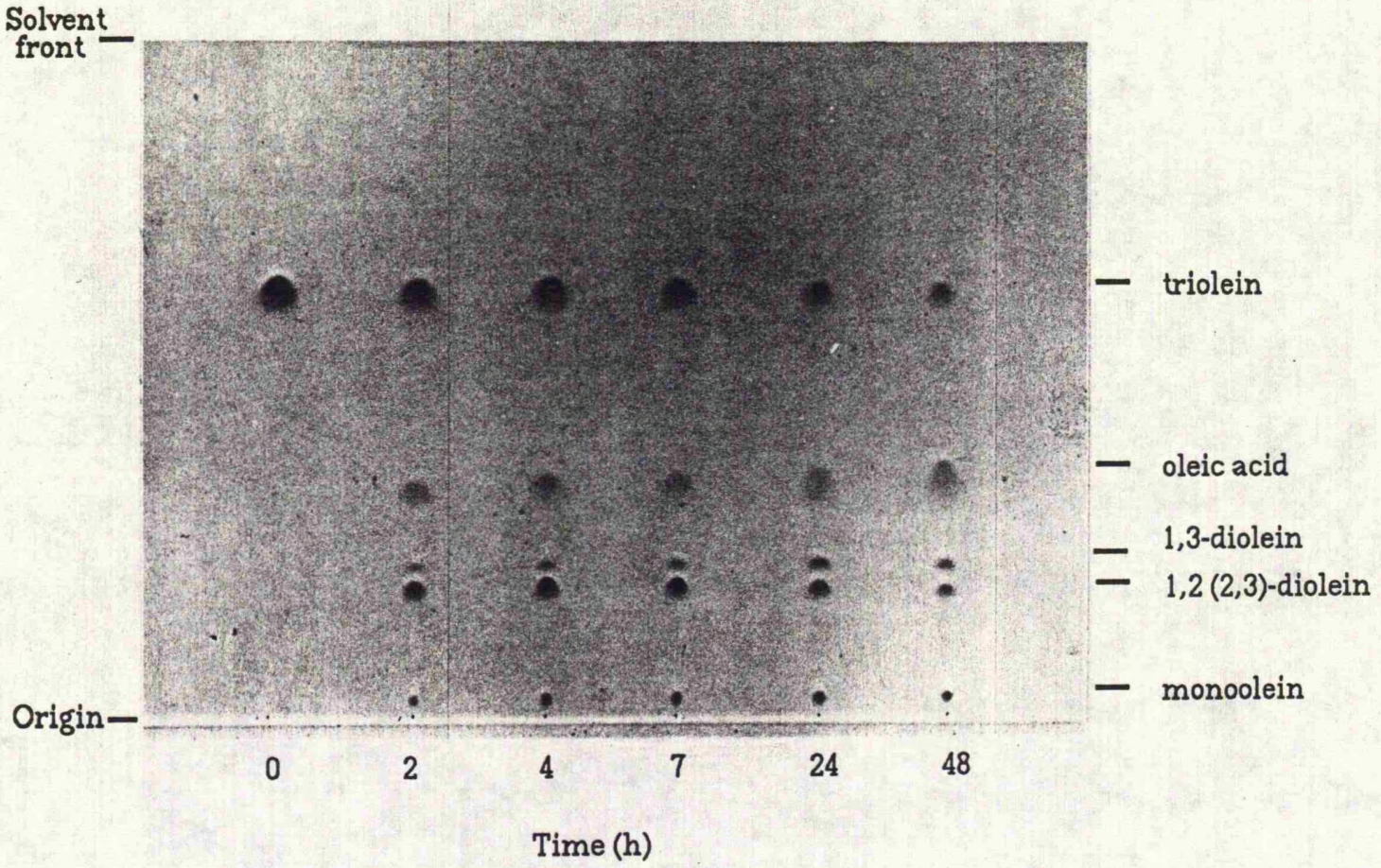
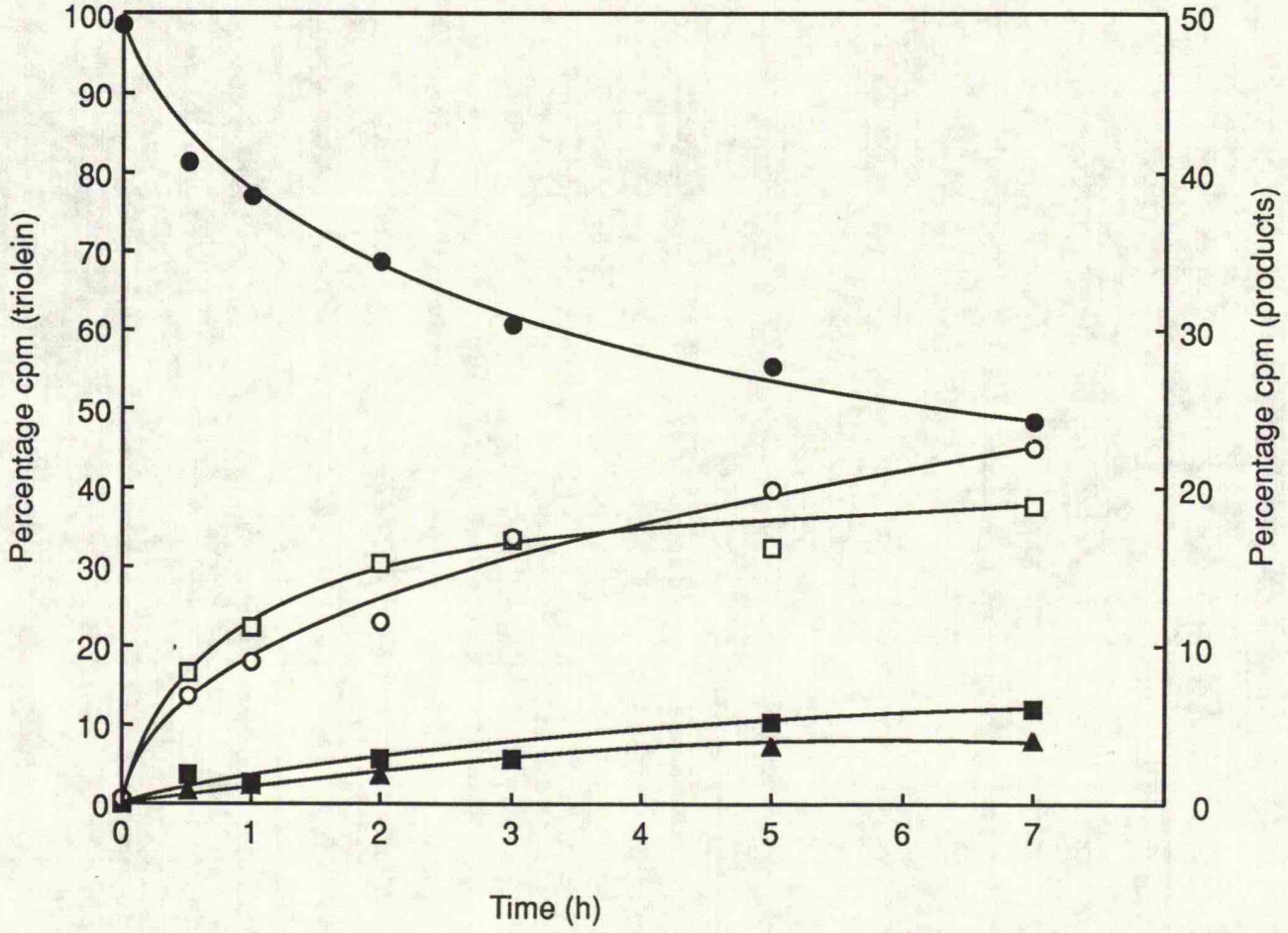


Figure 4.16. *Hydrolysis of [^{14}C] triolein by purified lipase.*

Lipase was incubated with [^{14}C]triolein (glycerol tri[1-^{14}C]oleate) at 37°C, pH 7.5 for up to 7h. Samples were removed, the products extracted and applied to a silica gel chromatography plate. The concentrations of radiolabelled triolein, dioleins, monooleins and oleic acid were determined by scintillation counting, as described in Methods.
●, triolein; □, 1,2 (2,3)-diolein; ■, 1,3-diolein; ▲, monooleins; ○, oleic acid.



that it discriminated between a mixture of unlabelled dioleins (85% 1,3-diolein, 15% 1,2 (2,3)-diolein), hydrolysing the 1,3-diolein much faster than the 1,2 (2,3)-diolein (Fig. 4.17).

4.5.7 *Amino-terminal amino acid sequence.*

The N-terminal amino acid sequence of the purified lipase was determined. Two molecular species were present, indicating exopeptidase degradation and/or non-specific proteolysis:

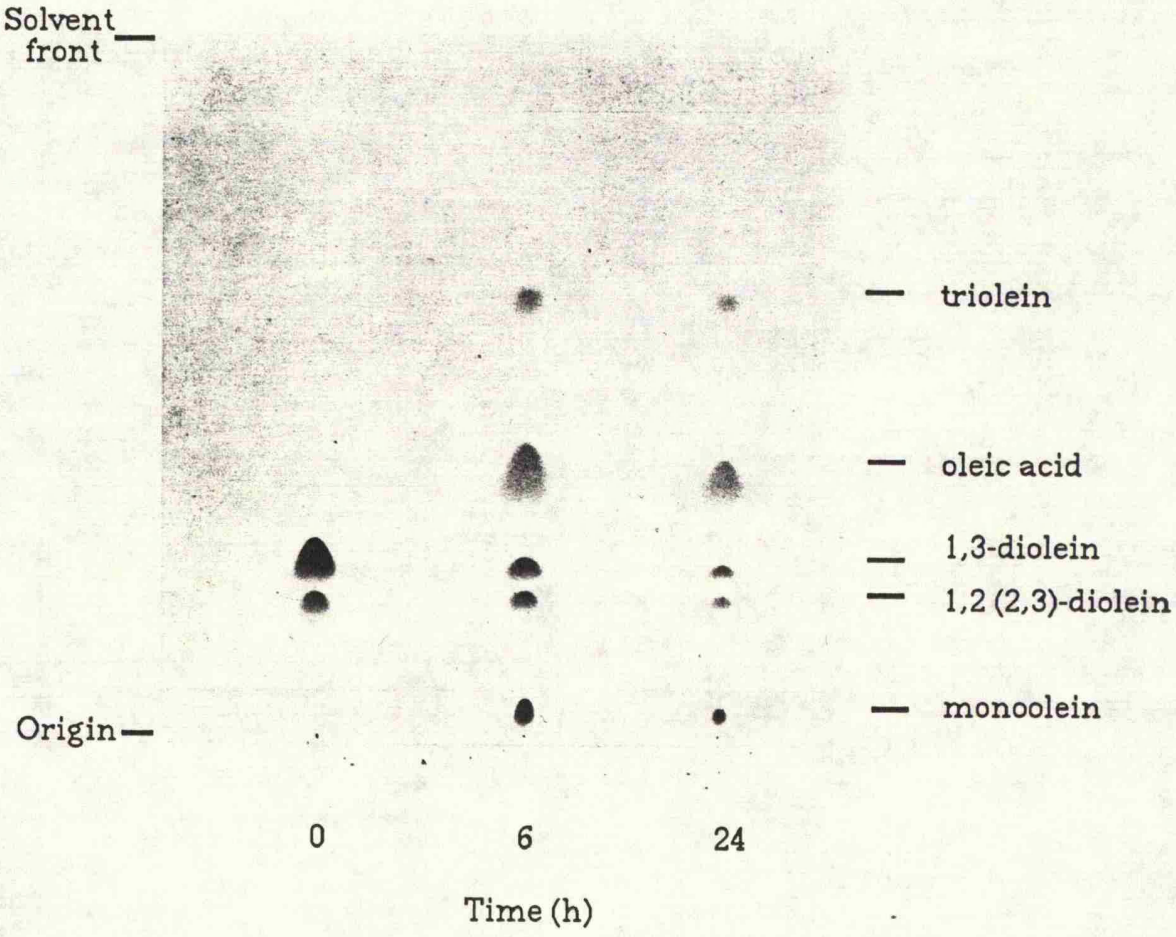
(1) (S) T Y T Q T Q Y P I V L A

(2) Y T Q T Q Y P I V L A

The first amino acid was thought to be a serine, although it was not unambiguously confirmed due to the presence of a tyrosine residue, which resulted from the superimposition of the two sequences. Species 1 comprised $\frac{1}{3}$ of the total protein.

Figure 4.17. *Hydrolysis of diolein by purified lipase.*

Lipase was incubated with diolein [85 % 1,3-, 15 % 1,2 (2,3)] at 37°C, pH 7.5 for up to 24 h. Samples were removed , the products extracted and applied to a silica gel chromatography plate, as described in Methods.



4.6 *Discussion.*

Lipase from *Pseudomonas aeruginosa* EF2 was purified 31-fold to homogeneity (99.5 % pure) by FPLC. The 18 % recovery (Table 4.1) was low compared to published data for other *Pseudomonas* lipases (Table 4.3). This resulted from the concomitant elution off the gel-filtration (Superose) column with a contaminant protein, which meant that a number of fractions were discarded, even though they contained a high concentration of lipase (determined by SDS-PAGE analysis and activity measurements).

The near-constant ratio of lipase to esterase activities (both *p*-nitrophenyl acetate and Tween 80 hydrolysis) throughout the purification procedure indicated the presence of a single lipase that exhibited weak esterolytic activity. The specific activity of the pure enzyme (6,606 LU mg^{-1}) was greater than those reported for *Ps. fluorescens* by Sztajer *et al.* (1991) (4,780 LU mg^{-1}), *Ps.* species ATCC 21808 by Kordel & Schmid (1990) (3,310 μmol min^{-1} $[mg]^{-1}$) and *Ps. cepacia* DSM 50181 by Dünhaupt *et al.* (1991) (1,030 μmol min^{-1} $[mg]^{-1}$), but less than for *Ps. aeruginosa* PAC1R by Stuer *et al.* (1986) (45,510 μ mol min^{-1} $[mg]^{-1}$), although they were assayed under slightly different conditions. Similarly, the catalytic constant (k_{cat}) was significantly lower than the *Ps. aeruginosa* PAC 1R lipase (3193 cf. 22,000 s^{-1}).

The isoelectric point of purified lipase was 4.95. This was commensurate with its ability to bind to the anion-exchange resin (Mono Q) during FPLC, and was similar to the lipase of *Pseudomonas* species ATCC 21808 (pI 4.5 - 4.6; Kordel & Schmid, 1990). The lipases of other *Pseudomonas* species were generally also acidic (Table 4.3).

Analysis of purified lipase by SDS-PAGE indicated a sub-unit M_r of 29,000, which was similar to lipases of other *Pseudomonas* species

Table 4.3. *Purification and properties of* Pseudomonas *lipases.*

Isolate	Yield %	Purification	Native M_r	Sub-unit M_r	pI	Comments	Reference
Pseudomonas aeruginosa EF2	18	31	Large	29,000	4.9	Failed to enter a native gel	-
Pseudomonas aeruginosa 10145	30 - 100	35	Large	-	-	Activity immediately after void volume (Sephadex G-200)	Finkelstein *et at.*, 1970.
Pseudomonas aeruginosa PAC 1R	15	1265	$>10^5$	29,000	5.8	Associated with lipopolysacchride	Steur *et al.*, 1986.
Pseudomonas aeruginosa YS 7	-	-	-	40,000	-	-	Shabtai & Daya-Mishne, 1991.
Pseudomonas fluorescens AFT 29	47	10	Large	16,000	-	-	Dring and Fox, 1983.
Pseudomonas fluorescens AFT 36	47	47	Large	-	-	Activity immediately after void volume 3 lipase peaks	Fox and Stepaniak, 1983.
Pseudomonas fluorescens MC 50	78	500	-	55,000	-	Hydrodynamic molecular radius 4nm	Bozoğlu *et al.*, 1984.
Pseudomonas fluorescens	21	4780	-	450,000	-	-	Sztajer *et al.*, 1991.
Pseudomonas fragi 22,39B	-	-	-	33,000	7.0	-	Nishio *et al.*, 1986.
Pseudomonas fragi NCDO752	40	-	-	i 25,000 ii 250,000	-	2 peaks probably due to single lipase	Lawrence *et al.*, 1967 b.
Pseudomonas cepacia DSM50181	30	55		-	7.1	-	Dünhaupt *et al.*, 1991.
Pseudomonas species	13	406	-	30,000	-	-	Yamamoto & Fujiwara, 1988.
Pseudomonas species ATCC 21808	35	260	110,000	35,000	4.5 - 4.6	-	Kordel & Schmid 1990. Kordel *et al.*, 1991 b.

(Table 4.3). Recently, the lipase genes from various *Pseudomonas* species have been cloned and the nucleotide sequences determined (Chapter 5). This has enabled the M_r of the mature enzyme to be calculated very accurately. The lipases of *Ps. fragi* IFO 12049 (Aoyama *et al.*, 1988), *Ps. aeruginosa* TE 3285 (Nishioka *et al.*, 1991) and *Pseudomonas* species ATCC 21808 (Hom *et al.*, 1991) were also very similar to the *Ps. aeruginosa* EF2 lipase (M_r 29,966, 30,144 and 32,943, respectively). The lipase of *Ps. fragi* IFO 3458 (Kugimiya *et al.*, 1986) was, however, much smaller (M_r 14,643).

The native enzyme was thought to aggregate in a non-specific fashion to form a high molecular weight complex. This may be due to self-association by hydrophobic interactions (Macrae, 1983) of the amphiphilic protein (Jaeger *et al.*, 1991) or by complexing with lipopolysaccharide (Stuer *et al.*, 1986; Feller *et al.*, 1990), although the latter could not be detected. The failure of the *Ps. aeruginosa* EF2 lipase to enter a non-dissociating (native) polyacrylamide gel was the similar to that observed with a *Ps. fluorescens* lipase (Sztajer *et al.*, 1991). Attempts to overcome this by electrophoresing in urea (to break any hydrogen bonds) or NaCl (to reduce hydrophobic interactions) were unsuccessful. These experiments indicated that the lipase molecules were strongly associated, but they were insufficient to identify the nature of the association. These results were in direct contrast to those obtained by sedimentation analysis of *Pseudomonas* spp. lipase of M_r 33,000, which showed that the enzyme did not aggregate under a wide variety of conditions (Simpkin *et al.*, 1991).

Lipase and esterase activities were permanently lost following exposure to low concentrations of the anionic detergent SDS. This was also observed for other *Pseudomonas* lipases, by Watanabe *et al.* (1977), Kawase *et al.* (1985) and Yamamoto & Fujiwara (1988). As formulation of the enzyme into a commercial detergent would require activity to be maintained in the

presence of both non-ionic and ionic surfactants, the potential usefulness of the *Ps. aeruginosa* EF2 lipase is therefore impaired.

The pH and temperature optima for lipase and esterase (Tween 80) activities corresponded to the conditions under which *Ps. aeruginosa* EF2 was isolated (pH 8.5, 50°C), and were similar to those of other *Pseudomonas* lipases (Table 4.4). It should be noted that these values reflect the effect of pH and temperature not only on the catalytic activity of the enzyme, but also on the physico-chemical properties of the assay substrate; the term 'optimum' should therefore used with caution.

The enzyme exhibited a bi-phasic loss of activity at 45°C and above, which was similar to that found with the lipases of *Ps. fluorescens* AFT 29 and 36 (Dring & Fox, 1983; Fox & Stepaniak,1983). A plot of the logarithm of the decimal reduction time (D) at various temperatures (to calculate a Z value) has been generally employed in the assessment of heat sterilisation techniques (Brock *et al.*, 1984) and provides a useful method of quantifying heat inactivation. Z values for the above lipases were typically around 40°C, whilst that of *Ps. aeruginosa* EF2 was 9.75°C, indicating it was much more thermolabile. However, the thermostability at 60°C ($t_{1/2}$ 17.5 min) of the *Ps. aeruginosa* EF2 lipase was greater than that of *Ps. fluorescens* AFT 29, AFT 36 and *Ps.* species ($t_{1/2}$ 1-10 min; Dring & Fox, 1983; Fox & Stepaniak, 1983; Yamamoto & Fujiwara, 1988). Notably, the *Ps. fluorescens* AFT 29 lipase exhibited an unusual double-peaked thermal inactivation curve, being relatively thermolabile at 70°C and 150°C, but stable between these temperatures. The lipases of Ps. fluorescens AFT 36 and *Ps. azotoformans* No. 400 (Kumura *et al.*, 1991) also showed similar properties to this, unlike the *Ps. aeruginosa* EF2 lipase, which was rapidly denatured at 100°C.

Table 4.4. *pH and temperature profiles of purified* Pseudomonas *lipases.*

Isolate	pH Optimum	pH Stability	Temperature Optimum (°C)	Thermo-Stability* (°C)	Reference
Ps. aeruginosa (EF2)	lipase 9.0 esterase 8.5	- -	50 50	360 min @ 45°C : 50% 17.5min @ 60°C : 50% 2.1min @ 70°C : 50%	-
Ps. aeruginosa (10145)	8.8 - 9.1	4.5 - 11	40	25 - 55	Finkelstein *et al.*, 1970.
Ps. fluorescens (AFT 29)	7.0	6.2 - 7.6	22	10 - 30 1min @ 50°C : 75% 1min @ 70°C : 0% 1min @ 100°C : 30%	Dring & Fox, 1983.
Ps. fluorescens (AFT 36)	8.0	6 - 9	35	100 - 150 Labile @ 60 - 80°C	Fox & Stepaniak 1983.
Ps. fluorescens (MC 50)	8.0 - 9.0	6 - 9	30 - 40	<40	Bozoğlu *et al.*, 1984.
Ps. fragi (22.39B)	9.0	6 - 11	65 - 70	<51 3 d @ 51°C : 50%	Nishio *et al.*, 1986.
Ps. fragi (22.39B)	9.5	5 - 11	75 - 80	20min @ 70°C : 95%	Watanabe *et al.*, 1977
Ps. nitroreducens var. *thermotolerans* (261.B)	9.5	5 - 11	50	20min @ 70°C : 95%	Watanabe *et al.*, 1977.
Ps. mephitica var. *lipolytica*	7.0	3.4 - 11.2	70	1.5-9h @ 70°C : 50%	Kosugi & Kamibayashi 1971.
Pseudomonas species	7.0	9 - 10	60	30min <35°C : Stable >40°C : Inactive	Yamamoto & Fujiwara,1988.
Pseudomonas species (ATCC 21808)	8.5	4.5 - 9	50	8h @ 45°C : 100% 8h @ 50°C : 60% 1h @ 21°C : 1%	Kordel & Schmid, 1990.
Pseudomonas species (various)	9.0 - 10.5	-	40 - 60	-	Pierce *et al.*, 1990.

*: % residual activity.

Classical Michaelis-Menten kinetics are not applicable to the lipolytic reaction, as it occurs in a heterogeneous medium. The rate of reaction depends predominantly on the available droplet surface area on to which the enzyme can bind (Benzonana & Desnuelle, 1965). It has been suggested that NaCl reduces interfacial charge and aids ionisation of long-chain fatty acid (reduces the pKa of oleic acid from approximately 9.5 to 6.7), whilst $CaCl_2$ may reduce fatty acid inhibition of lipase activity (reduces the pKa of oleic acid further, to 6.4; Benzonana & Desnuelle, 1968). Lipase activities were therefore determined in the presence of these salts at concentrations suggested by Brockerhoff & Jensen (1974), and optimum values obtained. The two-fold increase in lipase activity by $CaCl_2$ was a purely interfacial effect on the olive oil emulsion, as $CaCl_2$ was shown not to effect esterase (Tween 80) activity. This was further supported by the observation that esterase (*p*-nitrophenyl acetate and Tween 80) activities were not inhibited when pre-incubated with the divalent cation chelator EDTA. The *Ps. aeruginosa* EF2 lipase was therefore thought not to be a metalloprotein, in accordance with *Ps.* species No.33 (Kumura *et al.*, 1991); but in contrast to human pancreatic lipase which possesses a calcium-binding site (Gubernator *et al.*, 1991) and the lipases of *Ps. fluorescens* AFT 29 (Dring & Fox, 1983) and *Ps. fluorescens* MC 50 (Bozoğlu *et al.*, 1984).

Recent structural determinations of human and fungal lipases by X-ray crystallography (Section 1.3) have confirmed earlier observations that the nucleophile at the active site is the hydroxyl group of a serine residue. Indeed serine-specific inhibitors, such as diethyl-*p*-nitrophenyl phosphate (E_{600}), have been shown to irreversibly inhibit lipase and esterase activities from various sources (see, for example, Kordel *et al.*, 1991 b), whilst other group-specific inhibitors simply prevent binding of the enzyme to hydrophobic surfaces (see, for example, Lóokene & Sikk, 1991). Nucleotide

sequence analysis of a variety of lipase genes indicate that they contain a highly conserved amino acid sequence around the active serine (-G X S X G-) which is not thought to be exposed at the surface of the protein (Kordel & Schmid, 1991).

As the *Ps. aeruginosa* EF2 lipase was generally similar to other *Pseudomonas* lipases with respect to its biochemical properties, it is highly likely that it also contains an active-site serine residue. 3,4-dichloroisocoumarin (DCI) was chosen as the serine-specific inhibitor as it has been used successfully to inhibit serine proteases (Harper *et al.*, 1985; Rusbridge & Beynon, 1990), but its effect on lipases has not yet been reported. In addition, it was also far less toxic than E_{600}.

Lipase and esterase activities were only weakly inhibited by DCI; the apparent inhibition constant (k_{app}) was over a hundred-fold less than that reported for serine proteases (10 cf. 8920 $M^{-1}s^{-1}$; Harper *et al.*, 1985). The presence of an active-site serine could therefore not be confirmed. However, in view of the recent structural determinations of various lipases (albeit fungal and mammalian; Section 1.3) it is likely that the active site was inaccessible to the inhibitor, possibly due to the presence of a surface loop or flap. Alternatively, the possibility exists that the enzyme did not contain a serine residue at the catalytic centre. This would, however, be contrary to evidence regarding other lipases which have been gained from nucleotide sequencing of the respective structural gene, inhibition studies and X-ray crystallography of the purified enzymes.

The inhibition of lipolysis has been suggested to depend upon the sequence of addition of lipase, inhibitor and substrate, the extent and rate of inhibition varying with the system used (Verger, 1991). To overcome such problems, inhibition studies are best carried out using substrate mono-layer

techniques (Verger *et al.*, 1991). These should be considered if the inhibition of the *Ps. aeruginosa* EF2 lipase is to be further characterised.

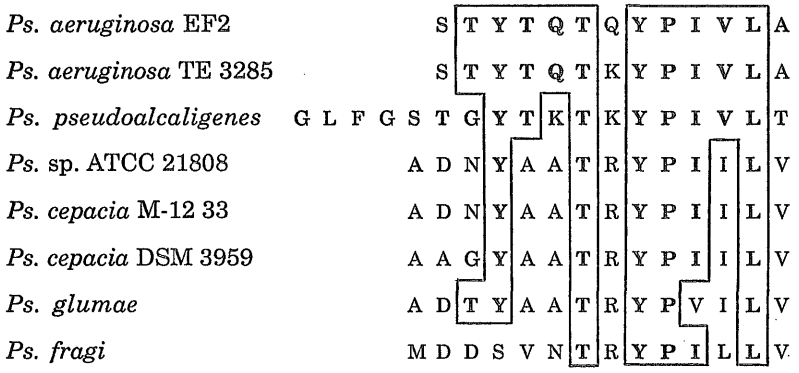
The *Ps. aeruginosa* EF2 lipase preferentially hydrolysed the 1,3-oleate residues of a triolein molecule. The experimental procedure employed could not determine whether this was an absolute specificity for the peripheral ester groups, or merely a general preference, due to acyl migration within the aqueous solution (Ergan & Trani, 1991). In order to clarify this, quantitation in organic solvents (where acyl migration does not occur) should be performed (Berger & Schneider, 1991).

Many bacterial and fungal lipases exhibit a 1,3-regio-specificity towards triacylglycerols (such as *Rhizormucor miehei* [Lipozyme] and *Ps. fluorescens*), as well as human pancreatic lipase (Macrae & Hammond, 1985). Others, such as *Geotrichum candidum*, show no positional specificity, although Yamaguchi and Mase (1991) reported a *Penicillium camembertii* U-150 lipase specific for only mono- and di-acylglycerols which showed no activity towards triacylglycerols.

The N-terminal amino acid sequence of the *Ps. aeruginosa* EF2 lipase, although not determined unambiguously, showed significant homology with published sequences for other *Pseudomonas* lipases (in particular to *Ps. aeruginosa* TE3285) derived from either peptide or nucleotide analysis (Fig. 4.18). All of the enzymes exhibited a consensus sequence (- T X Y P I X Y L -) close to the N-terminus, in which the first variable residue (X) was a basic amino acid (glutamine, arginine or lysine) and the second variable residue (X) was a hydrophobic amino acid (isoleucine, leucine or valine).

In summary, the biochemical and physico-chemical properties of the *Pseudomonas aeruginosa* EF2 lipase are very similar to those of other

Figure 4.18. *Comparison of the N-terminal amino acid sequence of* Ps. aeruginosa *EF2 lipase with that of other* Pseudomonas *lipases.*



References:

Ps. aeruginosa TE 3285	(originally called *Ps. fluorescens*; T. Nishioka,
personal communication);	Nishioka *et al.*, 1991.
Ps. pseudoalcaligenes ;	Andreoli *et al.*, 1989.
Ps. sp. ATCC 21808 ;	Kordel & Schmid, 1990.
Ps. cepacia M-12 33 ;	Nakanishi *et al.*, 1989, Jørgensen *et al.*, 1991.
Ps. glumae ;	Batenburg *et al.*, 1991.
Ps. fragi ;	Kugimiya *et al.*, 1986; Aoyama *et al.*, 1988.

Pseudomonas lipases, and all of these enzymes have a highly conserved N-terminal amino acid sequence. Further extensive evaluation will be required if this enzyme is to be considered for commercial exploitation. In particular, its relative thermostability at 50°C and 1,3-positional specificity, may be potentially useful in the enzymatic production of cocoa-butter equivalents (Macrae & Hammond, 1985). However, as the enzyme was sensitive to low concentrations of the anionic surfactant SDS, its incorporation into a detergent formulation (which contain a mixture of both non-ionic and ionic surfactants) may therefore be prevented.

CHAPTER 5.

THE MOLECULAR CLONING AND ANALYSIS OF THE LIPASE GENE FROM *PSEUDOMONAS AERUGINOSA* EF2.

5.1 *Abstract.*

The lipase gene of *Pseudomonas aeruginosa* EF2 was identified by Southern analysis of restricted chromosomal DNA using a synthetic oligonucleotide probe based on the N-terminal amino acid sequence of the purified lipase. The hybridizing *Eco*R I fragments were ligated into the *Pseudomonas/Escherichia* shuttle vector pKT230 (pJG1) and the M13-derived plasmid pUC18 (pJG5 and pJG31). These constructs were used to transform the lipase-negative *E. coli* JM109 and the resultant clones (JG1, JG5 and JG31) were able to grow on Tween 80. pJG5 was further analysed by restriction with various endonucleases and Southern blotting. This confirmed the presence of the 5' end encoding the N-terminal portion of the mature lipase and showed that it was contained on a 8.6 kbp *Eco*R I fragment. Restriction sites along the fragment were mapped and sub-clones of pJG5 constructed; pUC18 containing either a 4.3 kbp *Bam*H I, 2.5 kbp *Sal* I or 3.2 kbp *Sph* I fragment (designated pJG5.1, pJG5.2 and pJG5.3, respectively) failed to confer the lipase-positive phenotype to *E. coli* JM109, suggesting that the complete structural lipase gene and/or any *cis*-acting regulatory elements required for efficient expression were not present.

Esterase activity (measured as *p*-nitrophenyl acetate hydrolysis) of *E. coli* JG5, unlike that of *Ps. aeruginosa* EF2, was not subject to substrate-induction/end-product repression and was very low (possibly due to inefficient transcription/translation and/or secretion of the recombinant lipase). The latter possibility was supported by the observation that esterase activity remained largely cell-bound when *E. coli* JG5 was grown in a glucose/minimal salts medium, but was essentially extracellular when grown on Tween 80.

As the recombinant *Ps. aeruginosa* EF2 lipase was produced at low levels in *E. coli* JM109, a lipase-negative *Pseudomonas* strain was considered as an alternative host. The 8.6 kbp *Eco*R I fragment of pJG5 was ligated into pKT230 and used to transform *E. coli* NM522, which subsequently utilised Tween 80. The construct was then transferred into *Ps. putida* P2440 in an unsuccessful attempt to over-produce the recombinant *Ps. aeruginosa* EF2 lipase.

5.2 *Introduction.*

Up until 1988 very little was known about the molecular biology of microbial lipases. Since then the lipase genes from a variety of fungi and bacteria, such as *Rhizormucor miehei, Humicola lanuginosa* (Boel *et al.*, 1991), *Geotrichum candidum* (Shimada *et al.*, 1989) and *Staphylococcus hyicus* (Vogel *et al.*, 1990) have been successfully cloned and expressed in foreign hosts. Many fungi produce multiple forms of extracellular lipases (Jacobsen *et al.*, 1989 b), resulting from either post-translational modifications (Boel *et al.*, 1991) or expression of multiple genes (Sugihara *et al.*, 1990; Alberghina *et al.*, 1991; Kawaguchi & Honda, 1991; Shimada *et al.*, 1991). This is generally not the case with bacterial lipases. However, Feller *et al.* (1991) recently cloned and expressed three distinct lipase genes from a psychrotrophic *Moraxella* strain, whilst Colson *et al.* (1991) identified two *Bacillus subtilis* genes, one encoding a true lipase and the other an esterase.

Nucleotide sequencing of lipase genes has revealed a highly conserved pentapeptide sequence around the active site serine residue (Section 1.3). In many cases, an N-terminal secretory signal sequence has also been identified (for example, see Table 5.1). In addition, *Rhizormucor miehei* and *Staphylococcus hyicus* lipases are synthesized as pre-pro-peptides (Boel *et al.*, 1988; Götz, 1991, respectively) which require proteolytic cleavage to form active, mature enzymes.

During the past two years, the cloning, sequencing and expression of a variety of *Pseudomonas* lipase genes have been reported (Table 5.1). Most consist of a single open reading frame which encodes an N-terminal signal sequence (typically 20 to 40 amino acids in length) plus the functional lipase (ranging from 135 to 358 amino acids). A second gene has recently been

Table 5.1 *Cloned* Pseudomonas *lipase genes.*

Isolate	Host	Vector	Fragment size (kbp)	Comments	Reference
Ps. aeruginosa TE3285	*E. coli* JM109	pKK233-2	2.4	26 AA signal sequence, 258 AA lipase. Gene encoded within 933bp orf. High serine content (12% of AA) 66% GC. Expressed at low levels in *E. coli.*	Nishioka *et al.*, 1991
Ps. aeruginosa PAO2302	*Ps. aeruginosa* 6-1*	pKT248	3.1	Lipase locus mapped at 57 min on chromosome. Extracellular lipase secretion as wild-type donor.	Wohlfarth & Winkler, 1988
	E. coli	pKT230	3.1	Lipase not secreted.	
Ps. pseudoalcaligenes M-1	*E. coli* JM101	pTZ18R/19R	2.0	24 AA signal sequence, 289 AA lipase. Lipase activity not detected in *E.coli* Sub-cloning and site-directed mutagenesis in other *E. coli* strains produced lipase located in the periplasm.	Andreoli *et al.*, 1989
Ps. fluorescens SIK W1	*E. coli* BL12	pTTQ19	1.6	Lipase comprised 40% cellular protein when induced with IPTG. Produced as inclusion bodies in cytoplasm; solubilised by 8M urea.	Chung *et al.*, 1991.
Ps. fragi IFO3458	*E. coli* JM83	pUC9	2.0	18 AA signal sequence (but homology of this with N-terminal AA sequences *of other Pseudomonas* lipases (Fig. 4.18), 135 AA lipase. Gene encoded within a 1.1kbp fragment. Expressed at low levels in *E. coli.*	Kugimiya *et al.*, 1986.
Ps. fragi IFO12049	*E. coli* JM83	pUC9	3.3	Unidentified signal sequence 277 AA lipase. High % homology with *Ps. fragi* IFO3458.	Aoyama *et al.*, 1988, 1991.
Ps. cepacia M-12-33	*Ps. cepacia* HW10*	pFL100	2.9	2 genes identified: (i) *lipA*. 1092 nucleotides. 44 AA signal sequence, 320 AA lipase. (ii) *lipX*. 1032 nucleotides. Follows *lipA* gene tandemly. Associated with regulating lipase activity.	Nakanishi *et al.*, 1989, 1991.

Table 5.1 *(continued).*

Isolate	Host	Vector	Fragment size (kbp)	Comments	Reference
Ps. cepacia DSM3059	*E. coli* SJ2	pUC19	6.0	2 genes identified: (i) *lipA*. 40-44 AA signal sequence, 320 AA lipase (69% G/C). (ii) *limA*. 344 AA protein. Follows *lipA* gene tandemly. Associated with regulating lipase activity, possibly secretion. Acts in *trans*. Both genes required for secretion in *Bacillus subtilis* and *Streptomyceslividans*.	Jørgensen *et al*., 1991. Jørgensen, 1991.
Ps. glumae	*E. coli* JM101	pEMBL9	-	39 AA signal sequence, 358 AA lipase. Site-directed mutagenesis used to improve properties of lipase with respect to detergent formulations. Cloned and expressed in a variety of bacteria, fungi and yeast.	Batenburg *et al*., 1991
Ps. glumae	-	-	-	2 genes identified: (i) *lipA*. 39 AA signal sequence, 319 AA lipase. (ii) *lipB*. 353 AA protein. Follows *lipA*; co-trancribed from same promoter. Involved in secretion of the lipase.	Frenken*et* al., 1991.
Ps species ATCC 21808	*Ps. oleovorans*	pCP13 (cosmid) ATCC 8062	8.0	44 AA signal sequence, 319 AA Over-expressed by fusion with *tac* (*trp*/*lac*) promoter in *Ps.* species ATCC 21808.	Hom *et al*., 1991.

*, lipase-negative strains formed by mutagenesis.
AA, amino acid residues.

identified in *Ps. cepacia* and *Ps. glumae*, which was thought to be involved in the secretion and/or regulation of lipase production. *Ps. glumae* was originally classified as *Ps. gladioli*, although whether this represents a different species or it has simply been re-named was not clear in the literature. *Ps. gladioli* and *Ps. cepacia* are closely related as illustrated by the fact that they share significant rRNA and DNA homologies (Palleroni, 1984).

The genetics of *Pseudomonas aeruginosa* have been well characterised (Holloway *et al.*, 1979) and a detailed physical-genetic map now exists (Holloway *et al.*, 1987). Recent analysis of chromosomal DNA by pulsed-field gel electrophoresis indicated a large genome of 5862 kbp, second only in size to *Anabaena* species (7110 kbp; Ratnaningshi *et al.*, 1990). The DNA of *Pseudomonas* species contains a high genomic guanine plus cytosine ratio (67.2% cf. 48-52% for *E. coli*) with a strong bias for a cytosine in the third codon position (West & Iglewski, 1988) and this is thought to result from mutational pressure exerted on the genome (Osawa *et al.*, 1990). All reported *Pseudomonas* lipase genes were chromosomally-encoded and the lipase gene of *Ps. aeruginosa* PAO2302 was mapped at the 57 min position on the chromosome (Wohlfarth & Winkler, 1988).

In order to hydrolyse exogenous triglycerides, microbial lipases must be secreted into the external medium. In Gram-negative bacteria this involves transport across both the inner and outer membranes. The mechanism of protein secretion by Gram-negatives has been extensively researched (see for example reviews by Pugsley & Schwartz, 1985; Hirst & Welch, 1988; Wandersman, 1989; Pugsley *et al.*, 1990; Schatz & Beckwith, 1990), especially within the *Enterobacteriaceae*. However, knowledge of extracellular protein production in *Pseudomonas* species is somewhat limited. Nevertheless, biochemical and genetic analyses have recently shed light on the molecular mechanisms involved. Most mutations which affect

extracellular protein production (so-called *xcp* mutations) are pleiotropic, *ie.* they affect the secretion of several proteins, including lipase (Wretlind & Pavlovskis, 1984), and have been mapped to 3 loci within the chromosome (Filliaux *et al.*, 1989). Bally *et al.* (1991) recently identified the product of the *xcp*A gene as an inner membrane protein, in contrast to other *xcp* genes which are thought to be involved in regulating transport across the outer membrane (Filliaux *et al.*, 1989).

The secretion of *Pseudomonas* extracellular proteins is believed to be more complex than that in *Escherichia*. To produce recombinant *Pseudomonas* lipase successfully in *E. coli* requires not only efficient transcription and translation of the gene, but also recognition and correct processing of the foreign protein by the host (post-translational modifications). As lipases, by definition, interact with lipids, it is surprising that nowhere in the literature is the protection of host membrane lipids reported; if anything it is positively over-looked. In addition, their relatively hydrophobic nature may in some way facilitate export or require the use of an alternative transport system. Until recently it has been assumed that they follow the signal peptide-dependent route of other hydrophilic exo-proteins, but it is possible that a second gene product (see Table 5.1) might be involved.

The physiological regulation of lipase production by *Pseudomonas aeruginosa* EF2 was described in Chapter 3. Growth in batch, fed-batch and continuous cultures indicated the operation of a number of regulatory mechanisms (substrate-induction and product-repression). Analysis of the putative lipase operon (*ie.* by DNA sequencing) and other regulatory elements may confirm these observations and provide an insight into the mode of secretion of the extracellular enzyme, about which nothing is known.

The potential also exists to construct novel bacterial strains that over-produce lipase. This could be achieved by alleviating the physiological controls, such as product repression, or deletion of a regulatory gene; increasing transcript production above the non-repressed levels (*ie.* a stronger promoter or a higher gene dosage); or by improving the secretory pathway of the enzyme.

The biochemical properties of the purified lipase were similar to those of other *Pseudomonas* lipases (Chapter 4). The potential commercial application of the enzyme may require improvements in its stability towards thermal denaturation (Nosoh & Sekiguchi, 1990) or components of detergent formulations (such as proteases, detergents and oxidising agents; Batenburg *et al.*, 1991), both of which could be brought about by site-directed mutagenesis.

This chapter describes the molecular cloning and analysis of the *Ps. aeruginosa* EF2 lipase gene, and is discussed with reference to cloned lipase genes of other *Pseudomonas* species.

5.3 *Identification of the lipase gene by Southern analysis.*

Genomic DNA was prepared from an exponentially-growing culture of *Ps. aeruginosa* EF2 using the method described by Wilson (1990), which specifically removes proteins and polysaccharides by precipitation using CTAB. The DNA was then completely restricted with *Sal* I, *Bam*H I, *Pst* I, *Eco*R I and *Hin*d III endonucleases and the fragments separated by agarose gel electrophoresis. Following transfer to a nylon membrane (Hybond N) they were probed for the presence of the lipase gene by Southern analysis. A 20mer oligonucleotide probe based on the N-terminal amino acid sequence (Section 4.5.7) was used. This contained two inosyl residues to replace the four possible base combinations in the third positions of the second and fourth codons, thus reducing the total number of nucleotide degeneracies from 256 to 16:

N-terminal amino acid sequence	Y	T	Q	T	Q	Y	P
Probe (256 degeneracies)	TAC T	ACT C A G	CAG A	ACT C A G	CAG A	TAC T	CC
Inosyl probe (16 degeneracies)	TAC T	ACI	CAG A	ACI	CAG A	TAC T	CC

The probe was radio-labelled using ^{32}P and hybridized to the membrane-bound DNA at 37°C; a temperature calculated according to the following equation (Maniatis *et al.*, 1982):

$$\text{Hybridization temperature (°C)} = 69.3 + 0.41\,(G+C)\% - \frac{650}{L} - 12$$

where L is the length of the probe in nucleotides. Inosyl residues and mixed degeneracies were classed as (A+T).

$$\text{Hybridization temperature (°C)} = 69.3 + 0.41\,(30) - \frac{650}{20} - 12 = 37.1°C$$

Following hybridization, unbound probe was removed by repeated washes at 37°C and the membrane was subject to autoradiography. Hybridizing bands were not visible and it was assumed that this temperature was too high to enable the probe to effectively bind to the desired portion of DNA. The membrane was therefore re-probed at 30°C, with washes at room temperature. Again no hybridizing bands were visible following autoradiography using both standard X-Ray film and more sensitive pre-flashed film.

The inosyl probe was then replaced with the oligonucleotide containing the full complement of degeneracies. This was radio-labelled and used to probe the DNA bound to the Hybond N membrane at 30°C and washed at room temperature. Again no bands were visible following autoradiography for over 5 days. This led to the conclusion that hybridisation was extremely weak, due either to the presence of residual secondary structure in the chromosomal DNA probed, or to poor binding of the DNA to the nylon membrane.

At this stage, the quality of the newly-aquired Hybond N (Amersham) was checked. A positive control was performed using the cloned gene for the lactose-binding protein from *Agrobacterium radiobacter*. This was present on a 3.8 kbp *Pst*I fragment in pUC19 and was known to hybridize strongly with a 17mer oligonucleotide probe based on the N-terminal amino acid sequence of the protein (Williams *et al.*, 1990; S.G. Williams, personal communication). Uncut and *Pst*I-cut constructs were electrophoresed in duplicate and transferred on to both the newly-aquired Hybond N and an older batch. Subsequent probing and autoradiography of the two membranes

demonstrated clearly the difference in the ability of the two membranes to bind DNA (Fig. 5.1).

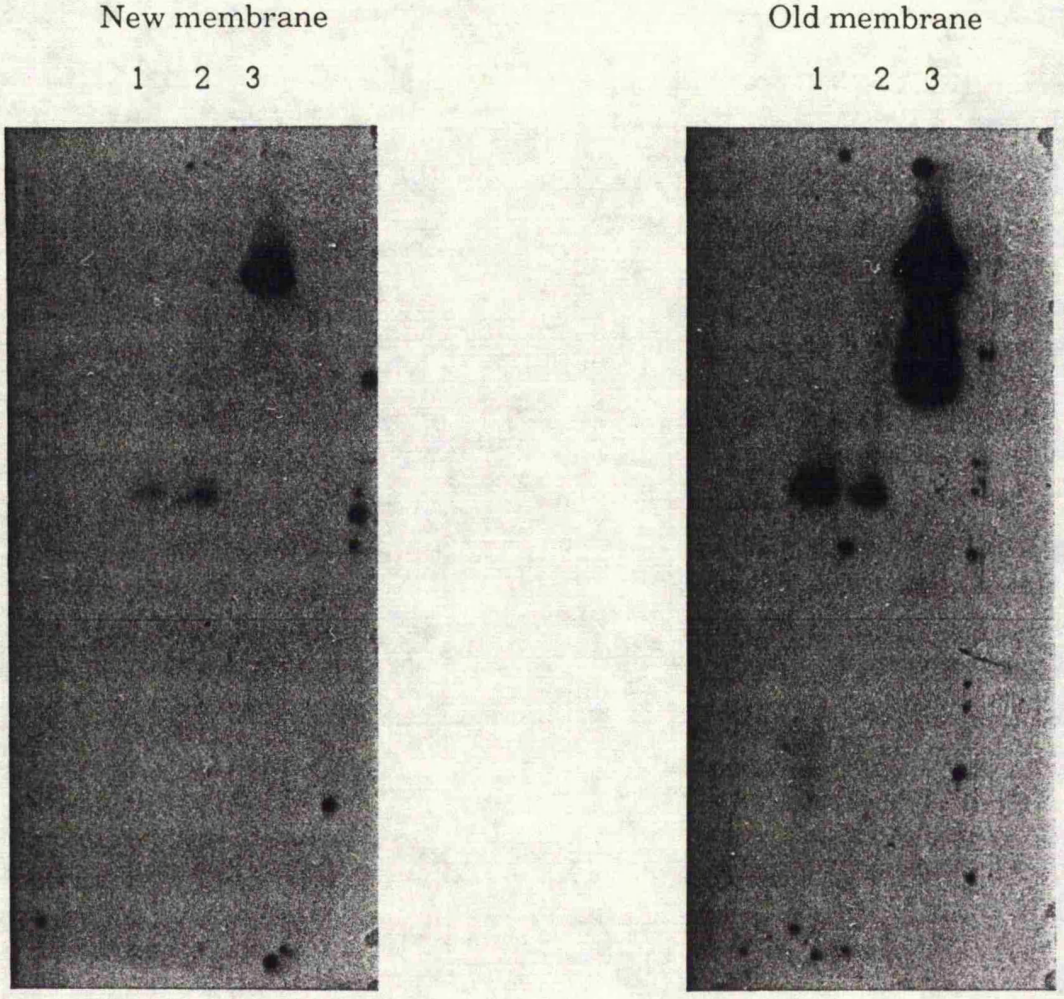
This suggested that the inability of the new batch of Hybond N to bind multi-copy pUC19 DNA sequences was probably also responsible for the failure to detect the lipase gene. As the lipase gene was probably present at only one copy per cell, it was therefore not surprising that hybridization to both the oligonucleotide probes was not observed.

Restricted *Ps. aeruginosa* EF2 genomic DNA was therefore transferred to the old-type Hybond N and re-probed at 37°C, with washes at room temperature, using the 256-degeneracy probe. Faint hybridizing bands were observed over a high background (due to non-specific binding) following autoradiography for over 50h. The probe hybridized with several fragments of the *Sal* I, *Bam*H I and *Pst* I digests, with a large (> 14 kbp) fragment of the *Hin*d III digest, and with a fragment of the EcoR I digest of approximately 7-9 kbp. The inosyl probe did not bind as strongly; the hybridizing bands were barely discernible above the background.

The lipase of M_r 29,000 was assumed to contain approximately 274 amino acids (assuming an approximate M_r of 106, calculated from a knowledge of the number of amino acids and M_r of the *Ps. aeruginosa* TE3285 lipase; Nishioka *et al.*, 1991). As each amino acid is encoded by three nucleotides, the expected size of the DNA fragment that contained the functional lipase gene (including all regulatory elements and possible N-terminal secretory peptide) was approximately 1 kbp. As a secondary regulatory gene similar to those in *Ps. cepacia* and *Ps. glumae* (Table 5.1) might have existed in *Ps. aeruginosa* EF2, a DNA fragment of at least 3 kbp was sought. The oligonucleotide probe bound to an *Eco*R I fragment of

Figure 5.1. *Southern analysis of the cloned lactose binding protein gene of* Agrobacterium radiobacter*: A comparison of the DNA-binding ability of old and new batches of Hybond N.*

Pst I-restricted and uncut pUC19 harbouring the cloned gene were electrophoresed in duplicate in a horizontal agarose gel. The DNA was then transferred on to a strip of either old or new-type nylon (Hybond N) membrane, hybridised with a 32P-labelled oligonucleotide probe and autoradiographed, as described in Methods. Tracks 1 & 2, *Pst* I-restricted plasmid; track 3, uncut plasmid.



approximately 7-9 kbp, which was therefore assumed to encode the functional lipase gene.

5.4 *Identification of potential hosts and screening for lipase-positive strains.*

Escherichia coli JM109 and a range of *Pseudomonas* species were evaluated as potential lipase-negative hosts into which the *Ps. aeruginosa* EF2 lipase gene could be cloned. The *Pseudomonas* species included one *Ps. putida* strain, one *Ps. cepacia* strain and six *Ps. aeruginosa* strains (including *Ps. aeruginosa* 6-1, a lipase-negative mutant of *Ps. aeruginosa* PAC 1R; S. Wohlfarth, personal communication; Wohlfarth & Winkler, 1988). This was carried out by screening their ability to grow on M9 minimal-salts-agar plates when supplemented with glucose or oleic acid (plus any necessary growth requirements), but not when supplemented with an olive oil emulsion. All strains, except *E. coli* JM109 and *Ps. putida* P2440, either failed to grow on all media (as their requirements for certain co-factors were unknown) or were lipase-positive (the latter included the supposed lipase-negative *Ps. aeruginosa* strain 6-1, which grew, albeit slowly, on olive oil). *Ps. putida* P2440 was therefore chosen as the pseudomonad host; a particularly useful choice since its non-pathogenic status was highly advantageous over *Ps. aeruginosa*, which is a well-characterised opportunistic pathogen (Palleroni, 1984).

The use of olive oil agar plates as a selective medium was somewhat problematic, as small bacterial colonies could often not be distinguished from oil droplets on the surface of the plate. This medium was therefore replaced by a similar medium which contained Tween 80 in place of olive oil and an increased amount of calcium chloride to precipitate any free oleic

acid. The lipase donor *Ps. aeruginosa* EF2 grew on this medium, whilst the hosts *E. coli* JM109 and *Ps. putida* P2440 did not. Similarly, they did not show any resistance to the antibiotics streptomycin, kanamycin or ampicillin which were used as plasmid selection markers. The method of Samad *et al.* (1989), which incorporated the pH indicator Victoria Blue B into the Tween 80 medium, was shown to be ineffective (*ie.* clear intensification of the indicator around lipase-positive colonies was not observed).

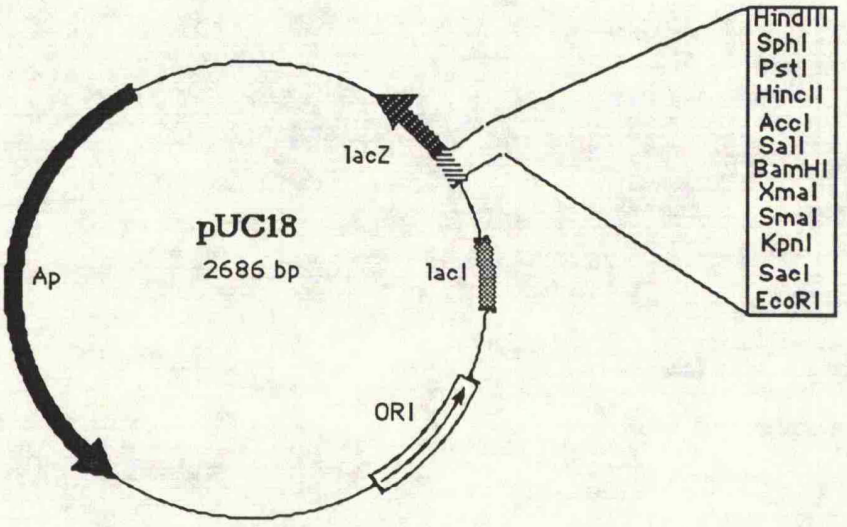
The use of a Tween 80 minimal salts medium to select for lipase-positive strains relied not only on expression of the recombinant lipase gene, but also secretion of the mature enzyme in sufficient quantities to support growth. In an attempt to overcome this, polyclonal antibodies raised against purified *Ps. aeruginosa* PAC1R lipase (K.-E. Jaeger, personal communication; Jaeger *et al.*, 1991) were used to probe purified lipases of *Ps. aeruginosa* PAC1R and EF2, and cell extracts of *E. coli* JM109 by Western analysis. The method had the potential to identify the production of low levels of recombinant lipase in *E. coli*, which were insufficient to adequately support growth on Tween 80-selection media. Unfortunately, the antibodies were shown to bind not only to the two lipase proteins, but also to a variety of *E. coli* JM109 cellular proteins. The identification of recombinant lipase by these antibodies was therefore not deemed to be feasible.

5.5 *Cloning of the lipase gene.*

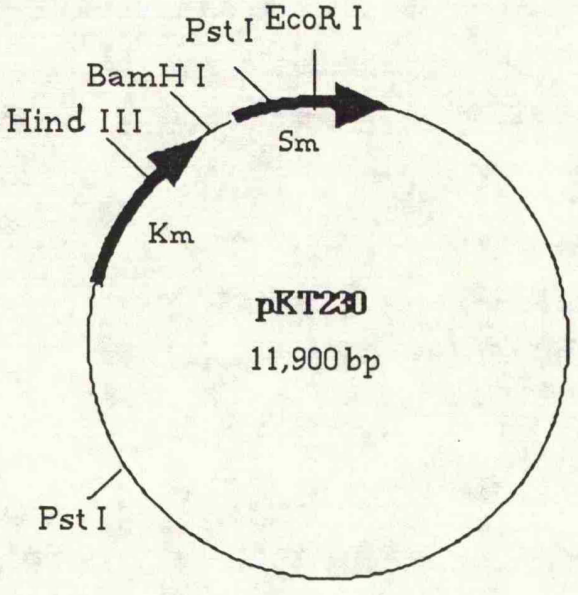
The fragments of *Eco*R I-restricted *Ps. aeruginosa* EF2 genomic DNA of approximately 5-9 kbp were ligated into the *Eco*R I site of the M13-derived plasmid pUC18 and of the *E. coli/Pseudomonas* shuttle vector pKT230 (Badgasarian *et al.*, 1981; Figs. 5.2a and 5.2b, respectively), which were then used to transform *E. coli* JM109. The transformation of *E. coli* JM109, made

Figure 5.2. *Endonuclease restriction maps of the plasmids pUC18 and pKT230.*

i. pUC18



ii. pKT230



Ap, Km and Sm denote the ampicillin, kanamycin and streptomycin resistance genes, respectively. The arrows indicate the direction of transcription.

competent by the method of Krushrer (1978), with plasmid DNA was shown to be superior to the recently-published method of Nishimura *et al.* (1990). Determination of transformation frequencies of uncut plasmids indicated that pUC18 was more amenable than pKT230 (3.4×10^5 cf. 4.5×10^4 transformants μg plasmid DNA^{-1}), probably as a result of its smaller size (2.7 kbp *cf.* 11.9 kbp).

5.5.1 *pKT230 clone.*

The 1:1 plasmid to insert ligation ratio resulted in the appearance of 43 kanamycin-resistant colonies (*ie.* were capable of growing on a nutrient agar-kanamycin selective medium), of which 8 contained inserts (*ie.* were streptomycin sensitive, due to insertional inactivation of the streptomycin resistance gene). Of these, only one grew on an M9 minimal salts-Tween 80-kanamycin medium. This construct was designated pJG1. No colonies expressing the desired phenotype were obtained with plasmid to insert ligation ratios of 1:2, 1:3 and 1:4.

5.5.2 *pUC18 clones.*

The 1:3 plasmid to insert ligation ratio yielded 61 ampicillin-resistant colonies (*ie.* were capable of growth on a nutrient agar-ampicillin agar medium) that did not hydrolyse X-gal (*ie.* produced white colonies, due to insertional inactivation of the *Lac Z'* gene encoding the α fragment of β-galactosidase, as described in Methods; Section 2.16.2). Of these, only two grew on an M9 minimal salts-Tween 80-ampicillin agar medium. These constructs were designated pJG5 and pJG31. No colonies expressing the desired phenotype were obtained with plasmid to insert ligation ratios of 1:0.1, 1:0.5 and 1:1.

5.6 *Characterisation of the recombinant plasmids pJG1, pJG5 and pJG31.*

5.6.1 *Growth on Tween 80.*

Growth of *E. coli* JG1, JG5 and JG31 on M9 minimal salts-Tween 80-antibiotic agar media was only apparent after incubation at 37°C for approximately 2 d, followed by incubation at room temperature for at least 5 d. This relatively slow growth rate was shown not to be due to any inhibitory effects of Tween 80 on the growth of *E. coli* JM109, since the growth of JM109 on a M9 minimal salts-glucose-Tween 80 agar medium was not significantly different to that on a M9 minimal salts-glucose agar medium. Figure 5.3 shows the growth of the clones on an M9 minimal salts-Tween 80 agar medium compared with the lipase-positive *Ps. aeruginosa* EF2 and the lipase-negative *E. coli* JM109 (containing uncut plasmids pUC18 and pKT230).

Clones pJG5 and pJG31 did not exhibit faster rates of growth when plated on to a M9 minimal salts-Tween 80-ampicillin plus IPTG agar medium, suggesting that the lipase gene was not located immediately downstream of the *lacZ* promoter, and was therefore not under its control.

5.6.2 *Estimation of the size of inserts in the recombinant plasmids.*

Restriction of the recombinant plasmids with *Eco*R I indicated that the pUC18-based constructs pJG5 and pJG31 both contained an 8.6 kbp *Eco*R I insert. Southern analysis (using the probe containing 256 degeneracies) confirmed that the 5' end encoding the N-terminal region of the mature lipase was contained within this region (Fig. 5.4). As *E. coli* JG5 and JG31 grew on Tween 80, the whole gene was therefore probably present.

Repeated attempts to restrict the pKT230-based construct pJG1 with *Eco*R I failed. All digests observed were partial, hence the size of the insert

Figure 5.3. *Comparative growth of* Ps. aeruginosa *EF2 and* E. coli *JM109 harbouring pUC18, pKT230, pJG1, pJG5 and pJG31 on a Tween 80-M9 minimal-salts agar medium.*

The agar plate was streaked with a single colony of the appropriate strain then incubated at 37°C over-night, followed by 5 d at room temperature, as described in Methods. Colony 1, *Ps. aeruginosa* EF2 ; colony 2, *E. coli* JM109; colonies 3 to 7, *E. coli* JM109 harbouring the plasmids: 3, pUC18; 4, pJG5; 5, pJG31; 6, pKT230; 7, pJG1.

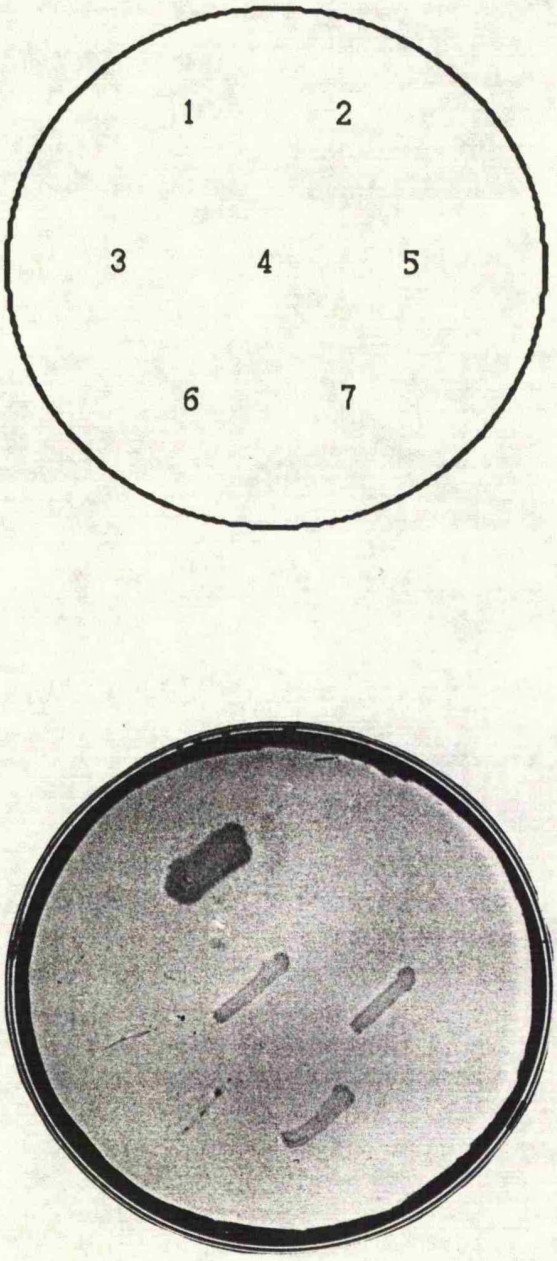
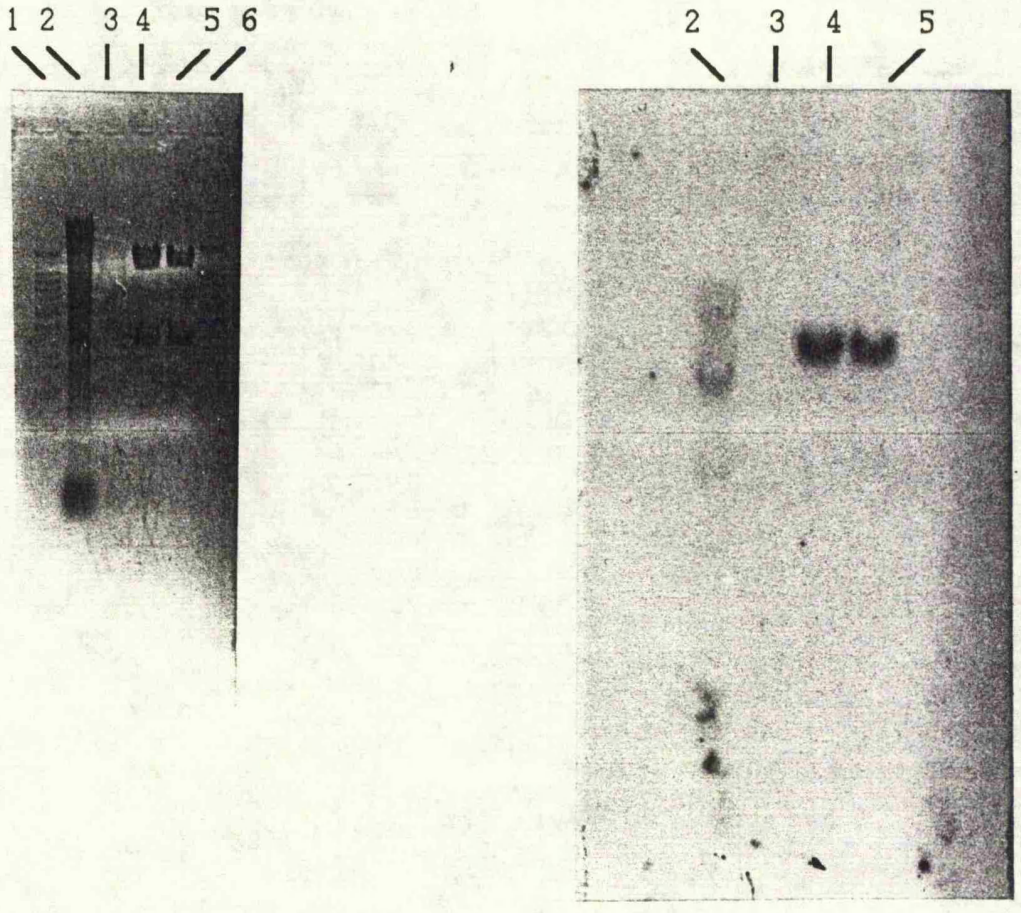


Figure 5.4. *Southern analysis of constructs pJG1, pJG5 and pJG31, restricted with* Eco*R I.*

Aliquots of chromosomal and plasmid DNA were digested with *Eco*R I and the fragments separated by horizontal slab agarose gel electrophoresis. The DNA was then transferred on to a nylon membrane, hybridised with a 32P-labelled oligonucleotide probe (256 degeneracies) and autoradiographed, as described in Methods. Tracks 1 & 6, molecular size standards; track 2, chromosomal DNA; track 3 pJG1; track 4, pJG5; track 5, pJG31.



could not be determined. This was probably due to the large size and low copy number of the construct, which made DNA manipulations difficult. No hybridizing band was observed upon Southern analysis (Fig 5.4). Therefore, even though the 5' end of the gene encoding the N-terminal portion of the mature *Ps. aeruginosa* EF2 lipase could not be identified, pJG1 probably contained the lipase gene, as *E. coli* JG1 grew on Tween 80.

On the basis of these results, pJG5 and pJG31, together with their relatively small size and high copy number, were chosen for further analysis.

5.6.3 *Restriction mapping of pJG5.*

pJG5 was digested with various endonucleases, and fragments containing the 5' end of the gene encoding the N-terminal portion of the mature lipase were determined by Southern analysis (Fig. 5.5 and Table 5.2). The estimated size of pJG5 was approximately 11.3 kbp, comprising the 2.7 kbp pUC18 plasmid and an 8.6 kbp chromosomal fragment inserted at the *Eco*R I site, thus confirming earlier observations (Section 5.6.2).

In order to construct a restriction map, a series of double digests were performed using *Eco*R I and one other restriction enzyme (Fig 5.6 and Table 5.3). In each case a 2.7 kbp fragment (corresponding to the pUC18 plasmid) was generated, together with fragments ranging in size from 0.6 to 8.6 kbp. The precise location of all the *Pst* I, *Sal* I, *Sph* I and *Sma* I restriction sites could not be determined, due to the small sizes of the generated fragments. The proposed restriction site map of pJG5 is presented in Fig. 5.7.

The orientation of the 8.6 kbp *Eco*R I fragment in pJG5 was compared to that in pJG31. Digestion of pJG5 with *Hin*d III yielded asymmetric fragments of approximately 7.6 and 4.1 kbp. If the 8.6 kbp fragment was

Figure 5.5. *Southern analysis of pJG5.*

Aliquots of pJG5 were digested with various restriction endonucleases and the fragments separated by horizontal slab gel electrophoresis. The DNA was then transferred on to a nylon membrane, hybridised with a 32P-labelled oligonucleotide probe (256 degeneracies) and autoradiographed, as described in Methods. Tracks 1&12, molecular size standards; track 2, *Eco*R I-cut *Ps. aeruginosa* EF2 chromosomal DNA; track 3, *Eco*R I-cut *Ps. aeruginosa* EF2 chromosomal DNA (5 to 9 kbp); tracks 4 to 11, restricted pJG5, with the size of the hybridising fragment indicated in brackets (in kbp): 4, *Hin*D III (7.6); 5, *Sph* I (3.2); 6, *Pst* I (4.0); 7, *Sal* I (2.5); 8, *Bam*H I (4.3); 9, *Sma* I (2.1); 10, *Sac* I (11.2); 11, *Eco*R I (8.6).

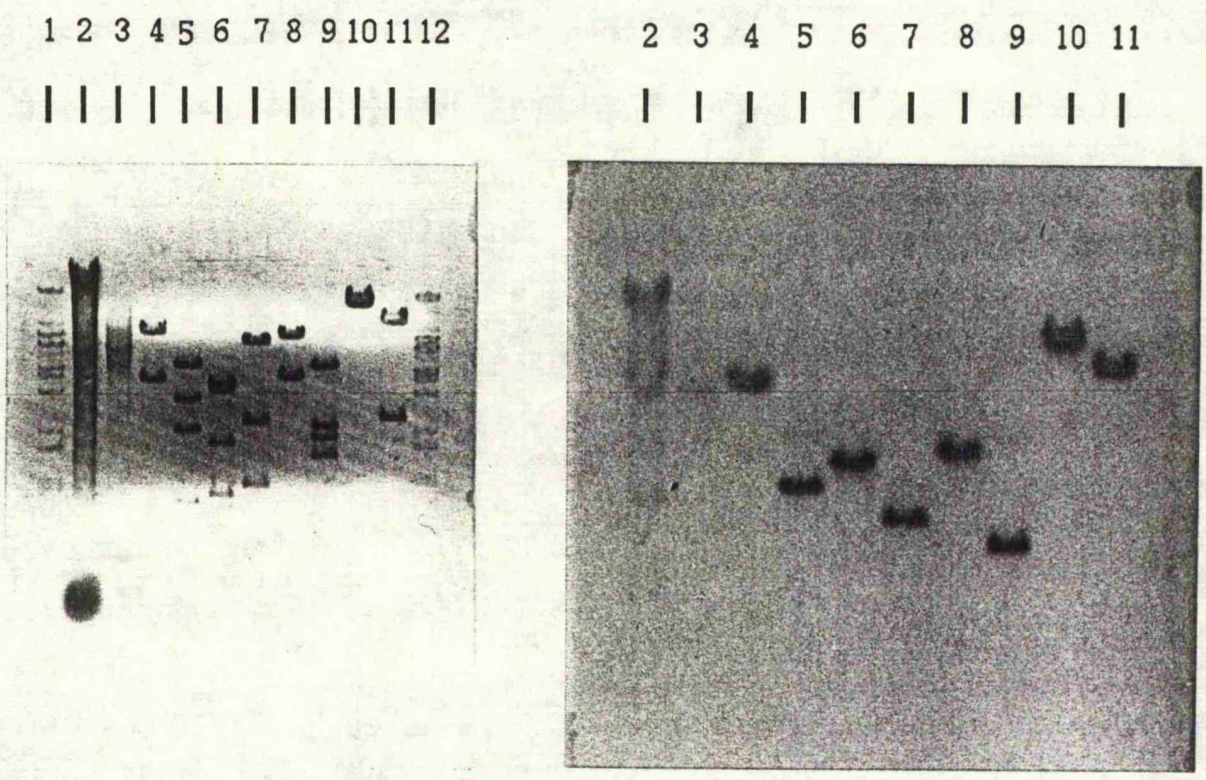


Table 5.2 *Single endonuclease restriction of pJG5.*

pJG5 was isolated from *E. coli* JM109 by the alkaline-lysis mini-preparation method, as described in Methods. Aliquots were digested with various restriction endonucleases and the fragments separated by agarose gel electrophoresis. Size estimations were made by comparisons with the migration of λ *Bst*E II molecular weight standards.

Restriction endonuclease	Size of fragments generated (kbp)					Total estimated size of construct (kbp)
*Hin*d III	7.6*	4.1				11.7
Sph I	5.0	3.2*	2.3	1.0		11.5
Pst I	4.0*	3.6	2.0	1.1	1.0	11.7
Sal I	6.6	2.5*	1.3	0.8	0.8	12.0
*Bam*H I	7.2	4.3*				11.6
Sma I	5.0	2.4	2.1*	1.8		11.3
Sac I	11.2*					11.2
*Eco*R I	8.6*	2.7				11.3

*, fragment shown to contain the 5' end of the gene encoding the N-terminal portion of the mature lipase.

Figure 5.6. *Restriction analysis of pJG5.*

pJG5 was digested for 1h at 37oC with *Eco*R I, then purified using Geneclean. Aliquots were then digested with various restriction endonucleases and the fragments separated by horizontal slab agarose gel electrophoresis, as described in Methods. Tracks 1 & 10, molecular size standards; tracks 2 to 9, *Eco*R I-cut pJG5 digested with: 2, *Hin*D III; 3, *Sph* I; 4, *Pst* I; 5, *Sal* I; 6, *Bam*H I; 7, *Sma* I; 8, *Sac* I; 9, *Eco*R I.

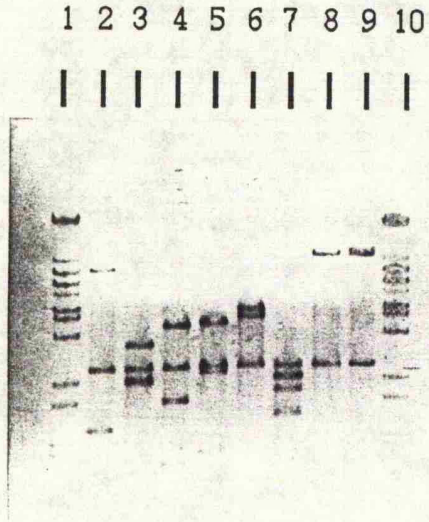


Table 5.3 *Double endonuclease restriction of pJG5.*

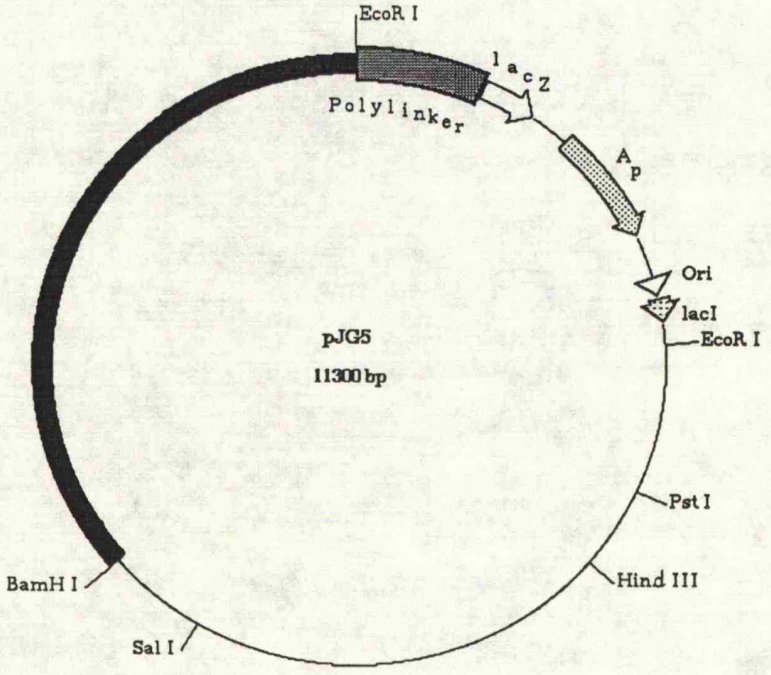
pJG5 was isolated from *E. coli* JM109 by the alkaline-lysis mini-preparation method, as described in Methods. Aliquots were digested with *Eco*R I plus another restriction endonuclease. The fragments were separated by agarose gel electrophoresis and the sizes estimated by comparisons with the migration of λ *Bst*E II molecular weight standards.

Restriction endonuclease	Size of fragments generated (kbp)						Total estimated size of construct (kbp)
*Hin*d III	7.7	2.7	1.5				11.9
Sph I	3.3	2.7	2.4	1.0			9.4
Pst I	4.0	2.7	2.0	1.1	1.0	0.9	11.7
Sal I	4.1	2.7	2.6	1.2	0.6		11.2
*Bam*H I	4.8	2.7	4.3				11.8
Sma I		2.7	2.4	2.1	1.7		8.9
Sac I	8.6	2.7					11.3
*Eco*R I	8.6	2.7					11.3

The 2.7 kbp fragment represents the pUC18 vector.

Figure 5.7. *Restriction map of pJG5.*

The construct comprises the 2.7 kbp pUC18 plasmid containing a 8.6 kbp *Eco*R I fragment of *Ps. aeruginosa* EF2 chromosomal DNA inserted at the *Eco*R I restriction site within the polylinker. The 4.3 kbp *Bam*H I to *Eco*R I region (shown as a thick black line) encodes the N-terminal region of the mature lipase.



inserted in the plasmid in the opposite orientation in pJG31, 9.9 and 1.4 kbp fragments would be expected upon restriction with *Hind* III (Fig. 5.8). This was shown not to be the case, as identical sized fragments to those from pJG5 were obtained.

5.6.4 *Sub-cloning of pJG5.*

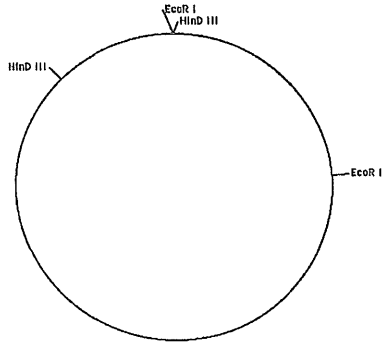
As the functional lipase gene of *Ps. aeruginosa* EF2 was calculated to be encoded on a minimum stretch of DNA of not less than 1 kbp (Section 5.3), the 8.6 kbp *Eco*R I fragment which conferred the lipase-positive phenotype on *E. coli* JM109 probably contained additional DNA that was not required. Subclones of pJG5 were therefore constructed that contained either the 4.3 kbp *Bam*H I fragment, the 2.5 kbp *Sal* I fragment, or the 3.2 kbp *Sph* I fragment (each of which was known to contain the 5' end of the gene encoding the N-terminal sequence of the mature lipase) ligated into pUC18. These were designated pJG5.1, pJG5.2 and pJG5.3, respectively. Restriction of these plasmids with *Bam*H I, *Sal* I and *Sph* I respectively (with or without *Eco*R I) confirmed the sizes of the inserts (Fig. 5.9).

These sub-clones grew on a nutrient agar-ampicillin medium, but failed to grow on a M9 minimal salts-Tween 80-ampicillin medium, even after prolonged incubation at 37°C and room temperature (Fig 5.10). It was therefore concluded that the complete functional gene(s) or *cis*-acting regulatory elements required to confer the lipase phenotype were not present in these constructs, despite the presence of the 5' portion encoding the N-terminal region of the mature enzyme.

An attempt to sequence this 5' region of the lipase gene in pJG5.1 and pJG5.2 using the 256-degeneracy oligonucleotide probe as a primer were unsuccessful. Short sections of DNA sequence were obtained, but band compressions prevented further reading. This was unfortunate, as it may

Figure 5.8. *Possible orientaions of the 8.6 kbp* Eco*R I fragment in pJG31.*

i. Restriction with *Hin*d III yields a 9.9 kbp and a 1.4 kbp sized fragment.



ii. Restriction with *Hin*d III yields a 4.1 kbp and a 7.6 kbp sized fragment.

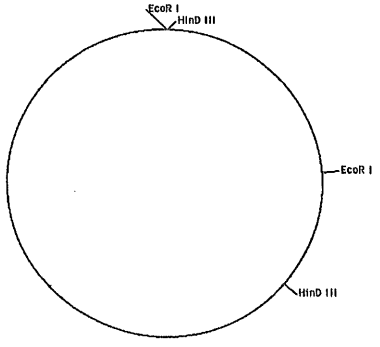


Figure 5.9. *Restriction analysis of the sub-clones pJG5.1, pJG5.2 and pJG5.3.*

Aliquots of plasmid were digested for 1.5 h at 37oC with either *Eco*R I or *Bam*H I, *Sal* I or *Sph* I for pJG5.1, pJG5.2 and pJG5.3, respectively. The fragments were then separated by horizontal slab gel electrophoresis, as described in Methods. Tracks 1 & 8, molecular size standards; track 2, *Bam*H I-cut pJG5.1; track 3, *Eco*R I-cut pJG5.1; track 4, *Sal* I-cut pJG5.2; track 5, *Eco*R I-cut pJG5.2; track 6, *Sph* I-cut pJG5.3; track 7, *Eco*R I-cut pJG5.3.

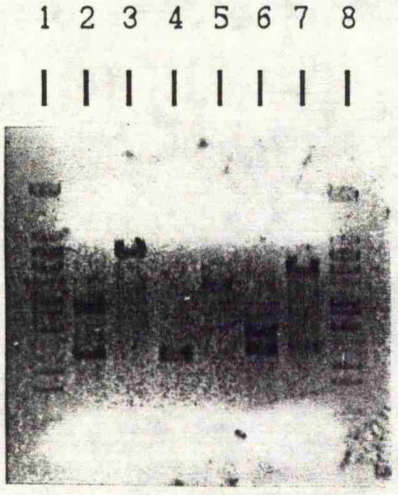
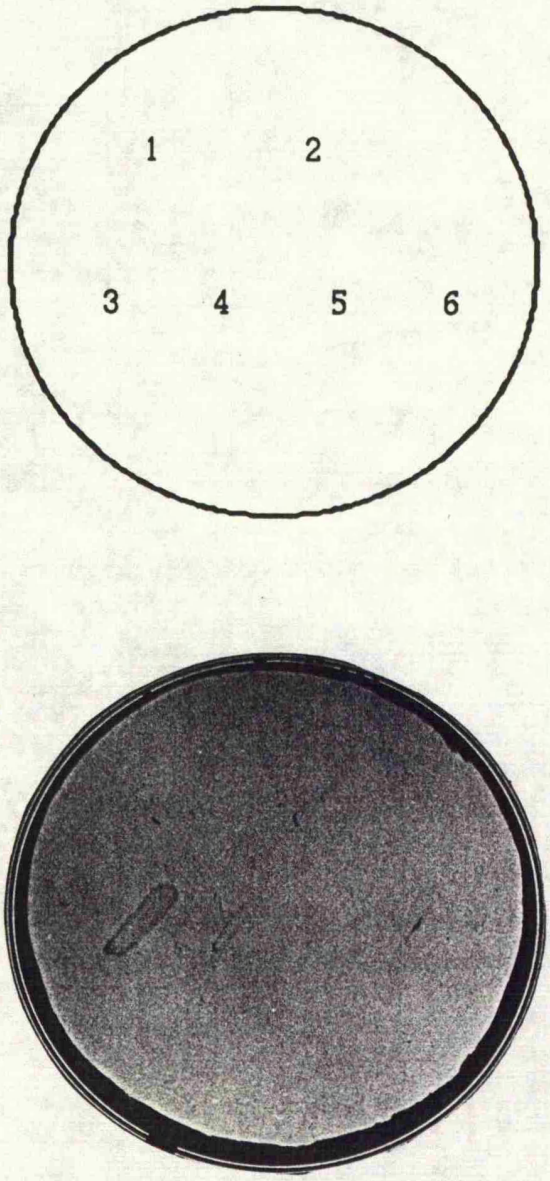


Figure 5.10. Comparative growth of E. coli JM109 harbouring pUC18, pJG5, pJG5.1, pJG5.2 and pJG5.3 on a Tween 80-M9 minimal-salts agar medium.

The agar plate was streaked with a single colony of the appropriate strain then incubated at 37°C over-night, followed by 5 d at room temperature, as described in Methods. Colony 1, *E. coli* JM109; colonies 2 to 6,*E. coli* JM109 harbouring the plasmids: 2, pUC18; 3, pJG5; 4, pJG5.1; 5, pJG5.2, 6, pJG5.3.



have enabled the structure of the lipase gene to be determined (hence the amino acid sequence of the protein to be deduced) and some of the regulatory elements to be identified

5.7 *Construction of pJG3.*

The very slow growth rates of *E. coli* JG5 and JG31 on M9 minimal salts-Tween 80 media indicated poor production and secretion of the recombinant lipase. The use of a *Pseudomonas* species host was deemed to be advantageous over *E. coli*, due to differences between the two genera, such as gene codon usage and protein secretion (Section 5.2). As the size of the insert in the pKT230-based construct pJG1 could not be determined, the 8.6 kbp *Eco*R I fragment was removed from pJG5 and ligated into pKT230. This was used to transform *E. coli* NM522 (shown to be lipase-negative, kanamycin- and streptomycin-sensitive). The presence of the fragment was confirmed by colony hybridisation using the purified 8.6 kbp *Eco*R I fragment recovered from pJG5 as a probe (C.A. Ambrose, personal communication). The construct was designated pJG3. *E. coli* NM522 containing pJG3 grew on a M9 minimal salts-Tween 80-kanamycin agar medium only after prolonged incubation.

Ps. putida P2440 was then transformed with pJG3, but the latter could not be recovered from the cells by the alkaline lysis mini-preparation method previously used successfully with *E. coli* JM109, due to the problems of excessive protein production (previously outlined; Section 2.17.1). The recombinant strain grew overnight at 37°C on an M9 minimal salts-Tween 80-kanamycin agar medium, indicating efficient expression and secretion of the recombinant lipase. However, wild-type *Ps. putida* P2440, although exhibiting sensitivity to both streptomycin and kanamycin, was subsequently

shown to grow on a Tween 80 agar medium. This was in contrast to earlier observations, which indicated that the strain was lipase-negative (Section 5.4; Fig 5.11). It was therefore doubtful whether the ability of *Ps. putida* P2440 to grow on a Tween 80-kanamycin plate was due soley to expression of the lipase (and kanamycin resistance) genes contained on pJG3.

5.8 *Lipase and esterase activities of* E. coli *JG5 and* Ps. putida *JG3 grown in batch culture.*

5.8.1 *Extent of lipase production.*

Lipase production by the clones was determined during growth in batch culture on M9 minimal salts medium supplemented with glucose or Tween 80 and an appropriate antibiotic (Table 5.4). *Ps. aeruginosa* EF2 exhibited far higher lipase and esterase activities following growth on Tween 80 then on glucose, thus confirming the inducible nature of lipase production (Section 3.4.3).

E. coli JM109 failed to grow on Tween 80, but the presence of pJG5 conferred the lipase-positive phenotype, enabling growth on the surfactant, albeit at a very slow rate (confirming previous observations of growth on an M9 minimal salts-Tween 80 agar medium). Both lipase and esterase activities were very low; the former being near the minimum limits of detection. By assuming a growth yield (Y) of *E. coli* JG5 on Tween 80 of 197 g cells (mol oleic acid)$^{-1}$ (Sections 3.4.3 and 3.6.3) growing at a specific growth rate (μ) of 0.09 h^{-1}, the *in vivo* rate of oleic acid utilisation (q_{OA}) can be calculated according to the following equation:

Figure 5.11. *Comparative growth of* E. coli *JM109 harbouring pJG5,* E. coli *NM522 harbouring pJG3 and* Ps. putida *P2440 harbouring pJG3 on a Tween 80-M9 minimal-salts agar medium.*

The agar plate was streaked with a single colony of the appropriate strain then incubated at 37°C over-night, followed by 5 d at room temperature, as described in Methods. Colony 1, *E. coli* JM109; colony 2, *E. coli* JM109 plus pJG5; colony 3, *E. coli* NM522; colony 4, *E. coli* NM522 plus pJG3; colony 5,*Ps. putida* P2440; colony 6, *Ps. putida* P2440 plus pJG3.

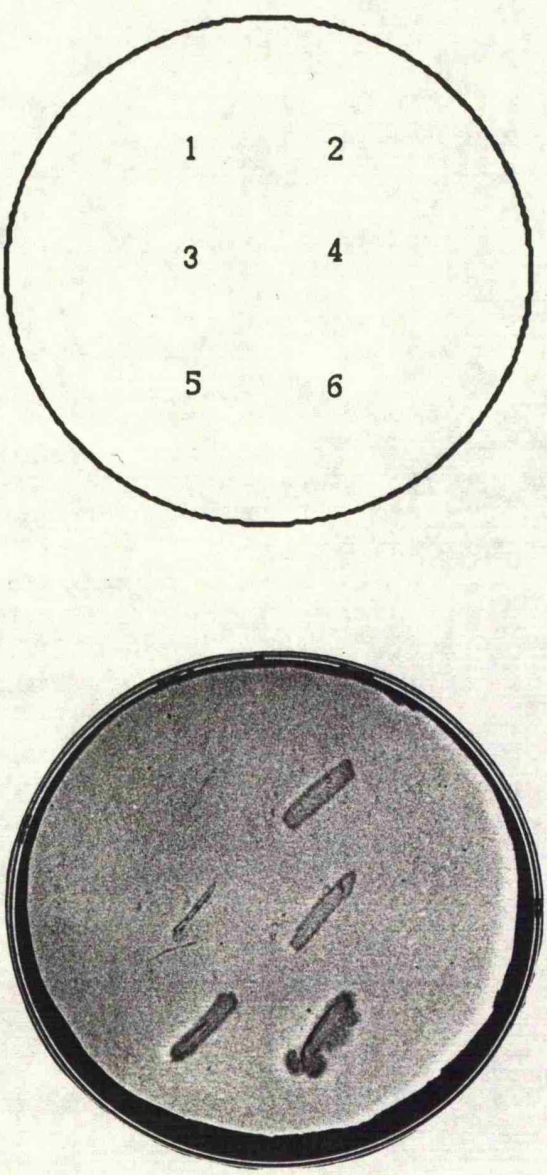


Table 5.4 *Lipase and esterase activities of various clones following growth in batch culture.*

Organisms were grown in batch culture in an M9 minimal salts medium, pH 7.4, supplemented with Tween 80 or glucose. Maximum lipase and esterase (*p*-nitrophenyl acetate as substrate) activities were determined as described in Methods.

Strain	Carbon source	μ_{max} (h^{-1})	Enzyme activities [LU or µmol min^{-1} (mg cells)$^{-1}$] Lipase	Esterase
Ps. aeruginosa EF2	Tween 80	0.63	10.8	2.63
	Glucose	1.16	0.3	0.22
E. coli JM109	Tween 80	0	ND	ND
	Glucose	0.52	ND	0.08
E. coli JM109 + pJG5	Tween 80	0.09	0.2	0.17
	Glucose	0.73	0.3	0.22
Ps. putida P2440	Tween 80	0.20	0.6	0.79
	Glucose	0.63	0.5	0.22
Ps. putida P2440	Tween 80	0.31	0.7	0.85
+ pJG3	Glucose	0.41	ND	0.17

ND; not determined.

$$q_{OA} = \frac{\mu}{Y} = \frac{0.09}{197}$$

$$= 4.57 \times 10^{-4} \text{ mol } h^{-1} \text{ (g cells)}^{-1}$$

$$= 7.6 \text{ nmol } min^{-1} \text{ (mg cells)}^{-1}$$

Therefore the lipase and esterase activities detected were over a twenty-times greater than the minimum rate of Tween 80 hydrolysis required to support support growth. Lipase and esterase activities of Tween 80- and glucose-grown cells did not differ significantly, suggesting that expression of the lipase gene did not require induction by Tween 80. Furthermore, lipase production (measured only as esterase activity) did not appear to be subject to product repression, as similar activities were detected during both the exponential and stationary phases of growth (Table 5.5). This differs from lipase production in *Ps. aeruginosa* EF2. The possibility exists that regulatory elements (such as a putative repressor protein) which govern lipase production in *Ps. aeruginosa* EF2 were not present in *E. coli* JM109; thus expression was probably constitutive.

As the lipase activities of *Ps. putida* P2440 and JG3 grown on both glucose and Tween 80 were near the minimum limits of detection, no conclusions could be drawn from these results. However, esterase activities were independent of the presence of pJG3, thus confirming the suspicion that the wild-type strain was indeed lipase-positive. Esterase activities were greater in Tween 80- than glucose-grown cultures, suggesting that, like *Ps. aeruginosa* EF2, they were induced by Tween 80 (even though there was a possibility that a separate lipase and esterase might have existed). Nevertheless, *Ps. putida* P2440 was a far less prolific producer of lipase and esterase activities than *Ps. aeruginosa* EF2.

Table 5.5 *Location of esterase activities produced by* Ps. aeruginosa *EF2 and* E. coli *JM019 harbouring pJG5.*

Esterase activities (*p*-nitrophenyl acetate as substrate) were determined in exponential and stationary phase cultures (and in various fractions derived from these) following growth in M9 minimal-salts medium, pH 7.4, supplemented with either Tween 80 or glucose, as described in Methods.

Strain	Carbon source	Growth phase	Esterase activities [μmol min^{-1} $(mg\ cells)^{-1}$]			
			Culture	Culture supernatant	Whole cells	Sonicated cells
Ps. aeruginosa EF2	Tween 80	E	0.21	0.15	0	0.02
		S	2.63	1.90	0.1	0.1
	Glucose	E	0.10	0.04	0	ND
		S	0.15	0.13	0	0
E. coli JG5	Tween 80	E	0.17	0.08	0	0
		S	0.14	0.14	0	0
	Glucose	E	0.22	0	0.24	0.09
		S	0.09	0	0.08	0.10

E, exponential phase; S, stationary phase; ND, not determined.

5.8.2 *Location of lipase.*

The distribution of esterase activity of *Ps. aeruginosa* EF2 and *E. coli* JG5 were compared following growth on Tween 80 or glucose (Table 5.5). The esterase activity of *Ps. aeruginosa* EF2 grown on both Tween 80 and glucose was detected predominantly in the supernatant fractions, thus confirming the extracellular nature of the enzyme (Section 3.4.3). However, although the recombinant lipase produced by *E. coli* JM109 grown on Tween 80 was also predominantly extracellular, the enzyme appeared to remain bound to the cells following growth on glucose. It was concluded that the *E. coli* host was unable to efficiently secrete the recombinant *Pseudomonas* lipase (Section 5.2) during growth on glucose; although whether this simply reflected the physical absence of the surfactant Tween 80 (Section 3.7) or that a second gene responsible for secretion of the lipase (such as that reported in *Ps. cepacia* and *Ps. gladioli*; Section 5.2) was induced by Tween 80 but not by glucose, remains to be determined.

The possibility also existed that *E. coli* JG5 produced large amounts of lipase that were not correctly processed and therefore remained in an inactive form (such as that noted by Chung *et al.*, 1991). SDS-PAGE of a glucose-grown culture of *E. coli* JG5 did not reveal the presence of a protein of M_r 29,000. This was hardly surprising, as it was barely detectable in supernatants taken from high lipase activity *Ps. aeruginosa* EF2 cultures.

5.9 *Discussion.*

The lipase gene of *Ps. aeruginosa* EF2 was identified by Southern analysis of *Eco*R I-restricted genomic DNA, despite the highly-degenerate oligonucleotide probe used and the problems encountered with the Hybond N membrane. In retrospect, an oligonucleotide probe based on the codon bias exhibited by *Pseudomonas* species (West & Iglewski, 1988) would probably have hybridized more strongly to the gene.

N-terminal amino acid sequence	Y	T	Q	T	Q	Y	P
Most probable nucleotide sequence	TAC	ACC	CAG	ACC	CAG	TAC	CCG

The lipase gene, contained on the plasmid vectors pKT230 (pJG1) and pUC18 (pJG5 and pJG31) were cloned in *E. coli* JM109; pJG5 was further characterised and a variety of restriction sites mapped. Sub-clones of the 8.6 kbp *Eco*R I fragment containing the functional lipase gene in pJG5 were generated (pJG5.1, pJG5.2 & pJG5.3). These all contained the 5' end of the gene encoding the N-terminal portion of the mature enzyme, and were all larger than the calculated minimum size of DNA required to encode the lipase gene. However, *E. coli* JM109 containing these plasmids failed to grow on Tween 80, indicating that either the functional gene was not present or was not expressed. Although part of the 5' end of the structural gene was known to be present (confirmed by Southern blotting) the presence of the 5' and 3' untranslated sequences necessary for transcription and translation, the complete N-terminal secretory signal sequence and the remainder of the structural gene, were unknown. Recently, a second gene responsible for efficient secretion and/or regulation of lipase production has been identified (Table 5.1). Although this has been confined to *Ps. cepacia* and *Ps. glumae,* there is a distinct possibility that a similar situation exists in *Ps. aeruginosa* EF2. This is supported by the observations that pJG5.1, pJG5.2 and pJG5.3

(containing 2.5 to 4.3 kbp fragments) all failed to confer the lipase phenotype (although the exact location of the structural gene on the fragments was unknown).

The slow growth of *E. coli* JG5 on an M9 minimal salts-Tween 80-ampicillin medium was similar to other *Pseudomonas* lipase genes cloned in *E. coli* (Table 5.1), which were also expressed at low levels. This may be due to poor transcription/translation of the lipase gene by *E. coli*, possibly due to the high guanine plus cytosine content of the *Pseudomonas* genome (which would be reflected in the codon bias) and/or the inability of the hosts secretory mechanisms to recognise and transport the foreign protein efficiently. The latter was supported by the observation that enzyme activities were predominantly extracellular when grown on Tween 80, whilst cell-bound when grown on glucose. Other recombinant extracellular *Pseudomonas* enzymes have been cloned and expressed in *E. coli*, but not secreted (see for example, Bever & Iglewski, 1988), probably due to differences in the secretory mechanisms employed by the two bacteria. Tween 80 has been shown to release enzyme bound to cell walls (Section 3.7) and may therefore facilitate the release of lipase. Alternative methods of isolating sub-cellular fractions (such as cytoplasm and periplasmic fractions, inner and outer membranes) should have been employed to confirm the exact location of the recombinant lipase.

To circumvent the inherent problems of efficiently expressing and secreting a recombinant *Pseudomonas* lipase in *E. coli*, the supposedly lipase-negative *Ps. putida* P2440 was transformed with pJG5.4. Unfortunately, contrary to earlier observations, the host strain exhibited the lipase-positive phenotype (the supplied sample was possibly contaminated) and was therefore unsuitable for further characterisation. Lipase production following growth in batch cultures did not depend upon the presence of the

recombinant plasmid. However, it confirmed the inducible nature of lipase production by Tween 80 and emphasized the superiority of *Ps. aeruginosa* EF2.

Production of the recombinant lipase in *E. coli* JG5 was not subject to the induction/repression controls which operated in *Ps. aeruginosa* EF2. This would obviously confer a process advantage were the lipase to be produced commercially; however, the problems outlined above would first have to be overcome to over-express and secrete the recombinant lipase efficiently. The pUC18-based constructs were not under control of the *lacZ* promoter, as no difference in growth on Tween 80-ampicillin media was observed upon induction with IPTG. The low levels of lipase produced by *E. coli* JG5 could therefore be improved by cloning the lipase gene directly down-stream of this promoter, or of a stronger promoter, such as the *tac* (*trp/lac*) promoter. This was recently described by Chung *et al.* (1991) who over-produced a recombinant *Ps. fluorescens* lipase in *E. coli*. The host failed to secrete the lipase, such that it comprised over 40% of the cellular protein, and accumulated within the cytoplasm as insoluble inclusion bodies.

Further characterisation of the cloned *Ps. aeruginosa* EF2 lipase gene would require the use of an alternative method to identify positive clones which produces rapid, clearly defined results (possibly after an overnight incubation) in contrast to growth on Tween 80-antibiotic plates, which were at best slow. This might involve an overlay technique, such as that described by Andreoli *et al.* (1989). Western analysis of *E. coli* JM109 cell extracts using polyclonal antibodies raised against *Ps. aeruginosa* PAC1R lipases indicated they were unsuitable as probes to detect recombinant lipase, as extensive cross-reactivity with other proteins was observed. This was unfortunate, as it provided a potentially simple method of determining the production of small

amounts of recombinant lipase within a heterologous host that might not be sufficient to support growth on a Tween 80 selective medium.

The production of the cloned *Ps. aeruginosa* EF2 lipase in a homologous host, such as *Ps. aeruginosa* 6-1 (Wohlfarth *et al.*, 1988) should be attempted, despite the problems associated with isolating *Pseudomonas* DNA. In addition, the recently described *Escherichia / Pseudomonas* shuttle vectors, based on pUC18/19 (pUCP18/19) have many potential advantages over pKT230; their smaller size (4.5 cf. 11.9 kbp), useful multiple cloning site and relatively high copy number (Schweizer, 1991) would make them much easier to manipulate.

CHAPTER 6.

GENERAL DISCUSSION.

6.1 Summary of achievements.

6.2 Possible industrial applications.

6.3 Future proposals.

6.1 *Summary of achievements.*

Prior to 1988, very little was known about the physiological regulation of bacterial lipase production. The initial aim of this thesis was to investigate lipase production in a newly-isolated strain of *Pseudomonas aeruginosa* (strain EF2), which involved the use of batch, fed-batch and continuous culture techniques. Growth on a variety of different carbon substrates in batch culture indicated that lipase activity was induced by growth on long chain fatty acyl esters (especially Tween 80) and repressed by long chain fatty acids (particularly oleic acid). This was confirmed by growth in fed-batch and continuous cultures and led to the subsequent optimisation of lipase production in a Tween 80-limited continuous culture using statistical response surface analysis. Although statistical approaches have been used to optimise lipase production (Harris *et al.*, 1990), the systematic study of lipase production under defined conditions using a soluble substrate has not. These observations confirmed the inducible nature of bacterial lipase production (Gowland *et al.*, 1987), but also indicated that production by *Ps. aeruginosa* EF2 was tightly controlled by a combination of substrate induction and product repression mechanisms.

The extracellular lipase of *Ps. aeruginosa* EF2 was isolated from a high activity continuous culture and purified to homogeneity. Various biochemical properties were then determined. Similar studies have been performed on other *Pseudomonas* lipases and the results varied considerably. An assessment of the properties of this novel enzyme was therefore necessary. Most notable was the high specific activity and catalytic constant of the purified enzyme. Other properties were similar to the lipases of other *Pseudomonas* species, in particular the extent of homology of the N-terminal amino acid sequence, many of which have only recently been published.

Despite initial difficulties experienced with Southern analysis, the structural gene of the *Ps. aeruginosa* EF2 lipase was cloned and expressed, albeit at low levels, in *E. coli* JM109. Sub-cloning of the original DNA fragment using the plasmid pUC18, indicated that the functional gene(s) were encoded on a large stretch of DNA. The poor expression of the extracellular *Pseudomonas* lipase in *E. coli* led to the transfer of the lipase gene(s), using the shuttle vector pKT230, into a supposedly lipase-negative *Ps. putida* strain (P2440), in an unsuccessful attempt to over-produce the recombinant lipase.

6.2 *Possible industrial applications.*

Although lipase production by *Ps. aeruginosa* EF2 was increased over a hundred-fold using statistical optimisation in a Tween 80-limited continuous culture (*cf.* growth on glucose in batch culture), the commercial production of the *Ps. aeruginosa* EF2 lipase under these conditions is unlikely. Legislation regulates the amount of surfactants permitted in effluent waste (Garner, 1991); an important consideration bearing in mind the surface-active nature of a Tween 80-limited culture and the presence of the hydrolysis product PEG, which contains a non-hydrolysable ether bond. In addition, the surfactant properties of Tween 80 would undoubtedly cause severe foaming problems within an industrial-sized fermenter, probably rendering the process unfeasible. However, as a fundamental understanding of the regulation of lipase production by this isolate has been determined, this would enable the formulation of an appropriate, industrially-viable growth medium, rather than one based purely on empirical observations.

The high activity and relative thermostability of the *Ps. aeruginosa* EF2 lipase may make it suitable for a variety of different applications. In particular, the 1,3-positional specificity of the lipase may be useful to either modify or selectively hydrolyse various triacylglycerols. Incorporation into a detergent formulation may be limited by the apparent sensitivity to the anionic surfactant SDS. However, an assessment of the substrate and stereo-specificities of the lipase might facilitate its use within the speciality chemicals industry.

Whatever its use, it seems likely that the *Ps. aeruginosa* EF2 lipase would be produced as a recombinant enzyme in a benign host. The large volumes of *Ps. aeruginosa,* a potential pathogen (Iglewski, 1989), required to produce sufficient quantities of the enzyme are undesirable. More specifically, the possible contamination of the preparation with the endotoxin lipopolysaccharide (Brock *et al.*, 1984) would, in all probability, ensure that it would not pass the extensive toxicological tests required, for use within the detergent or food industries (Towalski, 1986, 1987; Jensen & Eigtved, 1990).

6.3 *Future proposals.*

The use of batch, fed-batch and continuous culture techniques enabled the physiological control of lipase production by *Ps. aeruginosa* EF2 to be determined. The precise nature of these regulatory mechanisms (*ie.* whether positive and/or negative systems operate), however, remains unanswered. Nucleotide sequencing of the cloned structural gene and the 5' untranslated region, would enable the identification of possible promoter sites or regulatory protein binding sites, which are generally highly conserved in bacteria (see, for example, Busby, 1986). In addition, a comparative analysis with other *Pseudomonas* lipase sequences could be

made, especially with respect to the conserved pentapeptide sequence around the active site serine residue and a putative secretory signal sequence.

Recent papers have identified a second gene located directly downstream of the structural lipase gene in certain *Pseudomonas* species, which is thought to regulate secretion of the extracellular enzyme (Frenken *et al.*, 1991; Jørgensen, 1991; Jørgensen *et al.*, 1991; Nakanishi *et al.*, 1989, 1991). Sub-clones (ranging from 2.5 to 4.3 kbp in size) of the original lipase-encoding DNA fragment from *Ps. aeruginosa* EF2, failed to confer the lipase-positive phenotype upon *E. coli*. Bearing in mind the possibility of a *cis*-acting adjacent gene, this observation suggests that the functional *Ps. aeruginosa* EF2 lipase gene is encoded on a large stretch of DNA, therefore this region may also contain a secondary gene. The identification of a putative lipase operon would be unique within *Ps. aeruginosa*, and information obtained about the regulation and transcription/translation of the two genes would be valuable. In addition, as nothing is known about the secretion of this novel lipase (a factor that probably impedes production in *E. coli*), identification of the mechanisms involved would be highly advantageous.

A knowledge of the controls that regulate both the synthesis and secretion of the *Ps. aeruginosa* EF2 lipase would enable the construction of a high level expression system, designed to optimise recombinant lipase production. This might involve site-directed mutagenesis within the 5' untranslated regions to prevent binding of a putative repressor protein (to obtain constitutive lipase production), or to increase the strength of the promoter. Alternatively, the complete deletion of the activator/repressor binding sites and the insertion of a strong inducible promoter, such as the synthetic *tac* promoter for *E. coli*, might be feasible.

Due to the high guanine plus cytosine content of *Pseudomonas* species, the successful expression of the recombinant lipase gene(s) would almost inevitably require the use of either a homologous host, such as *Ps. aeruginosa* 6-1 (Wohlfarth & Winkler, 1988) or even *Ps. aeruginosa* EF2 itself, or a heterologous host with a favourable G/C % ratio, such as *Agrobacterium* (which would be advantageous bearing in mind its non-pathogenicity towards humans). In addition, the deletion of the region encoding the putative *Ps. aeruginosa* EF2 secretory signal sequence and replacement with the sequence of a well-defined secretion system specific for the intended host, might suffice.

The production of large amounts of lipase would enable further biochemical studies to be carried out. In particular, the growth of protein crystals for X-ray analysis might be possible, a procedure that would otherwise be limited by the small amounts of lipase secreted by *Ps. aeruginosa* EF2. If kinetic and biochemical studies were to be of interest, the use of site-directed mutagenesis to modify specific amino acid residues in the mature protein (*eg*. the proposed active site serine) might facilitate the elucidation of the catalytic mechanism of this enzyme and enable novel, potentially commercial applications to be found.

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