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Catabolism of wax esters
in
Acinetobacter calcoaceticus

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Thesis Submitted for the Degree of Doctor of Philosophy
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1990

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

*In the name of
Allah,
Most Gracious, Most Merciful.*

*To my parents, wife
and daughters, Maria and Sarah*

Acknowledgements

I would like to thank Professor Miles D. Houslay and the late Professor R.M.S. Smellie for making the facilities of this department available to me. My special thanks are due to the Pakistan Medical Research Council and the Ministry of Science and Technology, Government of Pakistan, for awarding me a scholarship.

I wish to express my utmost appreciation to my supervisor Dr. Leslie M. Fixter for invaluable guidance, constructive criticism, inspiring new ideas and his friendly nature, without whose supervision this work could not have been possible. My gratitude is also due to Professor Charles A. Fewson for his kindness and for providing me the opportunity to work in the Microbial Biotechnology Group. I have been greatly inspired by his laboratory administration, professional learning, critical discussions and suggestions throughout my training.

My gratitude is also extended to my colleagues in C24 for their friendship and for making the lab an enjoyable place to work. In particular, my grateful thanks to Darren Baker, Jacqueline Murray, Martin McAvoy, Atip Likidlilid, Iffat Hussain, Joe Okungbowa, Mohammad Yasin, and Anuj Markand Bhatt. I would especially like to thank Martin McAvoy for his invaluable help in proof reading of this manuscript. I am also indebted to Patrick Ferry, Jim Jardine, Alan Scott and Mrs. M.A. Cowan, for their technical advice.

Finally I wish to record my gratitude to my family; especially to my parents for their encouragement throughout my studies and to my wife for her unfailing patience and understanding.

Abbreviations

The abbreviations used in this thesis are those recommended by the Biochemical Journal, London in the Instruction to Authors [Biochem. J. (1989) 257, 1-21] , except for the following additions:

ATCC	-American Type Culture Collection
" "	-Names of bacterial species which are not on the approved List of Bacterial Names are given in inverted commas, [Int. J. Syst. Bacteriol. (1980) 30, 225-420 and later amendments]
BSA	-Bovine serum albumin
DTT	-Dithiothreitol
KDO	-2-Keto-3-deoxy octonic acid
MTT	-3-(4,5-Dimethyl thiazolyl-2) 2,5-diphenyl-tetrazolium bromide
NBS	- <i>N</i> -Bromosuccinamide
NCIB	-National Collection of Industrial Bacteria
PAGE	-Polyacrylamide gel electrophoresis
PMS	- <i>N</i> -Methylphenazonium methosulphate,
PMSF	-Phenylmethanesulphonyl fluoride
SDS	-Sodium dodecyl sulphate
TEMED	- <i>N N N 'N'</i> -Tetramethylethylenediamine
THL	-Tetrahydrolipstatin

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Summary

1. The rate of wax ester degradation was determined in *A. calcoaceticus* NCIB 8250 and the following results were obtained:

(a) The initial and final rates of wax degradation were 22.5 and 3.3 $\mu\text{mol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$ respectively. The initial rate of wax degradation was 85% higher than that of final rate (3-6 h).

(b) The rate of endogenous respiration was measured during wax ester degradation. The endogenous consumption of oxygen, 0.13 $\text{mmol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$, was reduced by 10% during the fast phase of wax degradation and by 20% after 3 h.

(c) The viability of the bacteria having a low or high wax content was determined during carbon and energy starvation condition. 50% of the bacteria with a low content of wax esters were not viable after 84 h but 50% of the bacteria with a high wax content were still viable after 154 h.

(d) From literature values for the maintenance energy requirements as oxygen consumption, the initial fast rate of wax esters degradation provides approximately 40% of the energy required for the maintenance of viability, and in the final slower rate would only provide about 7% of the maintenance energy required for the maintenance of viability.

2. Esterolytic activities were found with short and long chain nitrophenyl esters and short chain aliphatic esters. Esterolytic activities in *A. calcoaceticus* NCIB 8250 were studied in detail and the following results were obtained:

(a) A short chain fatty acid ester specific esterase and a long chain fatty acid ester specific esterase activity can be differentiated using serine reactive inhibitors, and these two activities were designated as esterase and lipase activities respectively.

(b) The esterase and lipase activities in *A. calcoaceticus* NCIB 8250 were intracellular enzymes and were found in the supernate and membrane (inner + outer) fractions with no such activity being found in the culture medium.

(c) Similar esterase and lipase activities were also found in other strains of *A. calcoaceticus*. The esterase activities in ATCC 23055, EBF 65/65, and the esterase and lipase activities in HO1-N and some mutants derived from HO1-N were determined. The esterase and lipase activities in these strains were lower than those of strain NCIB 8250.

3. Culture conditions were found to effect the distribution of esterase and lipase activities in *A. calcoaceticus* NCIB 8250:

(a) Significant differences in total esterase activities were observed in the supernate and membrane fractions of exponentially growing, carbon limited or nitrogen limited bacteria. Total esterase activity was higher (about 70% of the total activity) in the supernate fractions of exponentially growing and nitrogen limited bacteria than the supernate fractions of carbon limited bacteria (about 30% of the total activity). The crude membrane fraction (i.e. inner + outer membranes) from carbon limited bacteria showed a higher total activity (about 70%) than in exponentially growing (total activity about 30%) and nitrogen limited bacteria (total activity about 30%). There was no significant effect on the specific activities of esterase in the supernate caused by shifting the bacteria from carbon to nitrogen or nitrogen to carbon limitation.

(b) As with esterase activity, significant differences in total lipase activity were observed in the supernate and membrane fractions of exponentially growing, carbon or nitrogen limited bacteria. The total lipase activity was higher (total activity about 60%) in the supernate fraction of nitrogen limited than the supernate fraction of exponentially growing bacteria or carbon limited bacteria (total activity about 40%). The total lipase activity

was higher in crude membrane fractions of exponentially growing or carbon limited bacteria (total activity about 60%) than nitrogen limited bacteria (total activity about 40%). There was no significant difference in the specific activities of lipase in membrane fractions of any culture condition. There was no significant effect on the specific activities of lipase in the supernate by changing the culture conditions from carbon to nitrogen limitation or from nitrogen to carbon limitation.

(c) Both the esterase and lipase activity have high molecular mass by gel filtration chromatography. Esterase activity was eluted as a high (M_r about 10^6) and a low molecular mass form (M_r about 6×10^5) while the lipase activity was present only in a high molecular mass form M_r about 10^6 .

4. The inner and outer membranes of carbon and nitrogen limited bacteria were separated using a sucrose density gradient. The esterase and lipase activities were assayed in the inner and outer membrane fractions. The following results were obtained:

(a) The esterase activity was present in both inner and outer membrane fractions but predominantly in the inner membranes in both carbon and nitrogen limited bacteria.

(b) The lipase activity was also found in both inner and outer membrane fractions. The lipase activity was higher in the inner membrane fractions than the outer membrane fractions in nitrogen limited bacteria, while it was equally distributed in the inner and outer membrane fractions of the carbon limited bacteria.

5. The esterase activity did not resolve into distinct bands on native PAGE gels. A method was developed to demonstrate the esterase activity using SDS-PAGE gels. The esterase activity could be detected only after the SDS was removed from the gel by washing. The esterase activity detected by

activity staining on the gel was proportional to the amount of protein and to the incubation time with the substrate. The following results were obtained when supernates were separated using SDS-PAGE gel and stained for esterase activity:

(a) Esterase activities detected by this method resolved into three distinct components of M_r 38,000; 40,000 and 43,000 when stained with 1-naphthyl acetate. These components of esterase activity showed substrate specificity as only two components of M_r 38,000 and 43,000 were stained with 2-naphthyl acetate.

(b) The culture conditions effected the relative activity of the components. A major difference was observed in the supernates of carbon limited bacteria where the low molecular mass component (M_r 38,000) showed more activity than those in exponentially growing bacteria or nitrogen limited bacteria.

(c) The effect of serine inhibitors was also determined on the components of the esterase activity separated by SDS-PAGE. An inhibitory effect on all components was observed with eserine. The component of M_r 43,000 was the most effected by other serine reactive inhibitors used.

6. The role of esterase or lipase activity in wax ester degradation was investigated using inhibitors e.g. PMSF, NBS, 2-hydroxy-5-nitrobenzyl bromide and THL. The following results were obtained:

(a) There was no significant reduction in the amount of wax ester degradation when the esterase activity was inhibited using PMSF.

(b) Some reduction in the amount wax ester degradation was observed when both the esterase and lipase activities were partially inhibited with NBS and 2-hydroxy-5-nitrobenzyl bromide. It was observed that anaerobic conditions and sodium azide inhibited the degradation of wax esters

and it was found that NBS and 2-hydroxy-5-nitrobenzyl bromide caused the inhibition of oxygen consumption. The reduction in wax ester degradation was likely to be due to inhibition of the electron transport chain produced by these inhibitors. Therefore the reduction in the degradation of wax esters could not be definitely assigned to the inhibition of the lipase activity.

7. Fatty acid oxidation enzymes were assayed in the supernates of *A. calcoaceticus* NCIB 8250 and compared with *E. coli* K12. The activities of all enzymes were higher, except for β -hydroxy acyl-CoA dehydrogenase, in *A. calcoaceticus* NCIB 8250 grown on oleate than on succinate, but these activities were higher than in *E. coli* K12 grown on oleate suggesting that fatty acid oxidation enzymes are meso-constitutive in *A. calcoaceticus* NCIB 8250 unlike the enzymes of *E. coli* K12 which are inducible.

Chapter 1

Introduction

1.1 The genus *Acinetobacter*.

The genus *Acinetobacter* has had a confusing taxonomic history. Brisou and Prevot, (1954) were the first workers who named as *Acinetobacter*, the non motile variants of the genus "*Achromobacter*". Bacteria now recognized as *Acinetobacter* strains have also been termed "Vibrio 01" (Happold and Key, 1932) because the strains were gram negative, oxidase negative, unable to oxidize sugars or liquefy gelatin and thus they had similarities with other *Vibrio* strains. Later it was shown that these strains of *Acinetobacter* were different from true *Vibrio* 01 strains which are unable to grow on phenylalanine as carbon source (Chapman and Dagley, 1960, 1962; Fewson, 1967a). The characteristics of *Acinetobacter* derived from phenotypic tests were often negative and this probably played a role in making their classification difficult. *Acinetobacter* strains have also been considered as belonging to the genus *Moraxella* (Henriksen, 1973), another genus which has often included organisms which could not be allocated into other genera. Baumann *et al.*, (1968) analysed the *Moraxella-Acinetobacter* group phenotypically and proposed that the oxidase negative moraxella be included in a separate genus *Acinetobacter*. They proposed, *A. calcoaceticus*, *A. lwoffii*, and *A. hemolysans*, to be the species within the genus. Later the Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria (Lessel, 1971) agreed that the genus *Acinetobacter* should be the oxidase negative bacteria defined by the study of Baumann *et al.*, (1968).

Recently Van Landschoot *et al.*, (1986) have confirmed and extended the work of previous authors by investigating rRNA relationships between *Acinetobacter* and other bacterial groups. These authors have analysed the intergeneric taxonomic position of *Acinetobacter* by DNA : rRNA hybridization,

which provides a simple, fast and very reliable method for finding the taxonomic position of a genus. Their results have shown that the genus *Acinetobacter* was more closely related to rRNA super family II (*Pseudomonas fluorescens* complex, *Azomonas*, *Azotobacter*, etc.) than to rRNA superfamily I (*Enterobacteriaceae*, *Vibrionaceae*, *Pasteurellaceae*, etc.). Bouvet and Grimont, (1986) analysed genotypically 85 strains of *Acinetobacter* and on this basis they proposed dividing the genus *Acinetobacter* into twelve genospecies. Even now the genus *Acinetobacter* is being extended in terms of strains with new properties. Recently Nishimura *et al.*, (1988) have proposed another species for the genus, "*Acinetobacter radioresistens*". They showed that the levels of DNA homology between radioresistant *Acinetobacter* strains that they had isolated and reference strains of *A. calcoaceticus*, *A. lwoffii*, and three of the genospecies (*A. baumannii*, *A. haemolyticus*, and *A. johnsonii*) of Bouvet and Grimont, (1986) were low (15-44%). Furthermore, electrophoretic analysis of enzymes of radioresistant *Acinetobacter* showed a different pattern from other *Acinetobacter* strains and these strains can also be differentiated from *A. calcoaceticus* by the lack of assimilation of citrate and L-arabinose and by the absence of acid production from saccharides. In summary all organisms in the genus *Acinetobacter* are aerobic, gram negative, non motile (in some strains 'twitching' movement has been shown), catalase positive and oxidase negative, with a G+C content of 38-47 mol % (Henriksen, 1976; Juni, 1978). They constitute a distinct grouping in the genera of bacteria belonging to rRNA superfamily II. At present the number of species within the genus is not settled.

Perhaps due to their versatile nutritional abilities acinetobacters are widely distributed in nature. *Acinetobacter* strains have been isolated from soil, water and sewage (Baumann, 1968; Warskow and Juni, 1972; Henriksen, 1973). They have also been found in milk and dairy products

(Koburger, 1964). *Acinetobacter* strains have been isolated from human sources, probably due to contamination from the surroundings or the skin which has a temperature about 30-35 °C, the optimum growth temperature for many acinetobacter strains (Juni, 1972; Henriksen, 1973; Gaughan *et al.*, 1979). Many reports have appeared about the possible pathogenicity of *Acinetobacter* strains. Some of the strains have been found to be associated with septicaemia, urinary tract infections (Glew *et al.*, 1977; Retailiau *et al.*, 1979; Lowes *et al.*, 1980; Hoffman *et al.*, 1982), infected wounds (Asheley and Kwantes, 1961; Brodie and Henderson, 1964), ulcers (Asheley and Kwantes, 1961; Carter *et al.*, 1970), brain abscesses (Opsahl, 1961) and chest diseases (Daly *et al.*, 1962). Some authors (Juni, 1972; Markham and Telfer-Brunton, 1983) have considered *Acinetobacter* as an opportunistic pathogen in humans which can cause disease only when resistance to infection is low due to immunosuppression or malignancies (Green *et al.*, 1965). Similarly, acinetobacters have also been isolated from farm animals mostly as harmless commensals, but some pathogenic strains have also been identified (Henriksen, 1973; Dickie and Regnier, 1978). Although acinetobacters may only be opportunistic pathogens, they have been reported to be resistant to many antibiotics, as well as being less sensitive to the ionizing radiation used in medical equipment and food sterilisation. This resistance can make their detection and elimination vital. They have been found in processed fish (Lee and Harrison, 1968), poultry (Thornley *et al.*, 1960), meat products (Tiwari and Maxcy, 1972; Ito *et al.*, 1976) and in cotton tampons sterilized by gamma radiation (Kairiyama *et al.*, 1979). Therefore acinetobacters have some economic importance as they are opportunistic pathogen of humans and animals and they can cause spoilage in the processed food industry and contamination of "clean" medical products.

Acinetobacters degrade a wide variety of aromatic and aliphatic compounds (Fewson, 1967b). Strains of *Acinetobacter* that oxidise aliphatic hydrocarbons, have been isolated from oil polluted aquatic environments and soil (Gutnick and Rosenberg, 1977; Bartha and Atlas, 1977). Such strains of *Acinetobacter* produce waxes during alkane oxidation (Makula *et al.*, 1975) and some of these strains also produce an emulsan which serves to emulsify the alkane substrates which are not soluble in water. These emulsans are of biotechnological importance as they are being used to enhance the recovery of crude oil (for review, see Brown *et al.*, 1986) and are also used to alter its surface properties (for review, see Rosenberg, 1986). Simple wax esters and fatty alcohols may also have a biotechnological importance as they are valuable raw materials for the production of lubricants, cosmetics, surfactants etc. and are generally obtained at present from plants, sperm whale oil or petroleum products. However, as there is a search for cheaper alternative sources of wax esters and fatty alcohols, the ability of the genus *Acinetobacter* to produce simple wax esters is being investigated by biotechnologists as a bacterial substitute for jojoba oil and sperm whale wax. Geigert *et al.*, (1984) have patented strains of *Acinetobacter* for the production of wax for commercial purposes.

1.1.1 *Acinetobacter calcoaceticus* NCIB 8250.

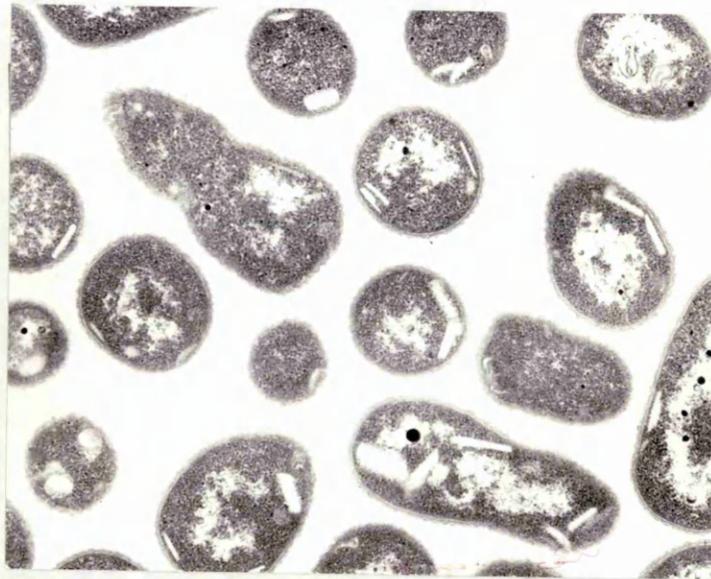
According to Bouvet and Grimont, (1986), to name strain NCIB 8250 as an *Acinetobacter calcoaceticus* strain is a misassignment in the strictest sense. The strain classifies into genospecies eleven rather than genospecies one which is *A. calcoaceticus sensu stricto* according to these authors. However, as there is no agreement on new species within this genus yet, strain NCIB 8250 will be referred to *Acinetobacter calcoaceticus* NCIB 8250 throughout this thesis.

A. calcoaceticus NCIB 8250 is unable to grow on carbohydrates (Cook and Fewson, 1973) but can grow on a wide variety of organic compounds, including aromatics, as sole energy source (Fewson, 1967b). The wax ester content of *A. calcoaceticus* NCIB 8250 is remarkably increased when grown under nitrogen limiting conditions with sufficient supply of carbon and energy source (see Figure 1.1). This organism can metabolise these wax esters to utilizable energy under starvation conditions (Fixter and Fewson, 1974; Fixter and McCormack, 1976; Fixter *et al.*, 1986).

1.1.2 *Acinetobacter calcoaceticus* HO1-N.

The history of *A. calcoaceticus* HO1-N clearly demonstrates the problem of classifying acinetobacters, as this strain was previously called "*Micrococcus cerificans*". Later it was shown to be an *Acinetobacter* sp (Baumann *et al.*, 1968). This strain has played an important role in the investigation of certain aspects of wax ester biosynthesis (Makula *et al.*, 1975). Mutants of *A. calcoaceticus* HO1-N have been isolated which produce about ten times more waxes than the parent strain, perhaps due to a reduced capacity to degrade wax esters by esterase or lipase activities (Geigert *et al.*, 1984).

Figure 1.1 A. *calcoaceticus* NCIB 8250 grown into stationary phase nitrogen limited conditions (A) and into stationary phase carbon limited conditions (B).



(A)



(B)

1.2 Wax esters.

In 1815 Chevreul showed that spermaceti oil from whale was different from common triacylglycerols as it had no glycerol as a constituent. On alkaline hydrolysis it gave a mixture of long chain fatty alcohols which were called "cetyl alcohols". Studies on wax esters have gained importance as they were shown to be present in plants, animals and in some species of bacteria (for reviews of earlier work on these subjects, see individual chapters in Kolattukudy, 1976a). It has been shown that waxes in plants and animals have a number of different functions, the two most important being to act as a barrier against water loss and to serve as an energy reserve (Kolattukudy, 1976b). This difference in function is often reflected in the difference in structure of the waxes (Kolattukudy, 1976b). The importance of these compounds has led to extensive studies on their metabolism and its regulation in animals and plants.

1.2.1 Structure and chemical properties of waxes.

Waxes can be classified into two types, simple and complex. Both types of waxes are found in nature.

1.2.1.1 Simple Waxes.

Simple wax esters are monoesters of straight chain fatty acids and long straight chain primary alcohols (Deuel, 1951) see Figure 1.2. A shorthand notation for waxes has been proposed in which the fatty alcohol precedes the fatty acid and chain length and number of double bonds of each residue is indicated e.g. cetyl palmitate (16:0 - 16:0), (Aasen *et al.*, 1971). Other examples of naturally occurring simple waxes are lauryl palmitate (12:0 - 16:0) and stearyl palmitate (18:0 - 16:0), which are usually present in marine animals

(Sargent *et al.*, 1976) and bacteria (Albro, 1976). Unsaturated waxes e.g. cetyl oleate (16:0 - 18:1), oleoyl palmitate (18:1 - 16:0), etc. are also found together with saturated waxes in marine animals and in some bacteria (Sargent *et al.*, 1976; Albro, 1976).

1.2.1.2 Complex waxes.

In complex waxes either the fatty acid or the fatty alcohol component or both may have complex structures. Typical of these are the waxes of *Mycobacterium tuberculosis* (for review see Asselineau, 1966) which are also known as cerides and are diesters of phthiocerols (C_{33} - C_{35} branched chain diols) with mycocerosic acids (C_{29} - C_{32} branched chain fatty acids), (see Figure 1.2). Complex diester waxes are also present in the lipids of animal skin surface (Downing, 1976). These waxes consist of two distinct types of diester. One type contains molecules of 2-hydroxy acid esterified with both a fatty alcohol and a fatty acid. A second type, which is more common, is a diester wax consisting of long chain 1, 2 diols in which both hydroxy groups are esterified to unsubstituted fatty acids (Nicolaidis *et al.*, 1970). In plants polyester waxes are also present e.g. cutin. This is the structural component of cuticle and consists of polyesters of hydroxy fatty acids. Another polyester wax, suberin, which covers the underground portions of plants and is a constituent of cork, contains fatty acids similar to those found in cutin but they are predominantly ω -hydroxy and dicarboxylic acids with phenolic materials (Kolattukudy *et al.*, 1976). The external waxes in plants and animals usually have longer carbon chains making them hydrophobic. Therefore, these external waxes help the epidermis to withstand the adverse effects of environmental conditions, such waxes serve as a barrier preventing water loss from the body or act as a water repellent surface (Kolattukudy, 1976b).

1.2.2 Properties of wax esters.

Triacylglycerols are commonly occurring non-polar lipids found in many biological systems and which can play role as barrier lipids and as stored energy. Simple and complex wax esters differ substantially in their physico-chemical properties from those of triacylglycerols because wax esters contain two long chain alkyl units per ester group which makes them more hydrophobic than triacylglycerols with their single long chain alkyl unit per ester bond. Due to this hydrophobicity wax esters are more difficult to emulsify with detergents than triacylglycerols and moreover this hydrophobicity means that they are more resistant to alkaline hydrolysis in polar solvents. The difference in hydrophobicity between wax esters and triacylglycerols is the basis for the separation of these types of lipids by thin layer chromatography or adsorption chromatography. The hydrophobicity of wax esters is very similar to that of the other major biological class of fatty esters of hydrophobic alcohols, the sterol esters, which are not usually present in bacteria. Wax esters do not form bilayers in water and thus are like triacylglycerols and cholesterol esters. They may form droplets or crystals (depending on their melting point) or they may insert at low concentration into already formed phospholipid bilayer membranes.

The internal waxes present in plants and animals can serve as an energy source. As waxes are highly reduced compounds, ^{they} produce more ATP (8.35 mol g atom⁻¹ carbon) than glycogen (6 mol g atom⁻¹ carbon) and slightly more than triacylglycerols (7.98 mol g atom⁻¹ carbon). Although there is only a small difference in ATP yield between wax esters and triacylglycerols, this may be an advantage for the organisms which use wax esters as energy reserves (Rosenberg, 1967; Miwa, 1971; Yermanos, 1975, Lee *et al.*, 1974). The specific gravity of wax esters is less than that of triacylglycerols, and therefore

wax esters will provide about 70% more upthrust, in marine animals, than the same volume of triacylglycerols and hence provide greater buoyancy (Lewis, 1970). Thus for marine animals, waxes as energy stores, have two advantages over triacylglycerols.

1.2.3 Distribution of wax esters in bacteria.

Simple wax esters have only been found in a few classes of bacteria (Albro, 1976). The presence of wax esters have been reported in *Bacillus cereus* (Kates *et al.*, 1962), *Nocardia* species (Laneelle *et al.*, 1965), *Corynebacterium* spp. grown on hydrocarbons (Bacchin *et al.*, 1974), *Moraxella*, *Acinetobacter*, *Branhamella* and *Neisseria* (Jantzen *et al.*, 1974, 1975; Gallagher, 1971; Thorne *et al.*, 1973; Fixter and Fewson, 1974; Makula *et al.*, 1975; Fixter and McCormack, 1976; Bryn *et al.*, 1977; Fixter *et al.*, 1986), *Clostridium* spp. (Hobbs *et al.*, 1971), *Brucella melitensis* (Thiele and Schwinn, 1973), "*Micrococcus cryophilus*" (Russell, 1974) and *Thiobacillus thioparus* (Christopher *et al.*, 1980). Although the presence of wax esters has been reported in these bacterial species, most of them contain only trace amounts of wax esters (0.02 to 0.2% of the total bacterial lipid). However many strains of *Acinetobacter*, "*Micrococcus cryophilus*" and *Moraxella* etc. contain significantly larger amounts of wax esters.

1.2.4 Biological importance of wax esters in bacteria.

There is very little information available to draw any general conclusion about the role of wax esters in bacteria. The waxes were initially investigated as a means of classifying certain bacteria (Gallagher, 1971), because they are not common and thus could have a certain value as a taxonomic tool (Bryn *et al.*,

1977; Fixter and McCormack, 1976). This work showed that although wax esters were found in most acinetobacters, they also occur in *Moraxella* and *Neisseria* etc. Thus wax esters could not be used to differentiate *Acinetobacter* strains from other bacteria in the *Moraxella - Neisseria - Branhamella* group.

The wax esters in *Acinetobacter* fulfil some of the criteria for an energy reserve described by Dawes and Senior, (1973) and they have been proposed to fulfil this role in *Acinetobacter* (Fixter and Fewson, 1974). In *A. calcoaceticus* NCIB 8250, the wax ester content increased substantially in nitrogen limited conditions when there was sufficient supply of carbon and energy source (Fixter and Fewson, 1974). The accumulated wax esters were degraded into water soluble compounds and energy (Fixter *et al.*, 1986). A possible reason for the use of wax esters as energy reserves rather than triacylglycerols in *Acinetobacter* is that except for a small group of strains, this genus does not metabolise glucose which yields glycerol easily (for review, see Juni, 1978). However this explanation is very unlikely as the phospholipids of *A. calcoaceticus* are based on glycerol (Makula *et al.*, 1975) and thus this genus has no great problems in generating glycerol units for biosynthesis.

The location of wax esters in *Acinetobacter* may help to explain their biological role. Thus it would be interesting to know whether the wax esters are components of membranes or occur in organelles within the cell. Thorne *et al.*, (1973) have detected wax esters in the outer membranes of *Acinetobacter* strain MJT/F5/199A and Russell (1978) also showed wax esters in the inner and outer membranes of "*Micrococcus cryophilus*". It was proposed that wax esters in bacteria may have a function analogous to that of cholesterol in other membrane systems in regulating membrane fluidity (Russell, 1974, 1978; Russell and Volkman, 1980). Later, Russell (for review, see Russell, 1984) proposed that the membrane fluidity in "*Micrococcus cryophilus*" is regulated by altering the

length of acyl chains rather than unsaturation. However, McGibbon *et al.*, (1985), in studies of the fluidity of membrane lipids from this organism showed that the presence of wax esters does not change the fluidity of the membrane lipids. Thus wax esters are unlikely to be important in regulating membrane fluidity.

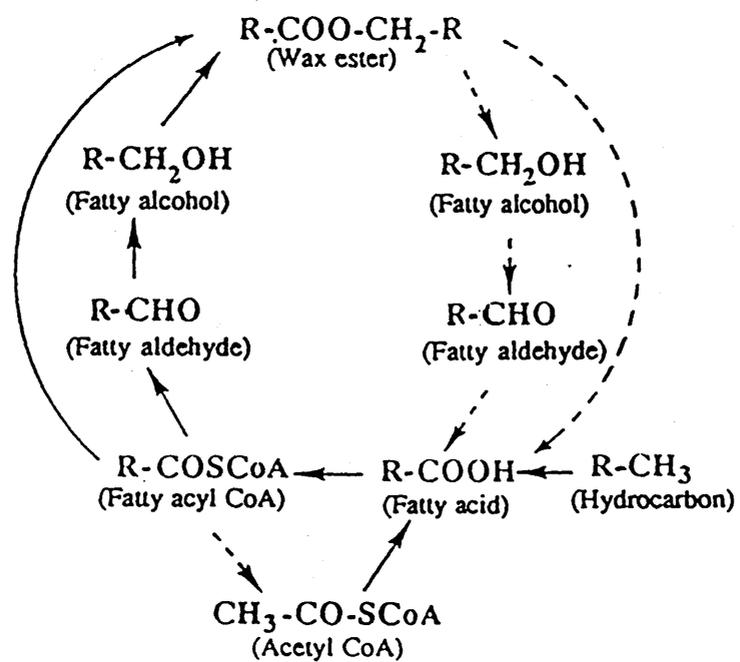
1.3 Biosynthesis of wax esters.

The biosynthetic pathway of wax esters from simple precursors has been studied in great detail in "*Micrococcus cryophilus*" (Lloyd and Russell, 1983) and in *A. calcoaceticus* NCIB 8250 (Nagi, 1981). The individual steps of wax ester biosynthesis follow the pathways already known in plants and animals. As fatty acids and fatty alcohols are the major constituents, the pathway of wax biosynthesis involves the production of fatty acids, their activation as CoA esters, the reduction of their CoA esters to aldehydes, reduction of the fatty aldehydes to alcohols and a final esterification of the fatty alcohol and fatty acid CoA ester. Biosynthesis of wax esters from radioactively labelled fatty acids and alcohols has been shown in *A. calcoaceticus* NCIB 8250 to follow this general pattern (Nagi, 1981)(see Figure 1.3).

Figure 1.3 Pathway of wax ester metabolism in *A. calcoaceticus* NCIB 8250

Biosynthesis ———

Catabolism ·····



1.3.1 Biosynthesis of fatty acids.

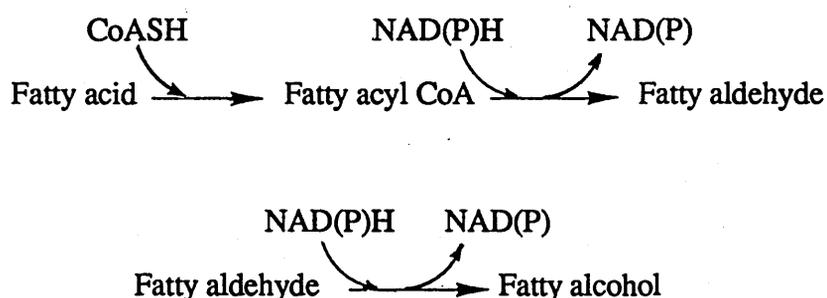
The acyl and alcohol moieties found in wax esters are derived from fatty acids. The biosynthetic pathway of fatty acids has been studied in detail in both eukaryotic and prokaryotic organisms. It is presumed that the biosynthetic pathway of saturated fatty acid in acinetobacters would not be different in any major aspect from the well studied corresponding pathway in *E. coli* (Volpe and Vagelos, 1976; for review see Cronan, 1978). Most studies of fatty acid biosynthesis in other gram negative bacteria have successfully used *E. coli* as a model. Hexadecanoic acid is the major primary fatty acid produced in many organisms, which can be further elongated, desaturated or derivatised (for review, see Schweizer, 1989). In prokaryotes e.g. in *E. coli* the enzymes catalysing the individual reactions of fatty acid synthesis are found on separate monofunctional proteins but there are exceptions e.g. in *Mycobacterium smegmatis* and some other gram positive bacteria, the fatty acid synthesis enzymes are present as a multifunctional protein. The mycobacterial fatty acid synthase is composed of six identical subunits and each subunit contains a multifunctional polypeptide which consists of the six reaction centres required for the synthesis of saturated fatty acids (for review, see Rock and Cronan, 1985). In contrast, eukaryotic synthesis of fatty acids is catalysed by a fatty acid synthase complex which in the case of yeast contains two multifunctional polypeptides, while in the case of mammalian and avian fatty acid synthetases all enzyme activities are located on a single polypeptide (for review, see Goodridge, 1985). In *E. coli*, fatty acid synthesis begins with the production of malonyl CoA by the carboxylation of acetyl CoA which is catalysed by acetyl carboxylase. Acetyl carboxylase is composed of three individual proteins, biotin carboxylase, carboxyl carrier protein, and carboxyl transferase, and appears to be an important regulatory enzyme of fatty acid biosynthesis in many eukaryotes

(for review, see Goodridge, 1985). Acetyl-ACP and malonyl-ACP, the primers of fatty acid synthesis are formed by the action of acetyl and malonyl transacylases respectively. The elongation reaction starts with the condensation of malonyl-ACP with acetyl-ACP catalysed by 3-ketoacyl-ACP-synthase. The keto ester is then reduced by an NADPH-dependant 3-ketoacyl-ACP reductase. Water is removed by 3-hydroxyacyl-ACP dehydrase and finally a saturated acyl-ACP is produced by the action of enoyl-ACP reductase (for review, see Rock and Cronan, 1985).

Studies on the regulation of biosynthesis of fatty acids have been confined to the regulation of synthesis in terms of producing acyl units for the membrane phospholipids of *E. coli* (for review, see Rock and Cronan, 1985). Experiments in *E. coli* have shown *in vivo* that the condensation of acetyl-ACP with malonyl-ACP to produce acetoacetyl-ACP is the rate limiting step in the fatty acid biosynthetic pathway (Jackowski and Rock, 1987). Thus studies in *Acinetobacter*, where fatty acids can be used for both phospholipid and wax ester biosynthesis, may well reveal differences in the regulation of fatty acid biosynthesis from the *E. coli* system. Only a few minor studies on the regulation of fatty acid biosynthesis have been undertaken in *A. calcoaceticus* HO1-N grown on long chain hydrocarbons (Sampson and Finnerty, 1974). It was observed that in hexadecane grown bacteria *de novo* fatty acid biosynthesis was reduced significantly and there was preferential assimilation of long chain fatty acids derived from hexadecane oxidation into cellular lipids (Sampson and Finnerty, 1974). This could indicate that there is feedback inhibition of fatty acid synthesis by exogenously supplied fatty acids.

1.3.2 Biosynthesis of fatty alcohols.

Fatty alcohols are key intermediates in wax ester biosynthesis. Therefore biosynthesis of fatty alcohol is of great importance in the metabolism of wax esters. For instance it is known that the rate of wax ester biosynthesis in *Euchaeta norvegica* is increased by supplying fatty alcohols (Sargent *et al.*, 1974). The general pathway for the biosynthesis of fatty alcohols is:



In marine animals, it was shown using radioactively labelled precursors that fatty alcohols are synthesised *de novo* from glucose, amino acids and palmitic acid (Sargent and Lee, 1975). The formation of fatty alcohols from fatty acyl CoA derivatives catalysed by an NADPH-fatty acyl CoA reductase was demonstrated in marine animals (Sargent and Gatten, 1976; Sargent and Falk-Petersen, 1988). In cell free extracts of the protozoan, *Tetrahymena pyriformis*, the production of cetyl alcohol from labelled palmitate in the presence of added CoA, ATP, Mg^{+2} and NADH has been shown (Kapoulas and Thompson, 1969). Similarly an enzymatic reduction of fatty acyl-CoA to primary alcohol through an aldehyde intermediate was demonstrated with cell free extracts from etiolated *Euglena gracilis*, which also required ATP, CoA and NADH (Kolattukudy, 1970).

1.3.3 Biosynthesis of fatty alcohols in microorganisms.

Fatty alcohols can be produced in wax synthesizing microorganisms by two different mechanisms, by oxidation of hydrocarbons or by enzymatic reduction of fatty acids.

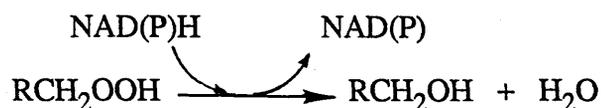
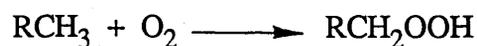
1.3.3.1 Oxidation of hydrocarbons.

The bacteria which can grow on n-alkanes produce fatty alcohols by the terminal hydroxylation of these compounds. Terminal hydroxylation of alkanes is common among many microorganisms like coryneforms, pseudomonads and acinetobacters. This reaction is critically important for such microorganisms as it is the first step in the pathway yielding fatty acids. *Acinetobacter* strain HO1-N accumulated wax esters (cetyl palmitate) in the medium, when it was grown on n-hexadecane-mineral salts medium (Stewart *et al.*, 1959, 1960; Geigert *et al.*, 1984). Confirmation of the pathway from alkane to alcohol was obtained by isotopic studies using [1-C¹⁴] hexadecane (Finnerty and Kallio, 1964).

Two mechanisms, hydroxylation and hydroperoxidation have been proposed for the initial oxidation of alkanes in microorganisms (for review, see Finnerty, 1989). Hydroxylation, in the presence of molecular oxygen, catalysed by mixed-function oxidases converts the alkane to a primary alcohol:



Hydroperoxidation, catalysed by a dioxygenase forms an n-alkyl hydroperoxide which is then reduced to a primary alcohol:



The enzymes which catalyse the oxidation of alkanes are linked to various electron-carrier systems. For example, in *Pseudomonas putida* grown on octane there is an enzyme system which comprises a rubredoxin, an NADH-rubredoxin reductase and an ω -hydroxylase which catalyses the hydroxylation of alkanes or the ω -hydroxylation of fatty acids (Peterson *et al.*, 1966, 1967; Peterson and Coon, 1968). Many eukaryotic and prokaryotic microorganisms use cytochrome P₄₅₀ which may be either NADH or NADPH dependant systems for the terminal oxidation of alkanes (for review, Finnerty, 1989). Induction of rubredoxin has been reported in a strain of *Acinetobacter* grown on alkanes but has no apparent role in alkane oxidation by this strain (Aurich *et al.*, 1976). In other strains of *Acinetobacter* capable of oxidizing alkanes, the presence of P₄₅₀ has also been reported (Asperger *et al.*, 1981). These authors, on the basis of alkane induction and synthesis of P₄₅₀ in these strains, and the presence of P₄₅₀ in partially purified alkane hydroxylase from *Corynebacterium* have proposed that P₄₅₀ plays a role in hydroxylation of alkanes in *A. calcoaceticus* strain (Asperger *et al.*, 1981). In *A. calcoaceticus* HO1-N grown on hexadecane a new terminal oxidase was induced, cytochrome *d* rather than P₄₅₀ (Ensley and Finnerty, 1980). It was reported that these enzymes were potentially able to function as terminal oxidases in this organism (Ensley and Finnerty, 1980). These contradictory reports indicate that much work remains to be done to characterise the alkane hydroxylation mechanism(s) in *Acinetobacter*.

1.3.3.2 Reduction of fatty acids.

Fatty acids are normally activated before the reduction to alcohols. The activation occurs in a condensation step catalysed by acyl-CoA synthetase in which the fatty acid is linked with CoA to produce the corresponding acyl-CoA. The activation of long chain fatty acids was first reported in eukaryotes (Kornberg and Pricer, 1953; Ailhaud *et al.*, 1962; Pande and Mead, 1968) and then in prokaryotes (Henneberry and Cox, 1970; Trust and Millis, 1971; Calmes and Deal, 1973).

Fatty acyl CoA's produced by the activation of fatty acids can be reduced enzymatically to corresponding alcohols (through an aldehyde intermediate) by microorganisms (Day *et al.*, 1970; Wang *et al.*, 1972). Cell free extracts from *A. calcoaceticus* NCIB 8250 were able to incorporate radioactively labelled palmitic acid in the presence of Mg^{+2} , ATP, and CoA into wax esters (Nagi, 1981). It was found that activated fatty acids in the form of CoA derivatives were required for wax biosynthesis, fatty alcohols were produced through an aldehyde intermediate and that the fatty alcohols were later esterified to give wax esters. In "*Micrococcus cryophilus*" wax ester biosynthesis was proposed to occur either from endogenous fatty acids using a cytoplasmic pool of fatty acyl CoA thioesters and alcohols or from an exogenous supply of fatty acids using a membrane bound pool of fatty acyl CoA thioesters and alcohols (Lloyd and Russell, 1983).

It is known that acyl-ACP can be used instead of the CoA derivatives in phospholipid biosynthesis in *E. coli* (Rock and Jackowski, 1982) and that fatty acyl-ACP derivatives are also used by bioluminescent bacteria to produce fatty aldehydes, a reaction which is catalysed by an NADPH-reductase (Wall *et al.*, 1986). Although there is evidence from *in vitro* studies that acyl-CoA thioesters

are used for wax ester synthesis in *A. calcoaceticus* NCIB 8250 (Nagi, 1981) and in "*Micrococcus cryophilus*" (Lloyd and Russell, 1983), it is possible that acyl-ACP could be the preferred substrate *in vivo* for wax ester synthesis as it is the end product of *de novo* fatty acid biosynthesis.

1.3.4 The mechanism of esterification in wax ester biosynthesis.

Fatty acids can be esterified with fatty alcohols in one of three ways:

- i) reversal of an esterase reaction (Friedberg and Greene, 1967).
- ii) acyl group transfer from a phospholipid (Kolattukudy, 1967).
- iii) acyl group transfer from acyl-ACP (Stumpf *et al.*, 1978) or acyl CoA (Kolattukudy, 1967).

In *A. calcoaceticus* NCIB 8250 there is evidence for the involvement of acyl-CoA (Nagi, 1981). Firstly, ATP, CoA and Mg^{+2} were essential for the incorporation of fatty acids into wax esters and secondly palmitoyl CoA was incorporated into wax esters without the addition of other cofactors and the addition of cetyl alcohol stimulated this incorporation. (Nagi, 1981). Similarly the incorporation of stearoyl-CoA into wax esters by cell free extracts of "*Micrococcus cryophilus*" was reported by Lloyd and Russell, (1983).

1.4 Catabolism of wax esters.

It has been observed that endogenous wax esters in plants (Huang *et al.*, 1978) and animals (Patton and Benson, 1975) are initially hydrolysed to their constituents i.e fatty acids and fatty alcohols, before proceeding to further catabolism. There are reports of secreted bacterial enzymes hydrolysing exogenous wax esters (Lin and Kolattukudy, 1980; McQueen and Schottel, 1987) and there is some evidence for enzymes hydrolysing endogenous wax esters in bacteria based on detection of the products of wax ester hydrolysis, i.e. fatty acids and fatty alcohols (Fixter *et al.*, 1986).

1.4.1 Catabolism of endogenous wax esters in microorganisms.

A. calcoaceticus NCIB 8250 is able to metabolise fatty acids and fatty alcohols from waxes as these products were not accumulated during catabolism of wax esters (Fixter *et al.*, 1986). The production of labelled CO₂ from [¹⁴C] waxes was proposed to be due to oxidation of labelled fatty acids via β -oxidation followed by total oxidation of acetyl units by the Krebs cycle. The oxidation of cetyl alcohol to fatty acids was shown to occur via an aldehyde intermediate (see Figure 1.3). An NADPH-alcohol dehydrogenase activity was also found in *A. calcoaceticus* NCIB 8250 (Fixter and Nagi, 1984), and it has been proposed that this enzyme may have some role in the wax ester metabolism (Nagi, 1981).

Hydrocarbons are totally oxidised by bacteria after hydroxylation by the same mechanisms used for wax ester catabolism. For instance, in *A. calcoaceticus* HO1-N grown on hexadecane, fatty alcohols are oxidised to

fatty aldehydes by a reaction catalysed by a fatty alcohol dehydrogenases (Singer and Finnerty, 1985a) and the fatty aldehydes are oxidised to fatty acids by an NADP-dependant fatty aldehyde dehydrogenases (Singer and Finnerty, 1985b).

1.4.2 Catabolism of wax esters in other organisms.

The cotyledons of jojoba seeds contain 50 to 60% of their fresh weight as intracellular wax esters which are stored in structures called wax bodies (Yermanos and Duncan, 1976; Huang *et al.*, 1978). These intracellular wax esters are hydrolysed to fatty acids and fatty alcohols by a wax ester hydrolase present in the wax bodies (Huang *et al.*, 1978). The released fatty acids and fatty alcohols are converted into sugars, a transportable form of energy and carbon skeletons to support the growth of the embryonic axis during germination. (Moreau and Huang, 1977). Two oxidative enzymes, an alcohol oxidase, converting fatty alcohols to fatty aldehydes (utilizing molecular oxygen) and an NAD linked fatty aldehyde dehydrogenase, converting fatty aldehydes to fatty acids were detected in cotyledon extracts (Moreau and Huang, 1979).

During starvation of *Euchaeta japonica* and *Gaussia princeps* triacylglycerols were utilized faster than wax esters (Lee *et al.*, 1974; Lee and Barnes, 1975) and the same was true for *Euchaeta norvegica* and *Calanus helgolandicus* (Sargent *et al.*, 1976). An *in vitro* study may help to explain the reason for the faster utilization of triacylglycerols over the utilization of wax esters. It is known that the hydrolysis of methyl oleate by triacylglycerol lipase (EC 3.1.1.3) from mammalian pancreas is inhibited by fatty alcohols (Mattson *et al.*, 1970). It is possible that a wax esterase might experience a similar inhibitory effect with fatty alcohols. The oxidation of fatty acids, derived from triacylglycerol or wax ester hydrolysis, will increase ratio of NADH / NAD in

the cell and this will tend to limit the oxidation of fatty alcohols by an NAD linked fatty alcohol dehydrogenase. The increase in the concentration of fatty alcohols caused by inhibition of the alcohol dehydrogenase would inhibit the wax ester hydrolase (Sargent *et al.*, 1976). This may be the explanation for a slower rate of wax ester degradation than triacylglycerol degradation in calanoids. Efforts to demonstrate a wax esterase activity in calanoids have proved unfruitful, perhaps due to sub-optimal assay conditions (Sargent *et al.*, 1976).

In mammals (Hansen and Mead, 1965) and in various fish (Bauermeister and Sargent, 1979; Patton *et al.*, 1975; Tocher and Sargent, 1984) dietary wax esters have been reported to be hydrolysed to fatty alcohols and fatty acids. In other experiments, a labelled wax ester, n-[1-C¹⁴] hexadecylmyristate, was force-fed to seven species of marine fish. It was observed that fish hydrolyzed the wax esters, and again after oxidation of released alcohols to fatty acids the fatty acid residues were incorporated into acyl lipids (Patton and Benson, 1975). An NAD linked alcohol dehydrogenase has been purified from the liver and intestine of *Salmo gairdnerii*, and it is proposed that this enzyme is important in the catabolism of dietary wax esters (Bauermeister and Sargent, 1978).

1.4.3 Oxidation of fatty acids.

In marine animals and in some bacteria, fatty acids released by the hydrolysis of wax esters or produced from the oxidation of fatty alcohols, either are used for reesterification or are oxidised to produce energy (for review, see Sargent *et al.*, 1976; Fixter *et al.*, 1986). The fatty acids are normally oxidised via β -oxidation in animals and other eukaryotes and in bacteria such as *E. coli* (for review see, Schulz, 1985).

The oxidation of fatty acids begins with the formation of fatty acyl-CoA derivatives catalysed by acyl-CoA synthetase. Fatty acyl-CoA derivatives are then dehydrogenated by acyl-CoA dehydrogenase to produce 2-trans-enoyl-CoA. Enoyl-CoA hydratase converts 2-trans-enoyl-CoA to L-3-hydroxyacyl-CoA which is then dehydrogenated by L-3-hydroxyacyl-CoA dehydrogenase to form 3-ketoacyl-CoA. Finally a thiolase cleaves 3-ketoacyl-CoA to produce an acetyl-CoA and an acyl-CoA which is two carbon atoms shorter than the starting substrate. This cycle continues until all the acyl-CoA is converted into acetyl-CoA units.

Fatty acid oxidation enzymes in *E. coli* have a complex organisation. A protein has been purified which shows enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, cis- Δ^3 -trans- Δ^2 -enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase activities in a multienzyme complex (Binstock *et al.*, 1977). Acyl-CoA synthetase and acyl-CoA dehydrogenase activities were not found in this multienzyme complex but are separate enzymes. The complex has a native molecular^{mass} of about 260,000 and contains four subunits i.e. $\alpha_2\beta_2$. The relative molecular mass of α and β subunits are 78,000 and 42,000 respectively (Pawar and Schulz, 1981). The β subunit showed only 3-ketoacyl-CoA thiolase activity, the remainder of the enzyme activities were found on the α subunit (Yang and Schulz, 1983). Unlike eukaryotes, there is a single acyl CoA synthetase for medium and long chain fatty acids in *E. coli* (Kameda and Nunn, 1981). A 200-fold induction of the *E. coli* enzymes was obtained when *E. coli* was grown on long chain fatty acids instead of glucose as a carbon source (Overath *et al.*, 1967). Overath *et al.*, (1969) have isolated mutants which were unable to grow on long chain fatty acids and have carried out genetic studies on the enzymes of fatty acid oxidation using these mutants. The genes required for fatty acid oxidation are

found at five different locations on the chromosome and are arranged in a regulon, unlike the genes for lactose degradation system which are organized in an operon. In *E. coli*, fatty acid oxidation is regulated by a similar mechanism to that regulating lactose degradation and it was proposed that fatty acid oxidation enzymes are induced by the binding of a long chain acyl-CoA to a repressor protein, which causes the coordinate induction of all enzymes required (for review see Schulz, 1985). A mechanism of regulation of fatty acid oxidation similar to *E. coli* may be expected in *A. calcoaceticus*.

1.5 Lipolytic enzymes and the degradation of wax esters.

These enzymes belong to the major class of enzymes, the hydrolases. The classification number for these enzymes is EC 3.1 according to the Recommendations of the Nomenclature Committee of the International Union of Biochemistry (Webb, 1984). Both esterases and lipases are carboxylic ester hydrolases (EC 3.1.1). Esterases can hydrolyse esters which are in true or micellar solution as well as in an emulsified form. This broad specificity with regard to substrate form introduces problems in their differentiation from true lipases which preferentially attack emulsified substrates. Brockerhoff and Jensen (1974) gave a more practical definition of lipases and esterases. A lipase is an enzyme capable of hydrolysing esters of long chain fatty acids with twelve or more carbon atoms while an esterase is an enzyme able to hydrolyse esters of short chain fatty acids i.e. less than twelve carbon atoms. Carboxylic ester hydrolases have generally an essential serine residue at their active site and they are inhibited by organophosphorus compounds such as diethyl 4-nitrophenyl phosphate (Krisch, 1971) and other inhibitors which react with serine e.g. phenylmethanesulphonyl fluoride (PMSF) (Turini *et al.*, 1969) and eserine (physostigmine) (Froede and Wilson, 1971). Eserine which is an inhibitor of cholinesterases, can also inhibit the carboxyl esterases but generally a higher concentration of eserine is required to inhibit these enzymes than cholinesterases (Bernhammer and Krisch, 1965). The differentiation of esterase and lipase activity may be even more difficult because it is known that pancreatic lipase has in fact two active sites, one catalysing the the hydrolysis of short chain esters and the other catalysing the hydrolysis of long chain ester substrates. It is possible to inhibit the lipase activity by serine reactive reagents while it retains the short chain esterase activity (Chapus and Semeriva, 1976).

1.5.1 Esterases.

Aldridge (1953a,b) has classified esterases on the basis of their sensitivity towards reactive organophosphorus reagents and other compounds.

a) A-esterases (E.C 3.1.1.2).

These esterases are not inhibited by organophosphorus compounds and can in fact hydrolyse them. They are also known as aryleresterases because they hydrolyse aromatic esters such as phenyl acetate (Krisch, 1971).

b) B-esterases.

These esterases are inhibited by organophosphorus compounds and do not hydrolyse these compounds (Krisch, 1971). They have serine at their active site and due to their wide specificity these enzymes are also called non specific esterases. Acetylcholinesterases, cholinesterases and endopeptidases were also included in this subclass of carboxylic ester hydrolases due to their catalytic properties and behaviour with serine reactive inhibitors (Krisch, 1971). The B-esterases which are not endopeptidases are included in the EC classification EC 3.1.1.1, while the more specific acetylcholinesterases and cholinesterases in EC classes 3.1.1.7 and 3.1.1.8 respectively (Webb, 1984).

c) C-esterases.

These esterases are neither able to hydrolyse organophosphorus compounds nor are inhibited by such compounds and such an esterase was found in porcine kidney extract (Bergmann *et al.*,1957). Therefore they are included in a separate subclass the C-esterases.

1.5.2 Substrate specificity of esterases and lipases.

A wide variety of synthetic substrates are hydrolysed by nonspecific carboxyl esterases (Krisch, 1971) e.g. 4-nitrophenyl acetate, ethyl acetate, ethyl butyrate, tributyrin. Hydrolysis of thioesters by purified pig liver esterase (Stoops *et al.*, 1969) and hydrolysis of amides by liver and *Mycobacterium* esterases have also been reported (Myers *et al.*, 1957). Short or long chain triacylglycerols and esters of long chain fatty acids are the usual substrates of lipases. Triacylglycerol lipases are found in mammals e.g. hormone sensitive lipase, in shellfish e.g. Surf clam (*Spisula solidissima*) (Patton and Quinn, 1973), in fungi e.g. *Aspergillus niger* (Fukumoto *et al.*, 1963) and *Geotrichum candidum* (Tsujiyaka *et al.*, 1973), and in some bacteria e.g. *Pseudomonas fluorescens* (Sugiura *et al.*, 1977) and *Staphylococcus aureus* 226 (Muraoka *et al.*, 1982). Synthetic esters such as nitrophenyl or naphthyl esters of long chain fatty acids are also hydrolysed by some lipases e.g. lipase from *Pseudomonas aeruginosa* (Stuer, *et al.*, 1986), *A. calcoaceticus* 69V (Fischer *et al.*, 1987), *A. calcoaceticus* O16 (Breuil and Kushner, 1975a). Some lipases can hydrolyse the esters of long chain fatty acids and fatty alcohols e.g. pancreatic lipase (Mattson *et al.*, 1970), the lipase of *Rhizopus arrhizus* (Schmid and Schmid, 1978), carp hepatopancreatic lipase and the hydrolase from intestinal caeca of rainbow trout (Kayama *et al.*, 1979; Tocher and Sargent, 1984).

1.5.3 Microbial esterases.

Esterases are widely spread in microorganisms. To date, a number of extracellular esterases from microorganisms have been purified and studied. Esterases are produced as intracellular enzymes and in some cases most of the

activity is secreted into the environment. Therefore, the organisms which secrete their esterases will also contain them intracellularly, as newly synthesised enzyme awaits export from the intracellular site of synthesis (for review of enzyme/protein secretion in bacteria, see Benson *et al.*, 1985).

1.5.3.1 Extracellular esterases.

The extracellular esterases are involved in the hydrolysis of the exogenous substrates. For example, the esterases of *Streptomyces scabies* initiate hydrolysis of the waxy polyesters present in the cuticle of the underground parts of plants (McQueen and Schottel, 1987). Similar esterases are produced by some fungi which damage the waxy surfaces of plants (Dickman *et al.*, 1982). Bacterial extracellular esterases have also been studied from *A. calcoaceticus* O16 (Breuil and Kushner, 1975a) and *A. calcoaceticus* RAG-1 (Shabtai and Gutnick, 1985).

1.5.3.2 Intracellular esterases.

There are a number of reports on intracellular esterases in microorganisms. Most of these studies have simply reported the presence of esterase activity and its purification. None of them have investigated in depth their role in intracellular ester metabolism. Intracellular esterases have been reported in *Pseudomonas cepacia* (Shum and Markovetz, 1974), *Pseudomonas fluorescens* (Nakagawa *et al.*, 1984), *Bacillus stearothermophilus* (Matsunaga *et al.*, 1974) and *Staphylococcus aureus* (Branger and Goulet, 1987). Some of these esterases were constitutive while others were inducible by their substrates, and they are mostly specific for esters of short chain fatty acids. Perhaps, the solubility properties and low molecular weight of short chain esters

allows their rapid entry into the bacterium and there is no need to secrete an esterase to hydrolyse them.

1.5.3.3 Molecular mass of esterases.

The molecular mass of many esterases from mammalian sources have been determined. Most of the native molecular mass are in the range M_r 162,000–168,000 (Krisch, 1971) e.g. pig liver esterase (Barker and Jencks, 1969a,b) and ox liver esterase (Runnegar *et al.*, 1969). The relative molecular mass range reported for microbial esterases is not as high as that for mammalian esterases and is usually in the range M_r 35,000-50,000, e.g. esterases from *Bacillus stearothermophilus* (Matsunaga *et al.*, 1974), *Mycobacterium smegmatis* (Tomioka, 1983), *Pseudomonas cepacia* (Shum and Markovetz, 1974) and *Pseudomonas fluorescens* (Nakagawa *et al.*, 1984). However, a very high molecular mass form of esterase has been found in *A. calcoaceticus* RAG-1 (Shabtai and Gutnick, 1985).

1.5.3.4 Multiple forms of esterases.

Esterase activities from various mammalian organs (e.g. from liver, kidney and brain) are resolved into multiple forms upon starch gel electrophoresis (Read *et al.*, 1966; Schwark and Ecobichon, 1968). In rat liver as many as 13 and in rat kidney as many as 11 electrophoretically different forms have been reported. There have been numerous reports of multiple forms of esterases in the plasmas and sera of many species (Lawrence *et al.*, 1960; Margolis and Feigelson, 1963). Multiple forms of enzymes arise due to multiple gene loci or due to the presence of multiple alleles at a particular gene locus. Non genetic causes for multiple forms are:- alteration in the covalent structure, combination of enzyme with non protein material or aggregation of enzyme

molecules.

Multiple forms of esterase activity have also been reported in bacteria (Goulet, 1980). Some bacterial species have been characterized on the basis of the electrophoretic patterns of their esterases (Goulet, 1981). Branger and Goulet (1987) have reported three kinds of esterase components after gel electrophoresis in various strains of *Staphylococcus aureus*. These components were classified into three groups on the basis of their activity with various substrates and their resistance to di-isopropyl fluorophosphate. The molecular basis for this multiplicity of esterases is not yet clearly understood. Goulet (1973) suggests that esterases of identical electrophoretic mobility would have homologies in amino acid sequences and that the electrophoretic variants reflect an intraspecific differentiation of esterases which may have resulted from esterase evolution from an ancestral gene.

1.5.4 Microbial lipases.

Like microbial esterases, lipases are synthesised as intracellular enzymes but in many cases most of the activity is secreted into the environment as extracellular lipase. Of the lipases produced by various microorganisms, most are extracellular but there are intracellular lipases which are not apparently secreted.

1.5.4.1 Extracellular lipases.

There have been a number of reports of extracellular lipases in the literature, for example the lipases of *Bacillus subtilis* (Kennedy and Lennarz, 1979), *A. calcoaceticus* O16 (Breuil and Kushner, 1975a),

Staphylococcus aureus 226 (Muraoka *et al.*, 1982), *A. calcoaceticus* 69 V (Fischer and Kleber, 1987) and *Pseudomonas cepacia* (Lonon *et al.*, 1988). Most of the fungal lipases are triacylglycerol lipases and such extracellular lipases have been studied in detail in *Aspergillus niger* (Fukumoto *et al.*, 1963), *Geotrichum candidum* (Tsujisaka *et al.*, 1973) and *Rhizopus delemar* (Iwai and Tsujisaka, 1974). Culture conditions such as temperature, ratio of nitrogen to carbon source, availability of oxygen and concentration of inorganic salts effect the production of lipases in variety of organisms. In *Serratia marcescens*, glycogen and other polysaccharides enhance the production of lipase (Winkler and Stuckmann, 1979) and in *Bacillus subtilis* the lipase was induced by increasing the concentration of nutrients in a complex growth medium (Kennedy and Lennarz, 1979). Lipase production was also increased by the addition of lipids to the growth medium of fungi and bacteria (Iwai *et al.*, 1973; Kosugi and Kamibayashi, 1971; Aisaka and Terada, 1979). This indicates that extracellular lipases are inducible enzymes in many organisms.

1.5.4.2 Intracellular lipases.

Intracellular lipases have not been widely investigated in microorganisms although the lipases of *Mycobacterium phlei* (Paznokas and Kaplan, 1977), *Pseudomonas fluorescens* (Sugiura *et al.*, 1977), *Brochothrix thermosphacta* and *Lactobacillus curvatus* (Papon and Talon, 1989) were reported to be intracellular enzymes. Evidence was obtained for intracellular lipases in *A. calcoaceticus* O16 during a study of the extracellular lipase when some cytoplasmic lipase activity was also detected (Breuil and Kushner, 1975a). Similarly, *A. calcoaceticus* 69 V secretes an extracellular lipase and the lipase activity was also found in the inner and outer membranes (Fischer *et al.*, 1987). The most likely function of intracellular lipases is the metabolism of intracellular

lipids, as long chain lipids would not be expected to enter the bacterium readily.

1.5.4.3 Molecular mass of lipases.

The relative molecular mass of mammalian lipases e.g. rat pancreatic lipase and bovine pancreatic lipase were determined by gel filtration as being M_r 40,000 and 48,500 respectively (Morgan *et al.*, 1968; Julien *et al.*, 1972). The molecular mass of microbial lipases have been shown not to be greatly different from mammalian lipases. For instance, a number of the bacterial lipases have molecular mass in the range of M_r 25,000-50,000 e.g. *Micrococcus* and *Pseudomonas* lipases (Lawrence *et al.*, 1967), *Mycobacterium phlei* lipase (Paznokas and Kaplan, 1977) *Staphylococcus aureus* 226 lipase (Muraoka *et al.*, 1982).

The lipase from *Chromobacterium viscosum* has two different native molecular mass forms, one of higher molecular mass (M_r 120,000) and other of lower molecular mass (M_r 30,000) (Horiuti and Imamura, 1977). The lipase from *Acinetobacter calcoaceticus* 69V has a high molecular mass (M_r 300,000) determined by gel filtration but on SDS-PAGE the subunit molecular mass was M_r 30,500 (Fischer and Kleber, 1987). Partially purified lipase from *A. calcoaceticus* O16 was eluted in two forms from a gel filtration column, a very high molecular mass form and a lower molecular mass form lipase (Breuil and Kushner, 1975b). Similar high molecular mass was also found for the lipase of *Pseudomonas fragi* and "*Micrococcus freudenreichii*" (Lawrence *et al.*, 1967).

A majority of the purified fungal lipases are glycoproteins containing about 2-15% carbohydrate, however the carbohydrate portion of the glycoprotein is not required for the catalytic activity (for review, see Macrae, 1983). The

carbohydrate moiety may have some possible role in the passage of proteins through the microbial cell wall or may make them more resistant from attack by extracellular proteases (for review, see Macrae, 1983). The native lipase of *Pseudomonas aeruginosa* also contains carbohydrates but this was due to lipopolysaccharide being complexed with the enzyme. A subunit molecular mass of about M_r 29, 000 for the enzyme was determined by SDS-PAGE (Stuer *et al.*, 1986). From these studies on lipases it can be concluded that lipases in microorganisms are not usually of very high molecular mass and that high molecular mass lipases are probably be due to aggregation of enzyme or complexing of the enzyme with non protein molecules such as lipopolysaccharides.

1.5.5 Biological functions of esterases and lipases.

Studies on esterase and lipase activities in marine animals showed that the esterase or lipase activities were involved in the hydrolysis of exogenous ester lipids, for instance, lipases present in intestinal fluid of anchovy, *Engraulis mordax*, hydrolysed dietary triacylglycerols and wax esters (Patton *et al.*, 1975). The hydrolysis of wax esters by carp hepatopancreas extracts has been reported (Kayama *et al.*, 1979). Triacylglycerol, wax ester and sterol ester hydrolase activities were studied in intestinal caeca of rainbow trout fed diets rich in triacylglycerols and wax esters. It was found that a wax ester-rich diet increased the wax ester and sterol ester hydrolase activities compared with the triacylglycerol hydrolase activity (Tocher and Sargent, 1984). In mammals, the hydrolysis of triacylglycerol in adipose tissue is controlled by hormone sensitive lipase. Epinephrine, norepinephrine, glucagon and adrenocorticotrophic hormone regulate the activity of hormone-sensitive lipase by stimulating the adenylate cyclase of adipose cells which increases the cyclic AMP levels. A protein kinase

is stimulated by the increased levels of cyclic AMP and this kinase activates the lipase by phosphorylation (Stryer, 1988). Similarly, in seeds of jojoba, a wax ester hydrolase is present which hydrolyses the stored wax esters for the growth of the seedlings (Huang *et al.*, 1978). In *A. calcoaceticus* HO1-N, a decrease in esterase or lipase activities was suggested to be responsible for the accumulation of more wax esters in the mutant strains made for producing large amount of wax esters (Geigert *et al.*, 1984). The esterase or lipase activities may be present in *A. calcoaceticus* NCIB 8250 and the accumulation of wax esters might be regulated by such enzymes.

The extracellular esterases and lipases produced by microorganisms also function to hydrolyse the lipids in the environment and thus they enable the microorganism to utilize the released products as carbon and energy sources. The lipase of *Leptospirae* hydrolyses extracellular lipids which allows the organism to use the exogenous fatty acid esters as carbon and energy source (Patel *et al.*, 1964). The secretion of esterase or lipase activities by some bacterial species infecting human beings has focused the attention of scientists towards their role in many diseases. For instance, *Pseudomonas aeruginosa* produce extracellular lipases (Stuer *et al.*, 1986) and these authors consider that secreted lipases may be important as a virulence factor in cystic fibrosis patients suffering from *Pseudomonas aeruginosa* infection.

1.6 Aims of this project.

Wax esters have already been proposed as energy reserves in this organism (Fixter *et al.*, 1986). However one important criterion for an energy reserve is that it helps in maintaining the viability of the organism and this had not been investigated. Therefore the role of wax esters in maintaining viability and supporting endogenous respiration had to be explored initially. As *A. calcoaceticus* NCIB 8250 is able to degrade endogenous wax esters, there must be enzymes involved in the initial degradation of wax esters and in oxidation of the products of degradation. The aims of this project were to identify if possible these enzymes and study the regulation of their activity.

As will be seen from results and discussion, two types of ester hydrolysing activities were rapidly identified and therefore the possible role of these activities in wax ester degradation was explored by investigating the effects of culture conditions on the activity of these enzymes, distribution of the enzymes in the bacterium and an attempt was made to show their role in wax ester degradation by using selective inhibitors. It is known that metabolic pathways are often regulated by the enzymes catalysing the first committed reaction of the pathway. For instance, the hydrolysis of triacylglycerols in adipose tissue is regulated by a hormone sensitive lipase. Therefore, in *A. calcoaceticus* NCIB 8250 the esterase and lipase activities were investigated as a likely area important in the regulation of wax ester catabolism. Different culture conditions are known to have some effect on the catabolism of wax esters (Nagi, 1981) and there may be some effect on the activities of esterases or lipases under these conditions. The distribution of these esterases and lipases in the bacterium may give further insights into the regulation of wax ester breakdown. Fatty acids and fatty alcohols did not increase in concentration during the degradation

of wax esters (Nagi, 1981), emphasizing that possibly there is some efficient system such as the enzymes for fatty acid oxidation to metabolise these products of wax ester hydrolysis.

Chapter 2

Materials and methods

2.1 Materials: commercial sources.

All chemicals used were the best grade available commercially. The chemicals listed below were obtained from Sigma Chemical Co. Ltd., Poole, Dorset BH17 7NH.

Fatty acids, fatty alcohols, Florisil (60-100 mesh), Triton X-100, Brij 35, 4-nitrophenyl acetate, 4-nitrophenyl butanoate, 4-nitrophenyl hexanoate, 4-nitrophenyl octanoate, 4-nitrophenyl decanoate, 4-nitrophenyl dodecanoate, 4-nitrophenyl hexadecanoate, 4-nitrophenyl octadecanoate, 2-naphthyl acetate, deoxyribonuclease (bovine pancreas, Type IV), ribonuclease (bovine pancreas, Type IA), 2-keto-3-deoxyoctonate, lysozyme, Coomassie Brilliant Blue G250, Fast Blue salt RR, eserine, bis-*p*-nitrophenyl phosphate (sodium salt), *N*-bromosuccinamide, phenylmethanesulphonyl fluoride, mercuric chloride, iodoacetamide, iodoacetate, 2-hydroxy-5-nitrobenzyl bromide, acetoacetyl CoA, crotonyl CoA, palmitoyl CoA, MTT tetrazolium [3-(4,5-dimethyl thiazolyl-2) 2,5-diphenyl tetrazolium bromide], β -hydroxyacyl CoA dehydrogenase (porcine heart, Type III), thymol blue, ethyl butyrate, tributyrin and methyl esters of palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid.

The calibration proteins for gel filtration chromatography and for gel electrophoresis were purchased from Pharmacia LKB Biotechnology Inc. Uppsala, Sweden.

The chemicals obtained from British Drug Houses Ltd., Poole, Dorset BH12 4NN, were the following: glycine, periodic acid, sodium arsenite, thiobarbituric acid, 1-naphthyl acetate, 2-mercaptoethanol, deoxyribose, sodium fluoride, *N*-methylphenazonium methosulphate,

NNN'N'-tetramethylethylenediamine, oleic acid, potassium cyanide and all solvents used.

Silica gel H (Type 60, Merck) was obtained from MacFarlane Robson Ltd., Burnfield Ave., Thorneliebank, Glasgow G46 7TP.

Sodium dodecyl sulphate, acrylamide, *NN'*-methylenebisacrylamide and Folin & Ciocalteu's phenol reagent were obtained from FSA Laboratory Supplies, Loughborough, LE 11 0RG, England.

The bovine albumin (plasma albumin, fraction V) used as a standard in protein estimations was obtained from Wilfrid Smith Ltd., Gemini House, High Street, Edgware, Middlesex HA8 7ET.

Tetrahydrolipstatin was a gift from Prof. A. Fischli, F. Hoffmann-LaRoche & Co., Basle, Switzerland.

Pyronin Y was obtained from George T Gurr Ltd., London S.W. 6 England.

Tris (base), ATP (disodium salt), NAD⁺(free acid), NADH (disodium salt), dithiothreitol (Cleland's reagent) and Coenzyme-A were obtained from the Boehringer Mannheim House, Lewes, Sussex, U.K.

Cooked meat medium (CM440), Nutrient broth (CM1) and Nutrient agar (CM3) were obtained from Oxoid Ltd., Basingstoke, Hants RC24 OPW.

2.2 Microbiological techniques.

2.2.1 Microorganisms.

Acinetobacter calcoaceticus strain NCIB 8250 used in this work was obtained from the National Collection of Industrial & Marine Bacteria (NCIB and NCMB Ltd., Torry Research Station, Aberdeen AB9 8DG Scotland). *Acinetobacter calcoaceticus* strain ATCC 23055 was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, U.S.A.). Strains EBF 65/65 and RAG-1 were kind gifts from Dr. A.Vivan (Bristol Polytechnic, U.K.) and Dr. D.L. Gutnick (Tel Aviv University, Israel) respectively. *Acinetobacter calcoaceticus* strain HO1-N and mutants were gifts from J.C. Hunter-Cevera, Cetus Corporation, Emeryville, California. *Escherichia coli* strain K12 was obtained from the Genetics department, University of Glasgow.

2.2.2 Storage of the bacteria.

The bacterial strains used were maintained in Oxoid cooked-meat medium (CM 440) and stored at 4 °C. Subcultures were made into Oxoid nutrient broth (CM1) at 6-12 monthly intervals, and stored at 4 °C as the working stocks of strains.

2.2.3 Media.

2.2.3.1 Routine growth medium.

This medium was used to produce bacteria either for inocula for production of carbon or nitrogen limited bacteria or for producing small amounts of bacteria. The concentration of carbon and nitrogen source i.e. succinic acid

and ammonium sulphate respectively, were chosen to produce reasonable amounts of bacteria without the risk of limiting growth. One litre of this medium contained 1.18 g succinic acid, 1 g $(\text{NH}_4)_2\text{SO}_4$ and 2 g KH_2PO_4 and was adjusted to pH 7.0 before sterilization. Before inoculation 2% (w/v) sterile $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (20 ml litre⁻¹) was added (Fixter *et al.*, 1986). Bacteria were grown in this medium for about 14-16 h.

As strain HO1-N and the mutants derived from it did not grow well on the routine growth medium containing succinate, they were therefore grown in a medium where 10 mM acetate replaced the succinate. Therefore, for comparative purposes strain NCIB 8250 was grown on ^{the} same carbon source.

2.2.3.2 Carbon and nitrogen limited media.

Succinic acid was used as carbon and energy source and ammonium sulphate as nitrogen source. The media were those used by Fixter and Fewson (1974), except that the carbon limited medium was modified to obtain more bacteria per litre by increasing the amounts of ammonium sulphate and succinic acid. The compositions of the two limiting media were:

	g litre ⁻¹	
	C-limiting medium	N-limiting medium
KH_2PO_4	2.0	2.0
$(\text{NH}_4)_2\text{SO}_4$	1.5	0.1
Succinic acid	0.37	1.18

The pH was adjusted to 7.0 before sterilization. Prior to inoculation 2% (w/v) sterile $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (20 ml litre⁻¹) was added. Bacteria were

harvested after 2 h in stationary phase as shown in Figure 2.1. Routinely, samples of exponentially growing bacteria were obtained from cultures growing in N-limited medium by harvesting at times indicated in Figure 2.1.

In the studies of the effect of culture conditions on esterase and lipase activities, the bacteria grown in one condition were transferred to media of a different limitation. For instance carbon limited bacteria grown in carbon limited medium were harvested aseptically by centrifugation at $5 \times 10^3 \text{ g}_{\text{av}}$ for 20 min at $4 \text{ }^\circ\text{C}$ as described in Methods 2.2.7 and the pellet was washed twice by resuspending in approximately 20 ml of ice-cold 20 mM potassium phosphate buffer, pH 7.0. The bacterial suspension was then transferred to a nitrogen limited medium (starvation medium but without nitrogen). Similarly the bacteria grown under nitrogen limitation were transferred to carbon limited medium (starvation medium). After this change over the bacteria were kept in the media for 3 h at $30 \text{ }^\circ\text{C}$

2.2.3.3 Starvation medium.

One litre of this medium contained 2 g KH_2PO_4 , 1 g $(\text{NH}_4)_2\text{SO}_4$, and adjusted to pH 7.0 before sterilization. Prior to inoculation 2% (w/v) sterile $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (20 ml l^{-1}) was added.

2.2.3.4 Nutrient broth medium.

Nutrient broth (Oxoid CM1; 13 g l^{-1}) was dispensed into 10 ml universal bottles for storage of cultures. Different strains of *A. calcoaceticus* were also grown on 500 ml of nutrient broth (13 g l^{-1}) in 2 litre conical flasks.

2.2.3.5 M9-oleate mineral salts medium.

This medium was used to grow *E. coli* K12 and *A. calcoaceticus* NCIB 8250, to compare the activity of β -oxidation enzymes (Overath *et. al.*, 1969). One litre of this medium contained: 6 g Na_2HPO_4 , 3 g KH_2PO_4 , 0.5 g NaCl , 1 g NH_4Cl and was adjusted to pH 7.4 before sterilization. The media and a 1% (w/v) solution of Brij-35 were mixed in the ratio 4 : 1. The solution of Brij-35 was used to keep the fatty acid carbon source in a soluble form. Prior to inoculation 2% (w/v) sterile $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (20 ml l^{-1}), 1% (v/v) oleic acid (1 ml l^{-1}), 0.1% (w/v) thiamine (1 ml l^{-1}) were added.

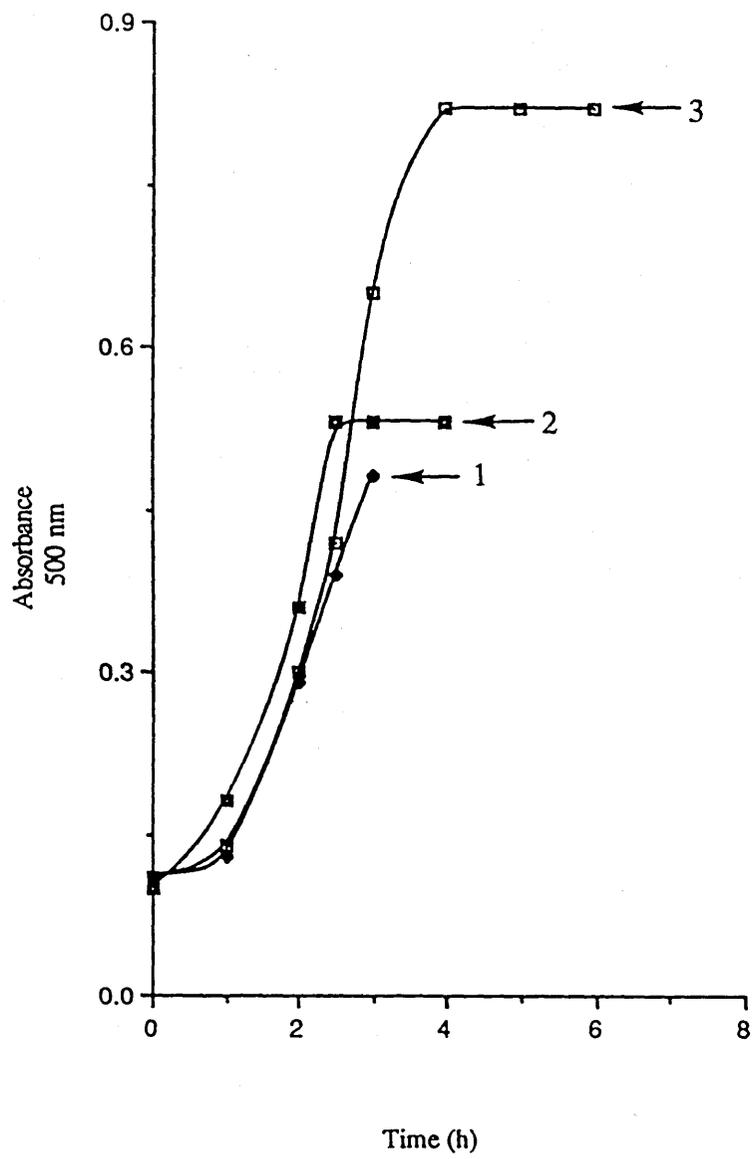
2.2.3.6 Solid medium.

Nutrient agar (Oxoid CM3; 28 g l^{-1}) was dissolved and sterilized. The sterile medium was cooled to $55 \text{ }^\circ\text{C}$ and poured into petri dishes (9 cm diameter, Sterilin Ltd., Teddington, Middlesex). The plates were left to dry at $30 \text{ }^\circ\text{C}$ for 24 h and stored at $4 \text{ }^\circ\text{C}$ prior to use.

Figure 2.1 Growth of *A. calcoaceticus* NCIB 8250 in different culture conditions.

A. calcoaceticus NCIB 8250 was grown on carbon and nitrogen limited media as described in Methods 2.2.3.2. The bacteria designated as exponentially growing were harvested in mid exponential phase, whereas carbon limited or nitrogen limited bacteria were harvested after 2 h in stationary phase. The arrows in the Figure show the time of harvestating.

1. Exponentially growing bacteria. ●
2. Carbon limited bacteria. ■
3. Nitrogen limited bacteria. □



2.2.4 Sterilization.

2.2.4.1 Moist heat.

Media containing carbon source and all other solutions were autoclaved at 5 psi and 109 °C for the times established by Fewson (unpublished results). Efficiency of sterilization was verified by using Browne's tubes Type 1 (A. Browne Ltd., Chancery Street, Leicester).

2.2.4.2 Dry heat.

Glass pipettes were packed in sterilization bags (DRG, Hospital Supplies, Dixon Road, Brislington, Bristol B54 5QY) and sterilized in an oven at 160 °C for 1.75 h. Each sterilization was checked by including a Browne's tube (Type 3).

2.2.4.3 Ethylene oxide.

Plastic pipettes were sterilized with ethylene oxide. They were packed in sterilization bags and exposed to ethylene oxide (Anprolene) for 12 h in a sterilizing box (AN 74; H.W. Anderson Products Ltd., Clacton-on-Sea, Essex). Sterilization was verified by an Anprolene exposure indicator (AN 85) or by a Steritest unit (AN 80). All the apparatus was aired in a fume cupboard for at least 24 h prior to use, to remove any residual ethylene oxide.

2.2.5 Growth of bacteria.

Cultures with volumes of 100 or 500 ml were grown in 250 ml or 2 litre Erlenmeyer flasks respectively. The flasks were stoppered with polystyrene

foam bungs (A. & J. Beveridge Ltd., 5 Bonington Road Lane, Edinburgh EH6 5BP) and shaken on a rotary shaker (MK V Orbital shaker, L.H. Engineering Co. Ltd., Stoke Poges) moving at about 180 oscillations min^{-1} in a room thermostatically maintained at 30 °C. Flasks were inoculated with 0.1-0.5 ml overnight bacterial culture grown on nutrient broth (see Methods 2.2.3.4).

Cultures with volumes of 2 or 4 litre were grown in 10 litre flat-bottomed flasks, plugged with non-absorbent cotton wool through which passed a cotton wool-plugged pipette (10 ml) for blowing air (200-300 ml min^{-1}) over the culture. The cultures were grown at 30 °C under conditions of vigorous aeration produced by a polypropylene-coated magnetic bar (45 mm) stirring at approximately 300 rpm on the apparatus described by Harvey *et al.*, (1968). These 2 and 4 litre cultures were inoculated by the sterile addition of 50 or 100 ml respectively of a 14-16 h grown bacterial culture on routine growth media (see Methods 2.2.3.1).

2.2.6 Turbidity measurement.

Bacterial growth was monitored by measuring the absorbance of the culture or a dilution of a culture relative to air at 500 nm in a 1 cm light-path cuvette using a Unicam SP8-100 Ultraviolet Spectrophotometer (Pye Unicam Instruments Ltd., 576 West Harbour Road, Granton, Edinburgh EH5 1PP) or a LKB Ultrospec II (LKB Instruments Ltd., L.H. Engineering Co. Ltd., Stoke Poges).

2.2.7 Harvesting and storage of bacteria.

Small volumes (50-250 ml) of bacterial cultures were harvested by centrifugation in 50 ml polypropylene centrifuge tubes at $8 \times 10^3 g_{av}$ for 30 min at 4 °C in an MSE 18 Highspeed centrifuge (MSE Scientific Instruments, Crawley, Sussex, RH10 2QQ). The pellet obtained was washed by resuspending and centrifuging twice, in 10 ml of ice-cold 50 mM-potassium phosphate buffer, pH 7.5 or 50 mM Tris-HCl, pH 8.5. The bacterial pellets were broken as described in Methods 2.2.8.2 for further studies without storage.

The bacterial cultures of large volume (500 ml-4 litre) were harvested by centrifugation in 750 ml plastic centrifugation bottles (MSE Ltd., Buckingham Gate, London) at $5 \times 10^3 g_{av}$ for 20 min at 4 °C in an MSE Mistral 6L centrifuge. Each pellet was washed twice by resuspending in approximately 20 ml of ice-cold 50 mM potassium phosphate buffer, pH 7.5 or 50 mM Tris-HCl, pH 8.5 and centrifuged in 50 ml polypropylene centrifuge tubes at $12 \times 10^3 g_{av}$ for 30 min at 4 °C in an MSE 18 Highspeed centrifuge. The bacterial pellets were broken as described in Methods 2.2.8.1 and used for further studies without storage.

The bacterial suspensions of known volume and A_{500} for lipid analysis were pelleted at $12 \times 10^3 g_{av}$ for 20 min at 4 °C in an MSE 18 Highspeed centrifuge and resuspended in 1 ml 20 mM potassium phosphate buffer, pH 7.5 and stored in a 1.5 ml polypropylene Micro-Centrifuge tubes (Scotlab, Righead Industrial Estate, Bellshill, ML4 3JQ U.K.) at -18 °C.

2.2.8 Disruption of bacteria.

2.2.8.1 Ultrasonic disruption.

Small volumes (0.5-1 ml) of bacterial suspensions (40-200 mg wet wt of bacteria) were placed in a 1.5 ml polypropylene Micro-Centrifuge tube surrounded by ice-NaCl slurry and disrupted with a 3 mm Dawe Soniprobe (Type 1130 A; Dawe Instruments Ltd., London) operating at 80 W for a total 4 min using a cycle of 30 sec sonication and 60 sec rest. The supernatant was separated from membranes by centrifugation at $8 \times 10^3 g_{av}$ for 10 min at 4 °C in an MSE Micro-Centrifuge.

2.2.8.2 Disruption by French pressure cell.

The bacteria were resuspended in 50 mM-potassium phosphate buffer, pH 7.5 or 50 mM Tris-HCl, pH 8.5, to a final concentration of 0.5 g wet wt ml^{-1} and broken by passing three times through a French pressure cell (Aminco, Silver Spring, Maryland, U.S.A.) at a pressure of 115 Mpa. The homogenate was centrifuged at $2 \times 10^3 g_{av}$ for 10 min at 4 °C in an MSE 18 Highspeed centrifuge to remove intact bacteria and large debris. The homogenate was then ultracentrifuged using a Ti 50 rotor in a Beckman L2-65B Ultracentrifuge (Spinco Division of Beckman Instruments Inc., Palo Alto, California, U.S.A.) at $1 \times 10^5 g_{av}$ for 2 h. The pellet was washed with buffer by centrifuging at $15 \times 10^3 g_{av}$ for 20 min at 4 °C in an MSE 18 Highspeed centrifuge and resuspended in the buffer used. The soluble fraction (this will be designated as supernate throughout this thesis) and the resuspended pellet were kept on ice for enzyme assays. The samples of supernate were stored at -18 °C until required for the separation of enzyme activity on SDS-PAGE and determination of protein concentration.

2.2.9 Determination of dry weight of bacteria.

Bacterial samples of known absorbance (A_{500}) were dried to constant weight at 105 °C and a calibration curve of dry weights against A_{500} was constructed.

2.3 pH measurement.

The pH values of all solutions were determined using a direct reading pH meter (Model 7010; E.I.L. Ltd., Cumbernauld, Glasgow G67 1AG) connected to a combined glass electrode (Type 224; Probion Ltd., Glenrothes, Fife KY6 3AE).

2.4 Protein estimation.

Protein content of bacterial extracts was measured by the modified Folin method described by Herbert *et al.*, (1971) using bovine serum albumin as the standard.

2.5 Chemical synthesis of wax esters.

The saturated and unsaturated wax esters were prepared synthetically in this laboratory for use as standard waxes for g.l.c. analysis (Table 2.1).

2.5.1 Saturated wax esters.

Saturated wax esters required for use as standards or as an internal standard were prepared according to the method of Baer and Fisher (1945) by reacting the fatty acyl chloride with the fatty alcohol at room temperature for 48 h.

2.5.2 Unsaturated wax esters.

Unsaturated wax esters used were synthesised by Nagi (1981) by the modification of the method described by Selinger and Lapidot (1966) for the preparation of fatty acid anhydrides.

2.5.3 Purification of synthetic wax esters.

The wax esters which were synthesised chemically were purified by the method of Carroll (1976) using a column (40 cm x 2 cm) of Florisil, hydrated with 7% (w/w) water. The column was filled with light petroleum and the hydrated Florisil (30 g) was added in small portions, and allowed to settle before the next addition. The excess solvent was drained out by opening the tap from time to time, as the addition of the Florisil raised the level of solvent in the column.

The wax esters (30 mg g^{-1} of the adsorbent) were dissolved in 2 ml of light petroleum (b.p. $40-60 \text{ }^{\circ}\text{C}$) and loaded carefully on the column and the tap released slowly to allow the the material to pass into the adsorbent. The wax esters were eluted with 100 ml of 5 % diethyl ether in light petroleum (b.p. $40-60 \text{ }^{\circ}\text{C}$). The solvent was evaporated and then light petroleum (b.p. $40-60 \text{ }^{\circ}\text{C}$) was added drop by drop to the dried material until a saturated solution formed. The saturated solution was kept at $-10 \text{ }^{\circ}\text{C}$ for 3-5 min. The crystallised wax ester was poured into a sintered glass funnel and the solvent containing any impurities was removed by suction. The wax ester crystals were dried *in vacuo*. The purity of wax ester was tested by analysing a sample by g.l.c. as described in Methods 2.9 and the purity determined was approximately 95%.

Table 2.1 Standard wax esters prepared synthetically.

Saturated and unsaturated wax esters were prepared as described in Methods 2.5. The number preceding the colon shows the number of carbon atoms either in wax esters or their constituent fatty acids and fatty alcohols, whereas the number following the colon shows the number of double bonds.

Wax ester	Fatty alcohol	Fatty acid
------------------	----------------------	-------------------

(a) saturated

1- 30:0	14:0	16:0
2- 32:0	16:0	16:0
3- 33:0	17:0	16:0
4- 34:0	18:0	16:0
5- 36:0	18:0	18:0

(b) mono-unsaturated

6- 30:1	18:1	12:0
7- 32:1	14:0	18:1
8- 34:1	16:0	18:1
9- 36:1	18:0	18:1

(c) di-unsaturated

10-32:2	14:0	18:2
11-34:2	16:0	18:2
12-36:2	18:0	18:2

2.6 Lipid extraction and purification.

Wax esters were extracted using a chloroform-methanol mixture (2 : 1) (v/v) as described by Folch *et al.*, (1957).

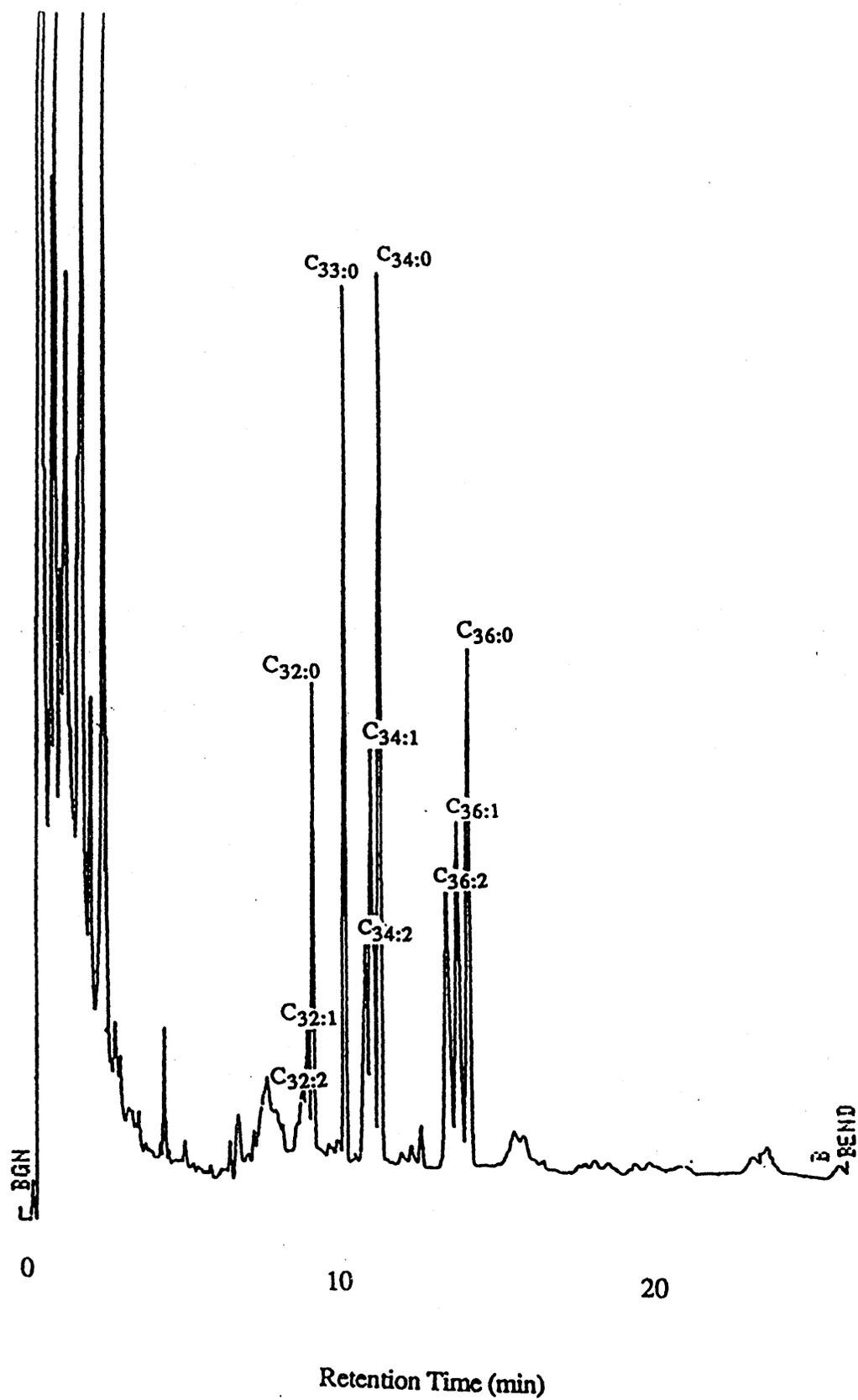
25 ml of the bacterial culture ($A_{500} = 1-1.5$) was centrifuged at $12 \times 10^3 g_{av}$ for 20 min to obtain the pellet, which was resuspended in 1 ml distilled water and mixed with 20 ml chloroform-methanol mixture (2 : 1, v/v). A calculated volume ($100 \mu l$, 0.3 mg ml^{-1} in chloroform) of internal standard, $C_{33:0}$ wax was added, mixed and the mixture allowed to stand for at least 30 min. Then 5 ml of 0.88% (w/v) potassium chloride was added, mixed thoroughly and allowed to stand for at least 30 min. The upper layer containing methanol and water was removed carefully by suction. The chloroform layer contained the extracted lipids. Then anhydrous sodium sulphate (2-3 g) was added to the chloroform layer to remove any traces of water present and was then filtered and dried as described in Methods 2.8.

The amount of $C_{33:0}$ wax (internal standard) required was calculated from the dry weight of bacteria used for wax extraction. It had been shown that bacteria grown in batch culture under nitrogen limited condition with an excess of carbon and energy source contained 50-60 mg wax $(\text{g dry wt bacteria})^{-1}$ (Fixter *et al.*, 1986) and that the wax esters were largely saturated $C_{32:0}$, $C_{34:0}$ and $C_{36:0}$. Therefore, $20 \mu g (\text{mg dry wt bacteria})^{-1} C_{33:0}$ wax was added and this would be about equal to one fifth of the total endogenous wax esters expected to be present. In this case the height of $C_{33:0}$ wax peak is comparable to other wax ester peaks which appeared on g.l.c. analysis as shown in Figure 2.2.

**Figure 2.2 Gas liquid chromatography of wax esters from
A. calcoaceticus NCIB 8250**

A. calcoaceticus NCIB 8250 was grown in nitrogen limited conditions and harvested as described in Methods 2.2.3.2 and 2.2.7 respectively. Wax esters were extracted as described in Methods 2.6 and analysed by g.l.c. as described in Methods 2.9.

Recorder Response



2.7 Separation of free fatty acids from other lipids.

The free fatty acid concentration in bacteria degrading wax esters in aerobic and anaerobic condition was determined after separating the free fatty acids from other lipids by thin layer chromatography. The methyl esters of the separated fatty acids were prepared and used for g.l.c. analysis. The lipids were extracted as described in Methods 2.6 and the internal standard, heptadecanoic acid (12 µg in 0.1 ml of chloroform), was added during lipid extraction of the bacteria.

2.7.1 Thin layer chromatography (t.l.c.).

The thin-layer plates (20x20 cm glass plates) with a 0.5 mm layer of silica gel H (Type 60) were made using a Quickfit spreader and plate holder (Quickfit instruments, Corning Ltd., Laboratory Division, Stone, Staffordshire ST15 0BG). The silica gel (60 g) was slurried with 130 ml of distilled water and spread over five plates with the spreader gap set at 0.5 mm and left for 3 h to set. The plates were activated before use by heating at 105 °C for a minimum of 3 h.

The samples were applied with a glass micropipette (10 µl) 2-3 cm from the bottom of the plate. The fatty acids present in the sample were located by running 20 µl of the standard, oleic acid (10 mg ml⁻¹ in chloroform) along side the samples.

2.7.1.1 Development of the chromatogram.

The thin-layer chromatograms were developed at room temperature in the ascending direction in a Shandon t.l.c. tank (Shandon Southern Instruments

Ltd., Firmley Road, Camberley, Surrey) containing the solvent mixture light petroleum 40-60 °C / diethyl ether / formic acid (150 : 50 : 1, by vol.).

2.7.1.2 Detection of fatty acids on thin-layer plates.

The lipids were visualized in ultra-violet light after the chromatogram had been sprayed with a solution of 0.1% (w/v) 2', 7'-dichlorofluorescein in 95% ethanol when the lipids appeared as yellow-green spots on a purple background. The area containing the fatty acids was carefully scraped off the plate and their methyl esters were prepared (see Methods 2.7.1.3) for further analysis by g.l.c (see Methods 2.10).

2.7.2 Preparation of fatty acid methyl esters.

The silica gel containing the separated fatty acids was transferred to a screw-capped pyrex tube and heated with 2 ml of 2% (v/v) sulphuric acid in dry methanol for 3 h at 70 °C in a heating block (Ori-Block OB-3. Techne, Ltd., Cambridge). The tube was cooled and diethyl ether (4 ml) and water (5 ml) were added. The contents of the tube were mixed thoroughly and the diethyl ether layer was removed into a stoppered tube and 5 ml of sodium carbonate (0.05% w/v) was added to neutralize any sulphuric acid. The ether layer was removed and dried over anhydrous sodium sulphate. The solvent was evaporated as described in Methods 2.8. The methyl ester of heptadecanoic acid was prepared by the method described above.

2.8 Evaporation of solvents.

All organic solvents were evaporated in a fume cupboard. Large volumes of solvents were removed from extracted lipids *in vacuo* under nitrogen (oxygen free) at 40 °C. Small volumes (10 ml) were transferred to conical glass tubes and heated at 40-45 °C on a heating block. A stream of oxygen free nitrogen was directed on to the surface of the solution to aid evaporation and avoid oxidation of the lipids.

2.9 Wax ester analysis.

After evaporating the extraction solvent, the lipid sample containing wax esters was dissolved in Analar toluene (4-6 µl) and 0.2-0.3 µl injected into a Perkin Elmer 8420 Capillary Gas Chromatograph (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire) using a 1 µl syringe, (Hamilton Co., Reno, Nevada. 89510, U.S.A.). The g.l.c. was equipped with an SGE capillary column (length 12 m, internal diameter 0.22 mm) coated with the BP1 stationary phase (0.25 µm thickness). The instrument was operated in the split mode (split ratio, 1 : 50). The temperature programme that gave the best resolution of the wax esters was one with an oven temperature of 250 °C rising immediately at 5 °C min⁻¹ to 300 °C with a final hold of 17 min. Both the injector and detector were at 350 °C. The carrier gas (oxygen free nitrogen) was passed through an oxygen trap (Alltech Associates Inc., New Street, Carnforth, Lancashire LA5 9BX) at a flow rate through the column of 1.5 ml min⁻¹. The instrument was attached to an integrator, which produced a table of retention times and calculated peak areas. A trace of a typical wax ester analysis on g.l.c. is shown in Figure 2.2. The amount of each wax component was calculated relative to the

internal standard according to the equation:

$$\text{mg wax ester (g dry wt bacteria)}^{-1} = \frac{\text{Area of wax component}}{\text{Area of internal standard}} \times \text{mg of internal standard added}$$

g dry wt of bacteria extracted

2.10 Fatty acid analysis.

The methyl esters of fatty acids prepared (see Methods 2.7.2.) were dissolved in 5 μl of toluene after the evaporation of the solvent. The sample (0.2 μl) was analysed as for wax ester analysis (see Methods 2.9) except the temperature programme was modified to an initial oven temperature of 180 $^{\circ}\text{C}$, holding for 17 min and then rising at 10 $^{\circ}\text{C min}^{-1}$ to 250 $^{\circ}\text{C}$ with a final hold of 20 min, and that the injector and detector were at 300 $^{\circ}\text{C}$. The amount of each fatty acid was calculated relative to the internal standard according to the equation described in Methods 2.9.

2.11 Enzyme assays.

2.11.1 Esterase assay with 4-nitrophenyl acetate.

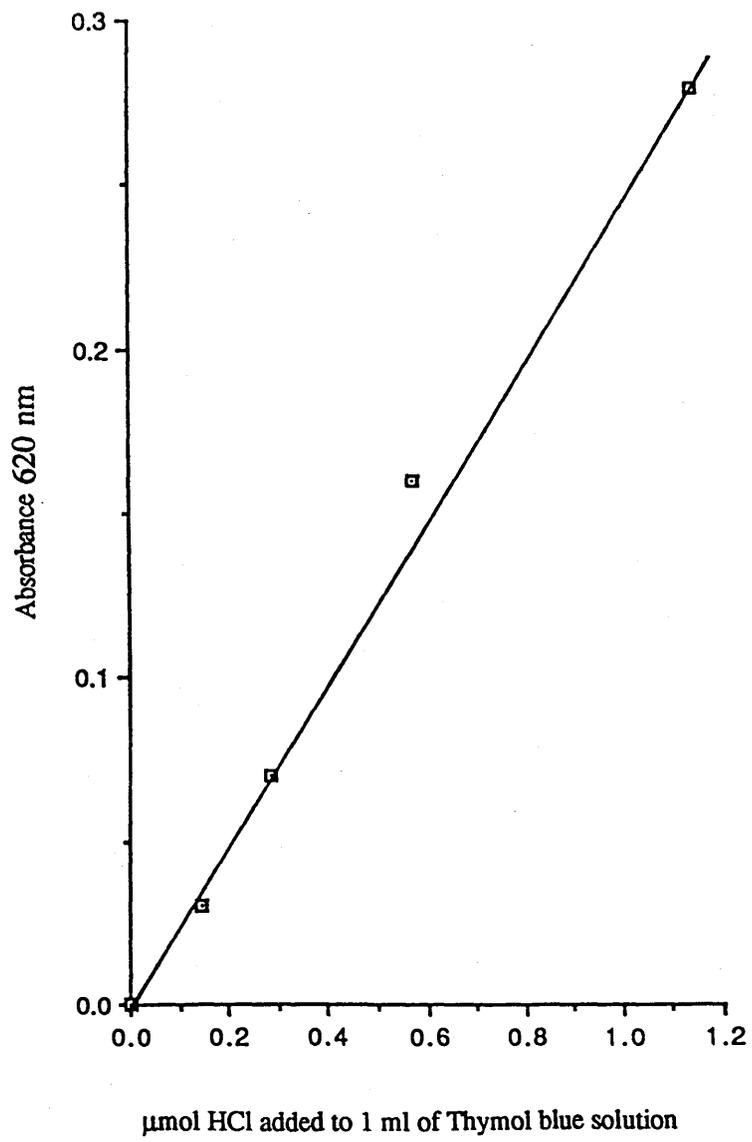
The substrate 4-nitrophenyl acetate was dissolved in 1 ml of methanol and this solution was then dispersed dropwise into 100 ml of 50 mM Tris-HCl, pH 8.5. The assay mixture contained: 0.3 mM 4-nitrophenyl acetate, 50 mM Tris-HCl, pH 8.5 in a final volume of 2.5 ml. The assay was initiated by adding 0.05-0.2 mg extract protein. The assay was carried out at 37 °C and nitrophenol production was measured spectrophotometrically at 400 nm. A calibration curve of nitrophenol was prepared at pH 8.5 to determine the amount of nitrophenol. The development of the assay for esterase activity is discussed in the Results section.

2.11.2 Esterase assay with ethyl butyrate.

Esterase activity with ethyl butyrate was assayed by monitoring the acid production spectrophotometrically using thymol blue. The assay mixture contained: 38 mM ethyl butyrate, 0.0025% (w/v) thymol blue, 5 mM Tris-HCl, pH 8.8 in a final volume of 1 ml. The assay was carried out at 37 °C. The assay was started by adding 0.05-0.1 mg supernate protein and the change in colour was monitored at 620 nm. Nanomoles of butyric acid produced by the action of enzyme were calculated from a calibration curve. The calibration curve was prepared using different amounts of hydrochloric acid in the assay without ethyl butyrate (see Figure 2.3).

Figure 2.3 Calibration curve for the amount of acid liberated in ethyl butyrate esterase assays.

0.0025% Thymol blue was prepared in 5 mM Tris HCl, pH 8.8. To 1 ml of the thymol blue solution different amounts of HCl (0.116 M) were added and absorbance was measured at 620 nm.



2.11.3 Esterase assay with tributyrin.

Esterase activity with tributyrin was assayed by a slight modification of the method used for ethyl butyrate. Tributyrin was dispersed in 5 mM Tris-HCl, pH 8.8 containing (0.1% w/v) deoxycholic acid. The assay mixture contained: 2.6 mM tributyrin, 0.0025% (w/v) thymol blue and 0.1% (w/v) deoxycholic acid, 5 mM Tris-HCl, pH 8.8 in a final volume of 1 ml. The assay was initiated by adding 0.05-0.1 mg supernate protein and the change in colour was monitored at 620 nm. The assay was carried out at 37 °C. Esterase activity was calculated as for ethyl butyrate.

2.11.4 Lipase assay.

Lipase activity was assayed using 4-nitrophenyl hexadecanoate. The substrate was emulsified using two different approaches. In the first method, which is slight modification of Winkler and Stuckmann (1979), the substrate (30 mg) was dissolved in 1 ml iso-propanol and then this solution was dispersed dropwise into 25 ml of 50 mM Tris-HCl, pH 8.5 containing 0.2% (w/v) deoxycholic acid and 0.1% (w/v) gum arabic. In the second method, a modification of the method of Paznokas and Kaplan (1977), the substrate (30 mg) was dissolved in 1 ml dimethyl formamide and this solution was dispersed dropwise into 25 ml of 50 mM Tris-HCl, pH 8.5, containing 1% (v/v) Triton X-100. The assay mixture contained 3.2 mM 4-nitrophenyl hexadecanoate and about 0.9% (v/v) Triton X-100 in a final volume of 1 ml. The assay was started by adding 0.1-0.25 mg of extract protein and was carried out at 37 °C. The production of nitrophenol was monitored spectrophotometrically and the amount of nitrophenol produced was calculated as described for esterase activity with 4-nitrophenyl acetate. The development of

the assay for lipase activity is discussed in the Results section.

2.11.5 Determination of the kinetic constants.

The K_m of the esterase and lipase activity in the supernates was determined by the measurement of initial velocities of enzyme activities with a range of substrate concentrations. The K_m was calculated by Lineweaver-Burk plot using an Enzpack computer programme (Williams, 1985).

2.11.6 Fatty acid oxidation enzymes.

2.11.6.1 Acyl CoA synthetase.

Acyl CoA synthetase was assayed by the method described by Kornberg and Pricer (1953). The assay mixture contained: 2.5 mM ATP, 0.75 mM CoA, 25 mM NaF, 7.5 mM $MgCl_2$, 2 mM oleic acid, 0.05% (v/v) Triton X-100, 500 mM NH_2OH (neutralized with NH_4OH to pH 7.4), 71 mM 2 mercaptoethanol, 60 mM Tris-HCl, pH 8.5 in a final volume of 2 ml. A 2 ml control assay was run without ATP and CoA. The samples were incubated at 37 °C for 1 h after the addition of the supernate protein (5 mg). Then 0.1 ml of bovine serum albumin (2.5 mg) was added and the enzymatic reaction was terminated by the addition of 0.2 ml of $HClO_4$ (70%, v/v). The samples were chilled on ice and centrifuged at $1.2 \times 10^3 g_{av}$ in a Beckman TJ-6 centrifuge. The pellet was washed with 1 ml of 3.5% (v/v) $HClO_4$ and the hydroxamic acid in the pellet treated with 2 ml of 10% (w/v) $FeCl_3$ in 0.2 M HCl. The absorbance of the hydroxamic acid-iron complex formed was measured at 520 nm. The calculation of the enzyme activity was done using an extinction coefficient of $1 \times 10^3 (M^{-1} cm^{-1})$ for the hydroxamic acid formed.

2.11.6.2 Acyl CoA dehydrogenase.

The assay used was the method developed by Arrigoni and Singer (1962) and modified by O'Brien and Frerman (1977). The assay mixture contained: 100 mM-potassium phosphate, pH 7.3, 0.16 mM palmitoyl CoA, 0.3 M MTT, 2 mM PMS, 19 mM potassium cyanide and supernate protein (0.05 mg) in 0.8 ml final volume. The assay was initiated by the addition of palmitoyl CoA. The assay was carried out at 27 °C and the increase in absorbance due to reduction of MTT was measured at 546 nm spectrophotometrically. The readings were corrected with a control assay which was run without palmitoyl CoA. A molar extinction coefficient for reduced MTT of 22,800 ($M^{-1} \text{ cm}^{-1}$) was used.

2.11.6.3 Enoyl CoA hydratase.

The assay used was the method of Wakil and Mahler, (1954). The assay mixture contained: 50 mM 2-amino-2-methyl 1,3-propan diol, pH 9.0, 5 mM mercaptoethanol, 1 mM NAD, 0.25 mM crotonyl CoA, 0.16 units of β -hydroxyacyl CoA dehydrogenase (porcine heart, type III) in a final volume of 0.8 ml. The assay was initiated at 27 °C by the addition of supernate protein (0.05 mg). The increase in absorbance at 340 nm due to formation of NADH was measured spectrophotometrically.

2.11.6.4 L-3-hydroxy-acyl CoA dehydrogenase.

The assay used was the method of Wakil *et al.*, (1954) modified by Weeks *et al.*, (1969). The assay mixture contained: 40 mM Tris-HCl pH 7.0, 80 mM acetoacetyl CoA, 0.16 mM NADH and the supernate protein (0.2 mg) in

a final volume of 1.26 ml. The assay was initiated at 27 °C by the addition of acetoacetyl CoA. The rate of oxidation of NADH was determined by measuring the decrease in absorbance at 340 nm spectrophotometrically.

2.11.6.5 Thiolase.

The assay used was the method of Stern, (1955) modified by Weeks *et al.*, (1969). The assay mixture contained: 150 mM Tris-HCl pH 8.1, 5 mM MgCl₂, 0.25 mM CoA, 4 mM mercaptoethanol, 50 mM acetoacetyl CoA in a final volume of 0.8 ml. The assay was initiated at 27 °C by the addition of supernate protein (0.05 mg). The decrease in absorbance due to cleavage of acetoacetyl CoA was measured at 303 nm spectrophotometrically. The readings were corrected with a control assay run without enzyme. A molar extinction coefficient for acetoacetyl CoA of 16.9×10^3 (M⁻¹ cm⁻¹) was used in calculations.

2.12 Gel permeation chromatography.

This was performed using a Pharmacia f.p.l.c. (Pharmacia LKB Biotechnology, Uppsala, S-751 82 Sweden) equipped with a 30 cm Superose 6 column. Supernate protein (5 mg) was loaded in 0.5 ml of elution buffer. The elution buffer was 50 mM Tris-HCl, pH 8.5 and 1 ml fractions were collected. The column was calibrated using thyroglobulin ($M_T = 669,000$), ferritin ($M_T = 440,000$) and catalase ($M_T = 232,000$).

2.13 Inhibition of esterase and lipase activities in supernates.

The supernates were prepared in 50 mM-potassium phosphate buffer, pH 7.5, as most of the inhibitors are effective at this pH. The supernate protein was treated with the different inhibitors at the concentrations listed in Table 2.2. These inhibitors were dissolved in the same buffer except for PMSF (dissolved in isopropanol, as described by Turini *et al.*, 1969), 2-hydroxy-5-nitrobenzyl bromide (dissolved in dried acetone, as described by Horton *et.al.*, 1965) and THL (dissolved in dimethyl sulphoxide as described by Hadvary *et al.*, 1988). The treatment of supernates with the inhibitors was performed at 0 °C, while that of the sulphhydryl reagents and THL was also carried out at 37 °C.

2.14 Inhibition of esterase and lipase activities in whole bacteria.

In studies of the effects of PMSF, NBS, 2-hydroxy-5-nitrobenzyl bromide and THL on esterase and lipase activities in whole bacteria, the bacteria (500 mg wet wt) were resuspended in 50 mM-phosphate buffer, pH 7.5 (1 ml) and treated with these inhibitors on ice (for concentrations and times, see Table 2.3). The inhibitor was removed by washing the bacteria four times with 50 mM-phosphate buffer, pH 7.5. The bacteria were broken by sonication and centrifuged to obtain the supernate and membrane fractions (see Methods 2.2.8.1). The esterase and lipase activities were assayed in both supernate and membrane fractions.

**Table 2.2 Inhibitors used to inhibit esterase and lipase activities
in supernates.**

The supernates were incubated with inhibitors at the concentrations indicated and the effect on esterase and lipase activities was determined as described in Methods 2.11.1 and 2.11.4 respectively.

Reagents	Concentration (mM)
Reagents which react with serine at the active site	
Phenylmethane sulphonyl flouride (PMSF)	0.5, 1 10
Bis- <i>p</i> -nitrophenyl phosphate	1, 5
Eserine (physostigmine)	0.05, 0.5 4
Reagents which react with sulphhydryl groups at the active site	
Iodoacetate	1, 10
Iodoacetamide	1, 10
Mercuric chloride	1, 10
Reagents which modify tryptophan at the active site	
<i>N</i> -bromosuccinamide (NBS)	5, 10
2-hydroxy-5-nitrobenzyl bromide	10, 20
Others	
Tetrahydrolipstatin (THL)	0.1, 0.4, 0.8

**Table 2.3 Inhibitors used to inhibit esterase and lipase activities
in whole bacteria.**

Whole bacteria were incubated with various concentrations of inhibitors and for the times indicated. The effect on esterase and lipase activities in the supernates and membranes were then determined as described in Methods 2.11.1 and 2.11.4 respectively.

Reagents	Concentration (mM)	Time (h)
PMSF	1, 2	1
<i>N</i> -bromosuccinamide (NBS)	5, 10	1
2-hydroxy-5-nitrobenzyl bromide	10, 20	1
Tetrahydrolipstatin (THL)	0.8, 1.6	2

2.15 Separation of inner and outer membranes.

The inner and outer membranes were separated by slight modification of the method described by Nishimura *et al.*, (1986). The bacteria were grown in carbon and nitrogen limited conditions and harvested (see Methods 2.2.3.2 and 2.2.7 respectively). The bacteria were washed three times by centrifugation with 50 mM Tris-HCl, pH 8.5. The bacteria (6 g wet wt) were resuspended in 12 ml of 50 mM Tris-HCl, pH 8.5 and broken by passing three times through a French pressure cell at 115 MPa (see Methods 2.2.8.2). Deoxyribonuclease 1 mg (g wet wt bacteria)⁻¹ and ribonuclease 1 mg (g wet wt bacteria)⁻¹ were added to degrade DNA and RNA. The suspension was incubated for 10 min at room temperature. The suspension was then centrifuged at $2 \times 10^3 g_{av}$ for 10 min to remove unbroken cells. Crude membranes were separated by centrifugation at $10^5 g_{av}$ for 2 h and were then resuspended and homogenized in 6 ml of the same buffer. Then 2 mg lysozyme was added and the suspension stirred with magnet at low speed for 10 min at room temperature.

Samples (1.5 ml) of the crude membranes were layered on the top of discontinuous sucrose density gradients which were prepared with 1 ml of 70% (w/v), 2 ml of 64% (w/v), 3 ml of 58% (w/v), 2 ml of 52% (w/v), 1.5 ml of 45% (w/v) and 1 ml of 35% (w/v) of sucrose in 50 mM Tris-HCl, pH 8.5. The gradients were centrifuged at $9.8 \times 10^4 g_{av}$ for 16 h using a Beckman SW 40 rotor. The separated bands were collected from top to bottom using a peristaltic pump and washed twice in 50 mM Tris-HCl, pH 8.5 by centrifugation at $10^5 g_{av}$ for 2 h. The purity of the membranes was determined by assaying the markers NADH oxidase for inner membranes and 2-keto-3-deoxy octonic acid (KDO) for outer membranes.

2.15.1 NADH oxidase assay.

NADH oxidase activity was assayed by following the oxidation of NADH at 340 nm (Kennedy and Fewson, 1968). Assay mixture contained: 0.1 mM NADH, 50 mM-potassium phosphate buffer, pH 7.5 in a final volume of 1 ml. The assay was initiated by adding 0.05-0.1 mg extract protein and the assay was carried out at 27 °C.

2.15.2 2-Keto-3-deoxy octonic acid determinations.

KDO was measured by the thiobarbituric acid method of Keleti and Lederer (1974) as described by McDougall *et.al.*,(1983).

Method

Stock solutions

- (a) H_2SO_4 : 0.05 M
- (b) Periodic acid : 0.0125 M in 0.0625 M H_2SO_4
- (c) Sodium arsenite : 2% (w/v) in 0.5 M HCl
- (d) Thiobarbituric acid : 0.3% (w/v) solution was made in warm water.
- (d) KDO : 0.2 $\mu\text{mole ml}^{-1}$
- (e) Deoxyribose : 0.2 $\mu\text{mole ml}^{-1}$
- (f) Butan-1-ol / HCl : 95 : 5 (v/v)

The samples (0.125 ml), the standard KDO (0.125 ml) and the standard deoxyribose (0.125 ml) were heated to 100 °C with 0.05 M H_2SO_4 (0.125 ml) for 20 min. After cooling to room temperature periodic acid (0.25 ml) was added and incubated at room temperature for 20 min. Sodium arsenite (0.5 ml) was added and left for 2 min at room temperature and thiobarbituric acid (2 ml)

was then added and heated to 100 °C for 10 min. The samples were then cooled to room temperature and butan-1-ol / HCl (3 ml) was added and mixed to extract the colour produced. The samples were centrifuged at $1.2 \times 10^3 \text{ g}_{\text{AV}}$ for 5 min in a Beckman centrifuge TJ-6 at 4 °C and the upper coloured butan-1-ol layer was collected carefully using a pasteur pipette. The absorbance was measured against a reagent blank at both 508 nm and 549 nm to eliminate the interference from deoxyribose.

2.16 Wax ester degradation.

2.16.1 In whole bacteria under aerobic conditions.

The degradation of endogenous wax esters was determined in the bacteria grown in nitrogen limited conditions (see Methods 2.2.3.2), which were starved of carbon and energy. The bacteria (approximately 2.5 g wet wt) were resuspended in 5 ml of 20 mM-potassium phosphate buffer, pH 7.0 and 1.25 ml of the bacterial suspension was inoculated into 100 ml samples of starvation medium (see Methods 2.2.3.3) in 250 ml conical flasks. The flasks were shaken on an orbital shaker at 30 °C for 6 h. The samples (one flask i.e. 100 ml culture) for wax ester analysis were collected at 0, 1.5, 3 and 6 h intervals.

2.16.2 In whole bacteria under anaerobic conditions.

The bacteria grown in nitrogen limited condition (see Methods 2.2.3.2) were starved of carbon and energy in anaerobic conditions to determine the rate of degradation of wax esters. The bacteria (2.5 g wet wt) were resuspended in 5 ml of 20 mM-potassium phosphate buffer, pH 7.0 and the suspension was inoculated into 500 ml of starvation medium (see Methods 2.2.3.3) in a 2 litre side arm flask (with a pipette passing through a rubber bung to gas oxygen free

nitrogen into the medium). The flask was gassed with nitrogen for 30 min prior to inoculation (to remove any oxygen) and the oxygen free nitrogen was streamed over the medium throughout the experiment. A control was prepared in the same way except it was gassed with air. The medium was stirred with a magnetic bar on a magnetic stirrer in a 30 °C room for 6 h. The samples (50 ml culture) for wax ester analysis were collected at 0, 1.5, 3 and 6 h intervals.

2.16.3 In cell free extracts under aerobic conditions.

The bacteria (approximately 5 g wet wt) grown in nitrogen limitation (see Methods 2.2.3.2) were resuspended in 10 ml of 20 mM-potassium phosphate buffer, pH 7.0 and broken by passing three times through French pressure cell (see Methods 2.2.8.2). The absorbance of the suspension at 500 nm was determined prior to disruption to determine the dry weight of bacteria. The cell free extracts (5 ml) were starved of carbon and energy source in 50 ml of starvation medium (see Methods 2.2.3.3) in a 250 ml conical flask shaken on a rotary shaker for 6 h at 30 °C. The samples (2.5 ml) for wax ester analysis were collected at 0, 1.5, 3 and 6 h intervals.

2.16.4 In cell free extracts under anaerobic conditions.

The cell free extracts (25 ml) were prepared as described in Methods 2.16.3 and starved of carbon and energy source in 250 ml of starvation medium (see Methods 2.2.3.3) in a 1 litre side arm flask anaerobically as described for whole bacteria (see Methods 2.16.2). A control was prepared in the same way except it was gassed with air. The medium was stirred with a magnetic bar on a magnetic stirrer in a 30 °C room for 6 h. The samples (2.5 ml) for wax ester analysis were collected at 0, 1.5, 3 and 6 h intervals.

2.16.5 In whole bacteria treated with sodium azide.

The effect of sodium azide on the degradation of endogenous wax esters was determined using bacteria grown in nitrogen limited conditions (see Methods 2.2.3.2). The bacteria (2.5 g wet wt) were resuspended in 5 ml of 20 mM-potassium phosphate buffer, pH 7.0 and 1.25 ml of the bacterial suspensions were inoculated into 100 ml samples of starvation medium (see Methods 2.2.3.3) containing 20 mM sodium azide in 250 ml conical flasks. A control was prepared without sodium azide. The flasks were shaken on a rotary shaker for 6 h at 30 °C. The samples (one flask i.e.100 ml culture) for wax ester analysis were collected at 0, 1.5, 3 and 6 h intervals.

2.17 The effect of inhibitors of esterase and lipase activities on wax ester degradation.

The bacteria (2.5 g wet wt) grown in nitrogen limited conditions (see Methods 2.2.3.2) were resuspended in 5 ml of 20 mM-potassium phosphate buffer, pH 7.0 and incubated with the following inhibitors: PMSF (1 mM), NBS (10 mM), 2-hydroxy-5-nitrobenzyl bromide (20 mM) and THL (1.6 mM) for 2 h at 0 °C. The bacteria were washed with 20 mM-potassium phosphate buffer, pH 7.0 four times by centrifugation to remove excess inhibitor and resuspended again in 5 ml of the same buffer. The bacterial suspensions (1.25 ml) was inoculated into 100 ml samples of starvation medium in 250 ml conical flasks. The flasks were shaken on a rotary shaker for 6 h at 30 °C. The samples (one flask i.e.100 ml culture) for wax ester analysis, esterase and lipase activities were collected at 0, 1.5, 3 and 6 h intervals.

2.18 Measurement of oxygen uptake.

Both the endogenous rate of oxygen consumption and the oxygen consumption with succinate as substrate were measured in the bacteria which were treated with PMSF (1 mM), NBS (10 mM), 2-hydroxy-5-nitrobenzyl bromide (20 mM), THL (1.6 mM) and sodium azide (20 mM). Measurements were carried out using a Clark type oxygen electrode (Rank Brothers, Bottisham, Cambridge, CB5 9DA) which produced a millivolt output dependent on the activity of oxygen in the solution. This output was measured via a chart recorder attached to the apparatus. The electrode was calibrated using 3 ml of 50 mM-potassium phosphate buffer, pH 7.0 which was saturated with air by stirring with a magnetic bar and then this buffer was made anaerobic by adding 2-5 mg sodium dithionite. The electrode was maintained at 30 °C by circulating water through the electrode cuvette jacket. 3 ml of 50 mM-potassium phosphate buffer, pH 7.0 and 20 µl of the bacterial suspension (0.44 mg dry wt bacteria) were pipetted into the incubation vessel. The perspex disc lid was carefully screwed down into vessel so that no air bubbles were trapped above the incubation mixture. The endogenous rate of oxygen uptake was recorded for at least 5 min after which 20 µl of 50 mM succinic acid, pH 7.0 was injected into the incubation mixture through a hole in perspex disc using a Hamilton syringe. The rate of oxygen uptake with succinic acid as a substrate was recorded for at least 10 min. A control of untreated bacterial suspension was used to compare the effect of inhibitors on the rate of oxygen uptake. A value of 234 nmol oxygen ml⁻¹ in the aerated buffer was used for calculation (Hodgman, 1960).

2.19 Poly acrylamide gel electrophoresis.

2.19.1 SDS-PAGE (discontinuous system).

The SDS-PAGE system used was that of Laemmli (1970) using a discontinuous Tris-glycine buffer, system.

2.19.1.1 Preparation of gels.

Stock solutions

Solution A : 3 M Tris-HCl, pH 8.5, 0.25% (v/v) TEMED

Solution B : (Running buffer): 25 mM Tris (base), 192 mM glycine, pH 8.8,
0.1% (w/v) SDS

Solution C : 28% (w/v) acrylamide, 0.735% (w/v) *N, N'* methylene
bisacrylamide (deionized with amberlite (0.1%,w/v) and filtered)

Solution D : 0.1 M Tris-HCl, pH 6.8, 0.8% (w/v) SDS, 0.25% (v/v) TEMED

Solution E : 20% (w/v) SDS

2.19.1.2 Gel plates.

Glass plates (9.5 cm x 20 cm x 0.15 cm) were washed with Decon-90 to remove any silicone grease. The plates were assembled with 1.5 mm teflon spacers and placed in a home-made gel making cast.

2.19.1.3 Separating gel (12.5% (w/v) acrylamide).

The main separating gel was prepared by mixing, 25 ml solution A, 89.3 ml solution C, 1 ml solution E and 82.2 ml distilled water (final volume 197.5 ml). The solution was degassed for 2 min in a 500 ml side arm flask with

a vacuum pump. Solid ammonium persulphate (150 mg) was added to initiate polymerisation and the mixture was degassed for a further 2 min. Then it was poured into four plates to approximately 1.5 cm below from the edge of the plates. The edge of the gel was smoothed by adding a thin layer of propan-2-ol on the top of the gel with a pasteur pipette immediately after pouring. After the gel was set, the top of the gel was washed with distilled water to remove the propan-2-ol and dried with a filter paper before pouring the stacking gel.

2.19.1.4 Stacking gel (5.2% (w/v) acrylamide).

The stacking gel was prepared by mixing 10 ml solution D, 17.5 ml solution C and 55 ml distilled water (final volume 82.5 ml). Polymerisation was initiated by adding 150 mg of solid ammonium persulphate, the mixture was degassed for 2 min and poured on the top of the separating gel. Teflon combs (4x18 track) were placed in the stacking gel before polymerisation started.

2.19.1.5 Preparation of samples and electrophoretic conditions.

Stock solutions

Solution F (Tracker dye) : To 10 ml of 0.5 M Tris-HCl, pH 8.8 were added 1.5 g SDS, 8 g sucrose and 100 mg Pyronin Y.

Solution G: 0.2 M DTT

The samples were prepared by mixing protein (40 µl), 25 µl of solution G and 10 µl of solution F together in a 1.5 ml polypropylene Micro-centrifuge tube. The amount of protein used for esterase activity was 250-300 µg per track and for other proteins, 20-40 µg. The samples were heated to 100 °C in a

boiling water bath for 2 min and then loaded on to the gel. The samples were electrophoresed at 80 mA per gel until the dye front was approximately 0.2 cm from the bottom of the gel. The gel was kept cool during electrophoresis by circulating ice cold water through the electrophoresis chamber.

2.19.1.6 Molecular mass determination on SDS PAGE.

The relative molecular mass (M_r) of unknown proteins was estimated by using standard proteins of known molecular mass along side the proteins of unknown molecular mass on SDS-PAGE. A calibration curve was prepared from electrophoretic mobilities (R_f) of the proteins of known molecular mass which allowed the M_r of the proteins to be determined.

$$R_f = \frac{\text{distance migrated by protein}}{\text{distance migrated by trackers dye}}$$

A stock solution of molecular mass markers was prepared by adding 400 μ l of distilled water and 100 μ l of tracker dye (section 2.19.1.5) to one vial of molecular mass markers. The contents were mixed and heated to 100 $^{\circ}$ C in a boiling water bath for 5 min and stored at -20 $^{\circ}$ C. The sample of molecular mass markers for loading on the gel was prepared by mixing 20 μ l of stock solution of molecular mass markers with 10 μ l of 0.2 M DTT. The sample was heated to 100 $^{\circ}$ C for 2 min and 10 μ l was loaded per track.

2.19.2 Non-denaturing gels.

2.19.2.1 Preparation of gels.

Stock solutions

Solution A: Same as for denaturing gel system (see Methods 2.19.1.1)

Solution B: (running buffer): 25 mM Tris (base) and 192 mM glycine, pH 8.5

Solution C: Same as for denaturing gel system (see Methods 2.19.1.1)

The gels (5% (w/v) acrylamide) were prepared by mixing 25 ml solution A, 36 ml solution C and 137 ml distilled water. Polymerisation was initiated by the addition of 150 mg solid ammonium persulphate. The solution was degassed for 2 min and poured into the casting box containing four plates. Teflon combs (4x18 track) were used to make wells for sample application.

2.19.2.2 Sample preparation and electrophoresis conditions.

The samples containing 250-300 μ g protein were mixed with 10 μ l of 0.05 % (w/v) Bromophenol Blue in 10 % (v/v) glycerol. The samples were loaded on the gel and electrophoresed at 40 mA per gel until the dye front was approximately 0.2 cm from the bottom of the gel. The gel was kept cool during electrophoresis by circulating ice cold water (4 $^{\circ}$ C) through the electrophoresis chamber.

2.20 Staining of gels.

2.20.1 Protein staining.

The gels were stained by immersing in a staining solution (0.1% (w/v) Coomassie Brilliant Blue G 250 in methanol / acetic acid / H₂O (50 : 10 : 40 by vol) for 1 h at 60 °C. Protein bands were visualized by destaining in methanol / acetic acid / H₂O (10 : 10 : 80, by vol) for about 2 h at 60 °C with several changes of the destaining solution.

2.20.2 Activity staining with 1-naphthyl acetate and 2-naphthyl acetate.

Activity staining of native gels was performed directly, but SDS containing gels were first washed by agitating for 2 h on a shaker at slow speed in two changes of 500 ml of 50 mM Tris-HCl, pH 8.5 at 23 °C. The activity stain contained either 0.2% (w/v) 1-naphthyl acetate or 0.2% (w/v) 2-naphthyl acetate, 0.1% (w/v) Fast blue salt RR in 50 mM Tris-HCl, pH 8.5. The gels were kept in 100 ml of the staining solution at 37 °C until stained bands developed (approximately 30 min). If there was any evidence of a coloured precipitate forming in the staining solution, it was replaced with fresh solution.

2.20.2.1 Effect of amount of protein loaded on the SDS gel on the esterase activity.

Different amounts of supernate protein (i.e 150, 200, 250 and 300 µg) were loaded on to an SDS-PAGE gel to determine the effect of amount of protein on esterase activity. The gel was electrophoresed and stained with 1-naphthyl acetate as described in Methods 2.19.1 and 2.20.2. The brown bands of

esterase activity were scanned as described in Methods 2.22.

2.20.2.2 Effect of time of incubation of SDS gel in the staining substrate on the esterase activity.

Same amount of supernate protein (250 μ g) was loaded in eight tracks of gel. The gel was electrophoresed and washed as described in Methods 2.19.1 and 2.20.2 respectively. Then the gel was cut into 4 pieces containing two tracks and stained the pieces for esterase activity for 0, 20, 40 and 60 min by incubating with 1-naphthyl acetate (see Method 2.20.2). The brown bands of esterase activity were scanned as described in Methods 2.22.

2.20.3 Activity staining with ethyl butyrate.

The gel was run in denaturing conditions except that 0.05% (w/v) thymol blue was added in the running buffer. The gel was washed as for the determination of naphthyl acetate esterase activity except that in 5 mM Tris-HCl, pH 8.8 containing 0.05% (w/v) thymol blue. The washing in the presence of thymol blue made a gel with a blue background. The esterase activity was developed by immersing the gel in the substrate solution (0.5% (v/v) ethyl butyrate in 5 mM Tris-HCl, pH 8.8 at 37 °C until yellow bands developed (approximately 45 min).

2.21 Inhibition of esterase activities on SDS-PAGE gels.

Equal amounts of supernate protein (250 μ g) were applied to 16 tracks of an SDS-PAGE gel and electrophoresed as described in Methods 2.19.1. The gel was washed in 50 mM Tris-HCl, pH 8.5 to remove the SDS (see

Methods 2.20.2). The gel was cut vertically into 8 pieces containing two tracks. One piece of gel was used as a control and the others were treated with inhibitors, PMSF (1 mM), bis-*p*-nitrophenyl phosphate (1 mM), eserine (10 mM) dissolved in 40 ml of 50 mM Tris-HCl, pH 8.5, for 1 h at 37 °C. The gels were washed 3-4 times with water to remove excess of inhibitor and soaked in 50 mM Tris-HCl, pH 8.5 for 10 min. The gels were stained for esterase activity with 1- and 2-naphthyl acetate (see Methods 2.20.2).

2.22 Scanning of gels.

After gels were stained, they were scanned using an LKB 2202 Ultrosan Laser Densitometer (LKB Producter AB Electrophoresis Products, Bromma, Sweden).

2.23 Determination of the viability of bacteria having low and high content of accumulated wax esters.

The bacteria were grown in carbon or nitrogen limited medium (see Methods 2.2.3.2). The bacteria (0.5 g wet wt) were resuspended in 2 ml of 20 mM-potassium phosphate buffer, pH 7.0 and 1 ml of the bacterial suspension was inoculated into 100 ml samples of starvation medium without ammonium sulphate in 250 ml conical flasks ($A_{500} = 0.6$). The flasks were shaken on rotary shaker for 240 h at 30 °C. The samples for bacterial viable counts were taken at the start and then after every 24 h. The bacterial count was carried out by diluting 0.1 ml of the sample was diluted logarithmically (1:100, 1:1000, 1:10000) into 9.9 ml of sterile distilled water and 0.1 ml of the diluted sample from each dilution was spreaded over duplicate nutrient agar plates. The bacteria were grown on agar plates at 30 °C for 36-48 h. The number of colonies on each plate were counted and multiplied by dilution to determine the number of

viable bacteria per ml of the original sample.

2.24 Photography.

Photography of the gels and other materials was done by members of staff at the Medical Illustration Unit, University of Glasgow.

2.25 Glassware.

The growth flasks were washed by autoclaving in 1 % (w/v) Hemosol solution (Meinecke & Co., Baltimore, U.S.A.). They were thoroughly rinsed with tap water followed by distilled water and then dried in an oven. All other glassware was cleaned by immersing in Hemosol overnight, rinsed thoroughly with tap and distilled water and then dried in an oven. All pipettes were cleaned by first soaking in "Kirbychlor" disinfectant solution (H & T Kirby & Co. Ltd., Mildenhall, Suffolk). They were unplugged and steeped in Hemosol solution overnight, rinsed thoroughly in tap water followed by deionized water and dried in an electrically heated pipette drier. All pipettes were plugged with cotton wool for safety reasons.

2.26 Safety.

The Guide Lines for Microbiological Safety issued by The Joint Co-ordinating Committee for the Implementation of Safe Practice in Microbiology were observed to avoid hazards from the microorganisms used in this study.

Bacterial cultures were killed by autoclaving before disposal. Any

bacterial spillage was immediately swabbed with 10% v/v propan-2-ol. All other general safety precautions taken are described in the University of Glasgow Safety Handbook.

2.27 Statistical methods.

Means and standard deviations were calculated using a Casio Scientific Calculator fx82A. Where the statistical significance of the results was required, it was determined by applying 'Student's' *t*-test. The versions of the *t*-test formula used were a standard *t*-test for randomized results or the *t*-test for paired results when appropriate (Wardlaw, 1987).

Chapter 3

Results

3.1 Wax ester degradation in *A. calcoaceticus* NCIB 8250.

The degradation of endogenous wax esters was determined in bacteria grown in nitrogen limited conditions by starving them of carbon and energy source. The rate of wax ester degradation in whole bacteria and cell free extracts is shown in Figure 3.1. It can be seen that wax esters were degraded in whole bacteria as well as in cell free extracts. The approximate initial and final rates of wax ester degradation were determined from such figures (see Table 3.1). The initial rate of total wax ester (saturated + unsaturated) degradation in cell free extracts was about 60% of that of whole bacteria, being about $14.82 \mu\text{mol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$ [$7.43 \text{ mg (g dry wt bacteria)}^{-1} \text{ h}^{-1}$], while the rate in whole bacteria which was $22.5 \mu\text{mol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$ [$11.50 \text{ mg (g dry wt bacteria)}^{-1} \text{ h}^{-1}$]. The final rate of total wax ester (saturated + unsaturated) degradation in whole bacteria ^{was} $1.63 \mu\text{mol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$ [$0.83 \text{ mg (g dry wt bacteria)}^{-1} \text{ h}^{-1}$], and in cell free extracts were $1.96 \mu\text{mol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$ [$1.0 \text{ mg (g dry wt bacteria)}^{-1} \text{ h}^{-1}$]. The major wax esters present were saturated C_{32} , C_{34} and C_{36} . The rates of degradation of the individual waxes $C_{32:0}$, $C_{34:0}$ and $C_{36:0}$ in cell free extracts were also about 60% those in whole bacteria (see Table 3.2). In whole bacteria, the initial rate of degradation of $C_{32:0}$ wax [$6.16 \mu\text{mol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$] was about 20% higher than that of $C_{34:0}$ wax [$5.11 \mu\text{mol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$] and the rate of degradation of the $C_{36:0}$ wax [$1.23 \mu\text{mol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$] was about 20% of the rate of the $C_{32:0}$ wax (see Table 3.2). In cell free extracts, the initial rate of degradation of $C_{32:0}$ wax [$4.79 \mu\text{mol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$] was about 25% higher than that of $C_{34:0}$ wax [$3.57 \mu\text{mol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$] and the rate of degradation of the $C_{36:0}$ wax [$1.27 \mu\text{mol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$] was about 20% of the rate of the $C_{32:0}$ wax (see Table 3.2).

Figure 3.1 Degradation of endogenous wax esters by whole bacteria and cell free extracts.

A. calcoaceticus NCIB 8250 was grown in nitrogen limited conditions and harvested as described in Methods 2.2.3.2 and 2.2.7 respectively. The whole bacteria (2.5 g wet weight) were resuspended in 5 ml of 20 mM-potassium phosphate buffer, pH 7.0 and 1.25 ml of the bacterial suspension was inoculated into 100 ml samples of starvation medium as described in Methods 2.16.1. The bacteria were incubated under these conditions for various times. 25 ml of the bacterial culture was used for the determination of wax esters. The dry weight of bacteria was determined from the absorbance of the culture using the calibration curve of dry weight against A_{500} . Wax esters were extracted as described in Methods 2.6 and analysed by g.l.c. as described in Methods 2.9.

Cell free extracts from the same bacteria used above were prepared as described in Methods 2.2.8.2. The cell free extracts were starved of carbon and energy source as described in Methods 2.16.3. Then samples (2.5 ml) were used for wax ester analysis. Wax esters were extracted as described in Methods 2.6 and analysed by g.l.c. as described in Methods 2.9.

Whole bacteria. □

Cell free extracts. ●

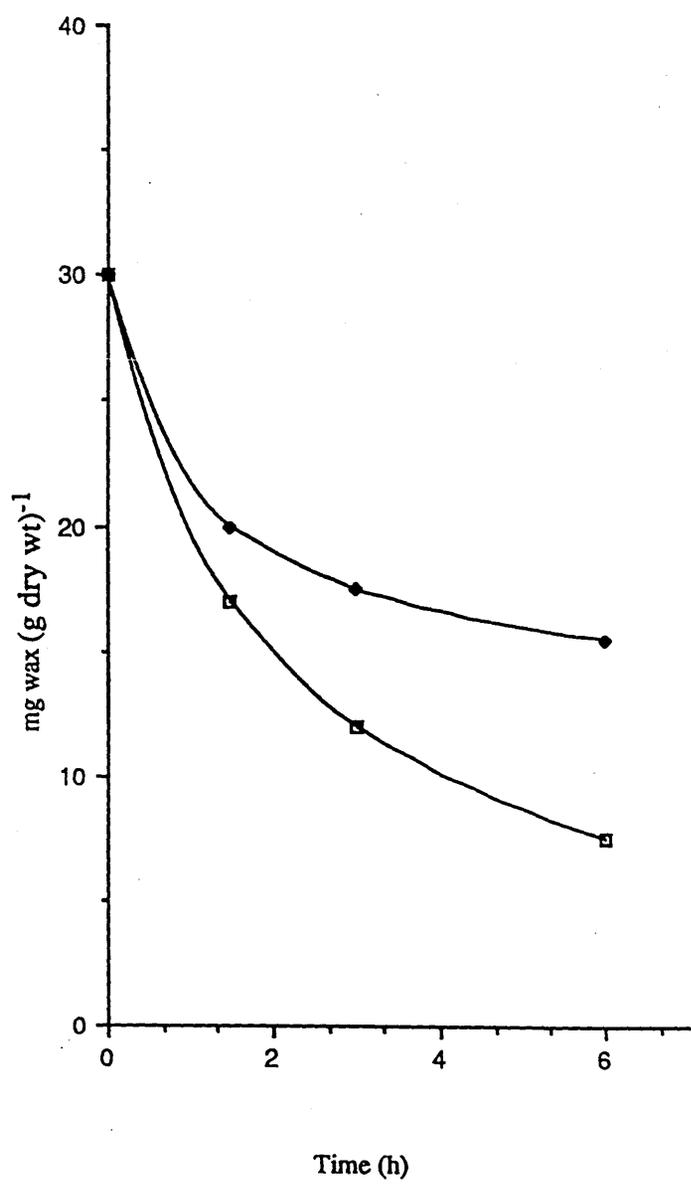


Table 3.1 Comparison of the initial and final rates of degradation of total wax esters in whole bacteria and cell free extracts.

The experimental details are described in the legend to Figure 3.1. The initial and final rates of degradation of total wax were calculated from the change in the amount of wax ester per hour. The results are the means of determinations on two separate bacterial cultures.

Rate of total wax degradation
 $\mu\text{mol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$

	Whole bacteria	Cell free extracts
Initial rate	22.50	14.82
Final rate	2.63	1.96

Table 3.2 Comparison of the initial rates of degradation of saturated wax esters in whole bacteria and cell free extracts.

The experimental details are described in the legend to Figure 3.1. The initial rates of degradation of saturated wax esters were calculated from the change in the amount of wax ester per hour. The results are the means of determinations on two separate bacterial cultures.

Initial rate of wax degradation
 $\mu\text{mol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$

	Whole bacteria	Cell free extracts
C_{32:0}	6.16	4.79
C_{34:0}	5.11	3.57
C_{36:0}	1.23	1.27

The degradation of wax esters in whole bacteria was determined in a number of experiments. The amount of total wax esters degraded is shown in Table 3.3. It can be observed from the statistical analysis of the results that the degradation of wax esters is highly significant either at 3 h or 6 h.

3.2 The effect of starvation on endogenous respiration in wax ester degrading bacteria.

The rate of endogenous respiration was determined in bacteria degrading endogenous wax esters during starvation. The rate of oxygen consumption by the exponentially growing bacteria and in the nitrogen limited bacteria harvested at stationary phase were about $0.19 \text{ mmol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$ and $0.13 \text{ mmol (g dry wt bacteria)}^{-1} \text{ h}^{-1}$ respectively. The wax ester content of the nitrogen limited bacteria was $18 \text{ mg (g dry wt bacteria)}^{-1} \text{ h}^{-1}$ (see Figure 3.2). It was observed that during carbon and energy source starvation the consumption of oxygen by the nitrogen limited bacteria was decreased by only about less than 10% in first 4 h, when the wax esters were being degraded rapidly (see Figure 3.2). While after 6 h, when about 80% of the wax esters have been consumed, the rate of oxygen consumption had only decreased by about 20% (see Figure 3.2).

Table 3.3 The rates of wax ester degradation in whole bacteria degrading wax esters.

The experimental details are described in the legend to Figure 3.1. The results were obtained from seven separate bacterial cultures. The value for the decrease in wax ester content between 0 h and 3 h was highly significant ($P = < 0.01$), using Student's 't' test (for paired data), similarly the decrease in the wax ester content between 3 h and 6 h is also highly significant ($P = < 0.01$)

mg wax (g dry wt bacteria)⁻¹

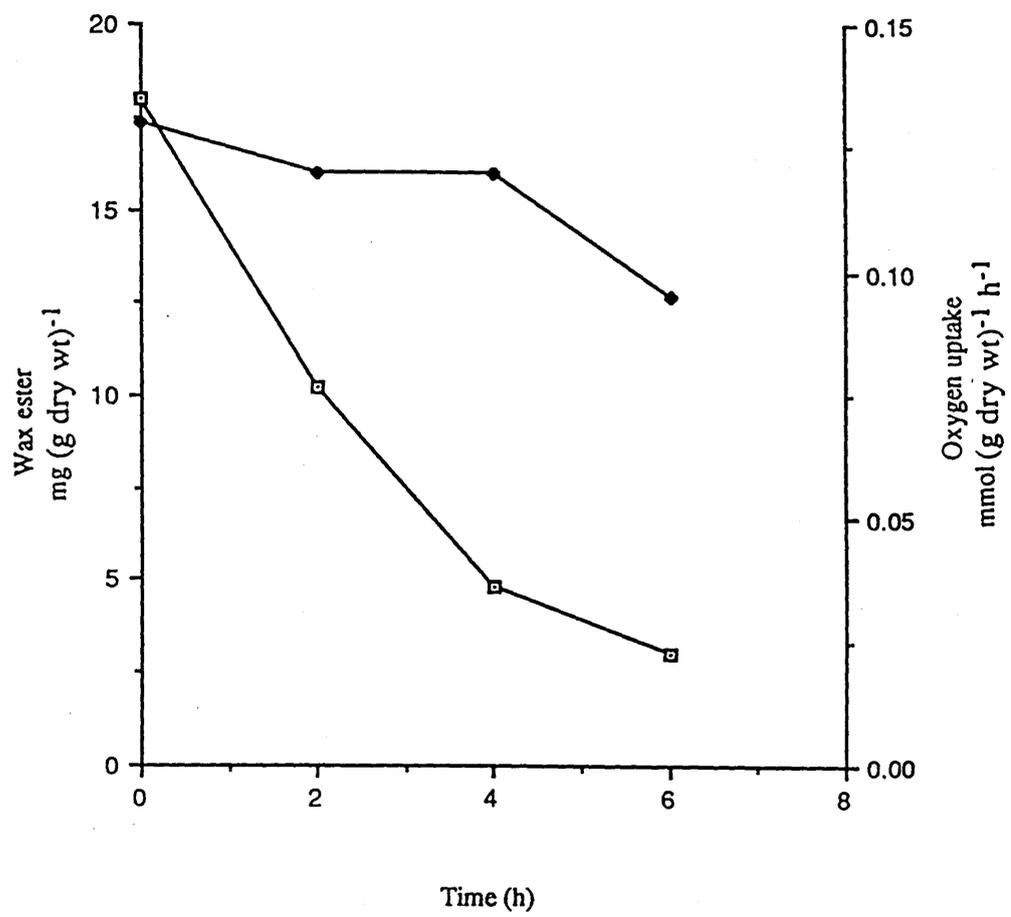
	0 h	3 h	6 h
Expt 1	23.4	10.2	4.4
Expt 2	28.2	8.0	3.1
Expt 3	28.1	12.5	8.2
Expt 4	26.4	12.4	10.0
Expt 5	12.4	6.1	5.1
Expt 6	20.2	11.3	8.1
Expt 7	35.0	22.5	17.2

Figure 3.2 The effect of carbon and energy source starvation on the endogenous consumption of oxygen by bacterial suspensions.

A. calcoaceticus NCIB 8250 was grown in nitrogen limited conditions and harvested as described in Methods 2.2.3.2 and 2.2.7 respectively. Whole bacteria (2.5 g wet weight) were resuspended in 5 ml of 20 mM-potassium phosphate buffer, pH 7.0 and 1.25 ml of the bacterial suspension was inoculated into 100 ml samples of starvation medium as described in Methods 2.16.1. The bacteria were starved of carbon and energy source for various times. 25 ml of the bacterial culture was used for the determination of wax esters as described in the legend to Figure 3.1. For the determination of endogenous consumption of oxygen, a sample of 25 ml of the culture was centrifuged and the pellet was resuspended in 0.5 ml of 20 mM-potassium phosphate buffer, pH 7.0. 100 μ l of the bacterial suspension [about 3 mg (g dry wt)⁻¹] was used to determine the consumption of oxygen as described in Methods 2.18.

Wax degradation □

Oxygen uptake ●



3.3 Comparison of the activities of β -oxidation enzymes in *A. calcoaceticus* NCIB 8250 and *E.coli* K12.

The activities of the β -oxidation enzymes were determined in the supernates of NCIB 8250 and *E. coli* K12 (see Table 3.4). The activities of all enzymes except β -hydroxy acyl CoA dehydrogenase were higher in NCIB 8250 grown on succinate or oleate than *E coli* K12 grown on oleate. But the activities of these enzymes were lower in NCIB 8250 grown on succinate than that of NCIB 8250 grown on oleate.

**Table 3.4 Fatty acid oxidation enzymes in *A. calcoaceticus*
NCIB 8250 and *E. coli* K12.**

A. calcoaceticus NCIB 8250 was grown on succinate and M9-oleate, while *E. coli* K12 was grown on M9-oleate (see Methods 2.2.3.1 and 2.2.3.5). The supernate^s were prepared as described in the legend to Figure 3.3. The enzymes were assayed in the supernate fractions (see Methods 2.11.6). The results are the averages of duplicate assays.

Specific activity
nmol min⁻¹ (mg protein)⁻¹

	NCIB 8250 on succinate	M9-oleate	<i>E. coli</i> K12 M9-oleate
β-oxidation enzymes			
Acyl CoA synthetase	4.5	41.4	2.3
Acyl CoA dehydrogenase	16.4	65.4	3.4
Enoyl CoA hydratase	850.5	1910.3	512.2
β-hydroxy acyl CoA dehydrogenase	25.6	35.5	453.4
Thiolase	160.3	165.6	67.2

3.4 Hydrolysis of chromogenic and aliphatic ester substrates by supernates and the assay of esterase and lipase.

The supernate of *A. calcoaceticus* NCIB 8250 grown on succinate (Methods 2.2.3.1) was able to hydrolyse nitrophenyl esters of short and long chain fatty acids and the aliphatic esters i.e. ethyl butyrate and tributyrin (see Table 3.5). The highest activity was shown with the water soluble substrates e.g. nitrophenyl acetate, and became progressively less as the acyl chain length of the ester increased. With the increase in chain length, i.e. from C₂ to C₁₈, the esters became more difficult to disperse in water, without using a detergent and therefore, deoxycholic acid and gum arabic were used to solubilise all substrates except 4-nitrophenyl acetate. The rate with 4-nitrophenyl octadecanoate was only about 1% of rate obtained with nitrophenyl acetate. The esterase activity assayed with ethyl butyrate was four to five times higher than that of 4-nitrophenyl acetate while the esterase activity with tributyrin was about double that of 4-nitrophenyl acetate.

The supernates from other strains of *A. calcoaceticus* which had been grown on succinate and nutrient broth were also investigated for esterase activity. The esterase activity in all strains tested was about same except for strain ATCC 23055 which had less than half of the activity of other strains when grown in nutrient broth (see Table 3.6). The esterase activities were about double when NCIB 8250 and RAG-1 were grown in succinate salts medium than those in nutrient broth (see Table 3.6).

Table 3.5 Esterase activity with different types of esters

The supernate used was prepared as described in the legend to Figure 3.3. The esterase activity with 4-nitrophenyl acetate, ethyl butyrate and tributyrin was determined as described in Methods 2.11. The substrates 4-nitrophenyl butanoate, 4-nitrophenyl hexanoate, 4-nitrophenyl octanoate, 4-nitrophenyl decanoate, 4-nitrophenyl dodecanoate, 4-nitrophenyl hexadecanoate, 4-nitrophenyl octadecanoate were prepared using deoxycholic acid and gum arabic as described in Methods 2.11.4.

Substrate	Conc (mM)	Esterase specific Activity nmol min⁻¹ (mg protein)⁻¹
Chromogenic esters		
4-nitrophenyl acetate	0.33	150.5
4-nitrophenyl butanoate	0.11	75.0
4-nitrophenyl hexanoate	0.18	40.4
4-nitrophenyl octanoate	0.18	23.0
4-nitrophenyl decanoate	0.16	51.0
4-nitrophenyl dodecanoate	0.16	5.2
4-nitrophenyl hexadecanoate	0.4	4.3
4-nitrophenyl octadecanoate	0.25	2.0
Aliphatic ester		
Ethyl butyrate	38	664.4
Tributylin	2.5	273.3

Table 3.6 Esterase activity in different strains of *A. calcoaceticus*

The bacteria were grown on succinate-minimal salts medium and nutrient broth as described in Methods 2.2.3.2 and 2.2.3.4. The bacteria (0.5 g wet weight ml⁻¹) were broken by sonication and supernates were prepared as described in Methods 2.2.8.1. The esterase activity was assayed as described in Methods 2.11.1.

- = not done

Strain	Specific Activity nmol min ⁻¹ (mg protein) ⁻¹	
	Nutrient broth	Succinate salts
NCIB 8250	42.5	102.8
EBF 65/65	30.3	-
ATCC 23055	10.0	-
RAG-1	55.8	122.4

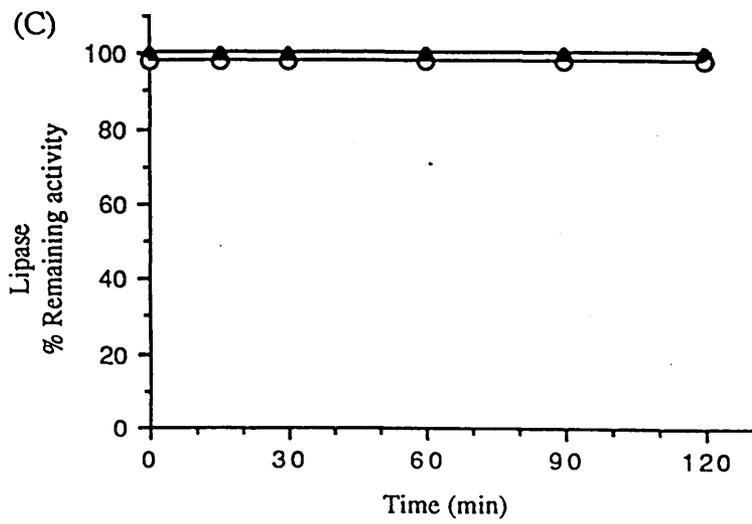
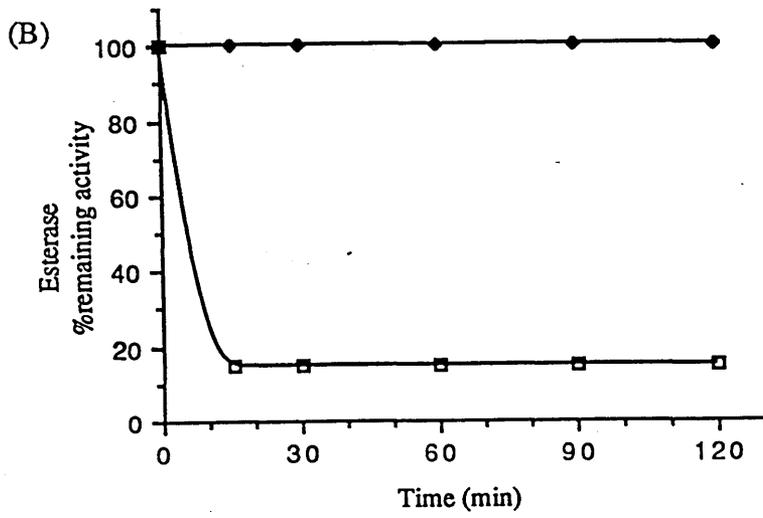
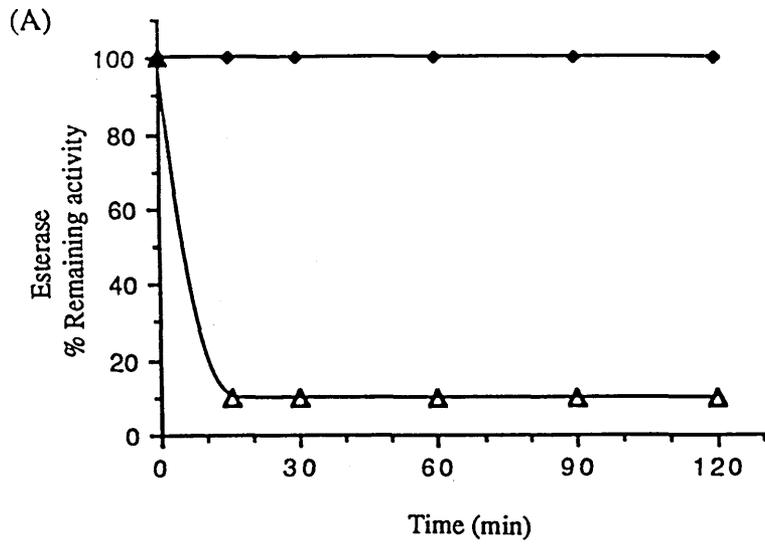
3.5 Characterization of esterase and lipase activities in *A. calcoaceticus* NCIB 8250.

The supernate of *A. calcoaceticus* NCIB 8250 was treated with PMSF (1 mM) and hydrolytic activities were assayed with 4-nitrophenyl acetate, ethyl butyrate and 4-nitrophenyl hexadecanoate. The hydrolytic activities, using 4-nitrophenyl acetate and ethyl butyrate as substrates were inhibited very rapidly by about 90%. After 15 min treatment the activities were inhibited irreversibly (see Figure 3.3 A and B), while the hydrolytic activity using 4-nitrophenyl hexadecanoate was totally insensitive to PMSF at this concentration during at least a 2 h treatment (see Figure 3.3 C). Even at higher concentrations of PMSF (10 mM), the activity using 4-nitrophenyl hexadecanoate was not significantly inhibited. These results helped to differentiate two enzyme activities, an esterase activity (assayed with 4-nitrophenyl acetate or ethyl butyrate) which is inhibited by PMSF and a lipase activity (assayed with 4-nitrophenyl hexadecanoate) which is insensitive to PMSF. Therefore separate assays were developed for determining esterase and lipase activities independently.

Figure 3.3 Characterization of esterase and lipase activities.

A. calcoaceticus NCIB 8250 was grown on routine growth medium and harvested as described in Methods 2.2.3.1 and 2.2.7. The bacteria were resuspended in 50 mM Tris HCl, pH 8.5 at 1 g wet wt ml⁻¹. French pressed and ultracentrifuged as described in Methods 2.2.8.2. The supernate (0.5 ml) was treated with PMSF (1 mM) as described in Methods 2.13. The esterase activity was determined using 4-nitrophenyl acetate (A), ethyl butyrate (B) and the lipase activity using 4-nitrophenyl hexadecanoate (C) as described in Methods 2.11.1, 2.11.2 and 2.11.4 respectively. The 100% values were 80.4 nmol min⁻¹ (mg protein)⁻¹, 660.5 nmol min⁻¹ (mg protein)⁻¹ and 18.5 nmol min⁻¹ (mg protein)⁻¹ for 4-nitrophenyl acetate, ethyl butyrate and 4-nitrophenyl hexadecanoate respectively with this extract.

- (A) 4-Nitrophenyl acetate, Control ●, PMSF treated ▲
- (B) Ethyl butyrate, Control ●, PMSF treated ■
- (C) 4-Nitrophenyl hexadecanoate, Control ●, PMSF treated ○



3.5.1 Development of an assay for esterase activity.

3.5.1.1 The effect of concentration of 4-nitrophenyl acetate on esterase activity.

4-nitrophenyl acetate was a suitable water soluble ester which could be used for esterase activity and therefore, 4-nitrophenyl acetate was used for esterase determinations throughout this work. Different concentrations of 4-nitrophenyl acetate were used to determine the concentration for maximum esterase activity. The supernates showed maximum activity above a concentration of 0.15 mM 4-nitrophenyl acetate (see Figure 3.4), therefore, 0.3 mM 4-nitrophenyl acetate was always used for assaying esterase activity. The K_m of esterase activity in the supernate was also calculated from the activity at different substrate concentrations. The results were calculated using the Enzpack computer programme (Williams, 1985), giving a calculated K_m of 0.12 mM.

3.5.1.2 The effect of time and protein concentration on esterase activity.

The production of nitrophenol from 4-nitrophenyl acetate by the action of esterase was linear from 0-5 min with 0.3 mM 4-nitrophenyl acetate at pH 8.5. Therefore, the enzyme activity was always calculated within this period (see Figure 3.5 A). The rate of production of nitrophenol was proportional to the amount of bacterial protein between 0.05-0.20 mg protein per assay (see Figure 3.5 B), therefore, protein concentrations within this range were used for esterase activity.

Figure 3.4 The effect of 4-nitrophenyl acetate concentration on esterase activity.

The supernate used was prepared as described in the legend to Figure 3.3. The supernate was used for the assay of esterase activity as described in Methods 2.11.1. The esterase activity is plotted against substrate concentration.

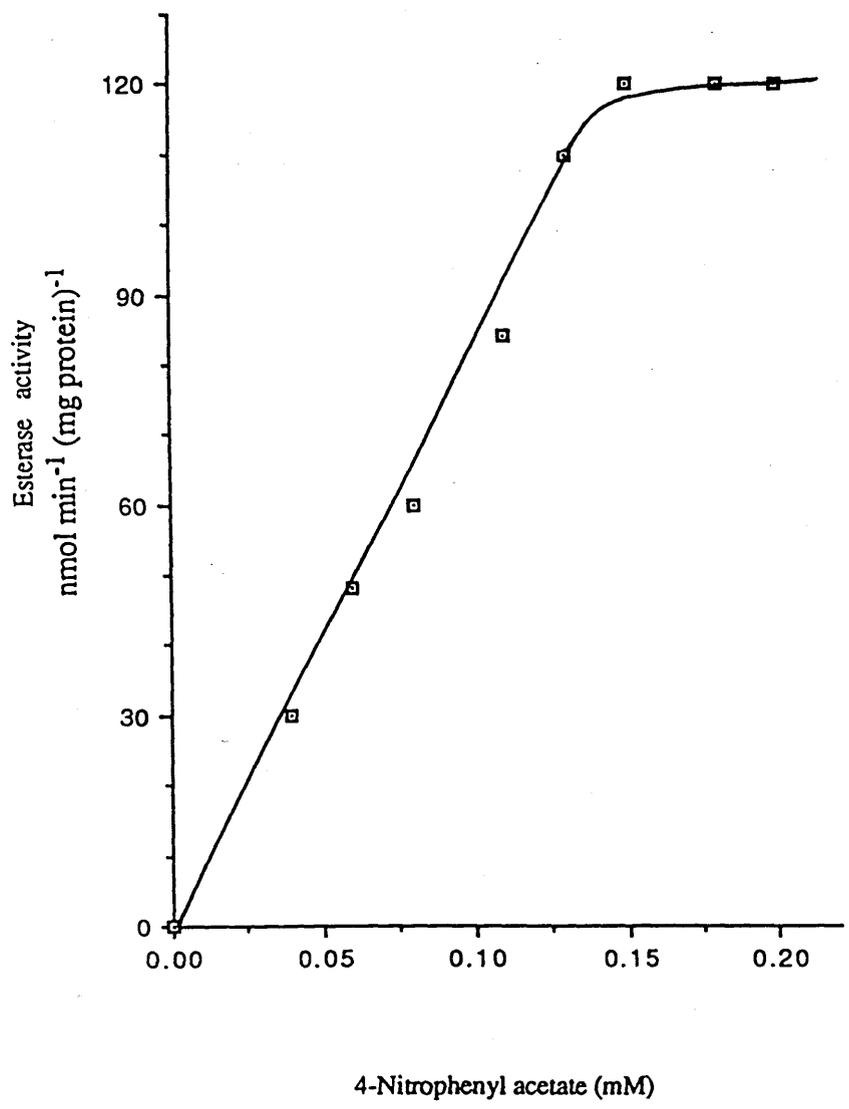
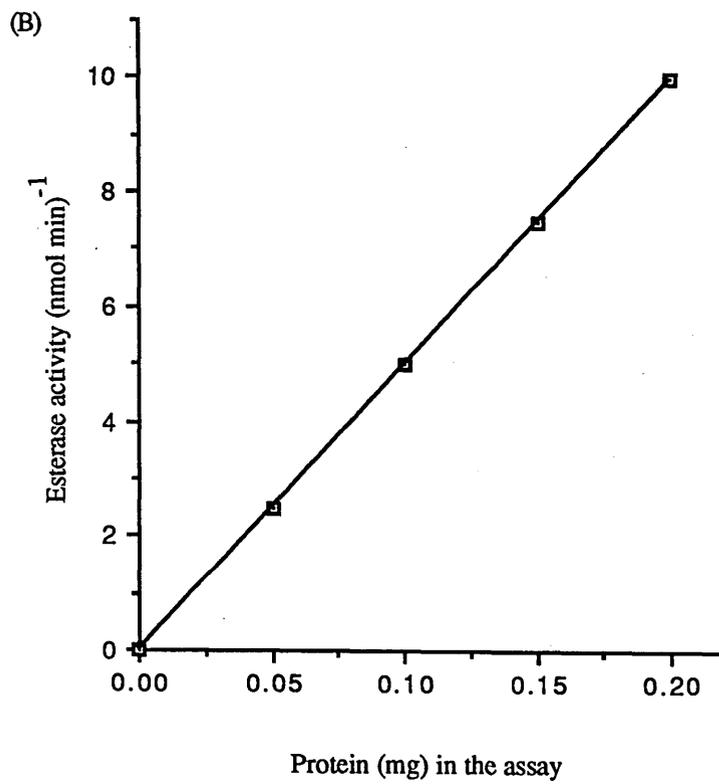
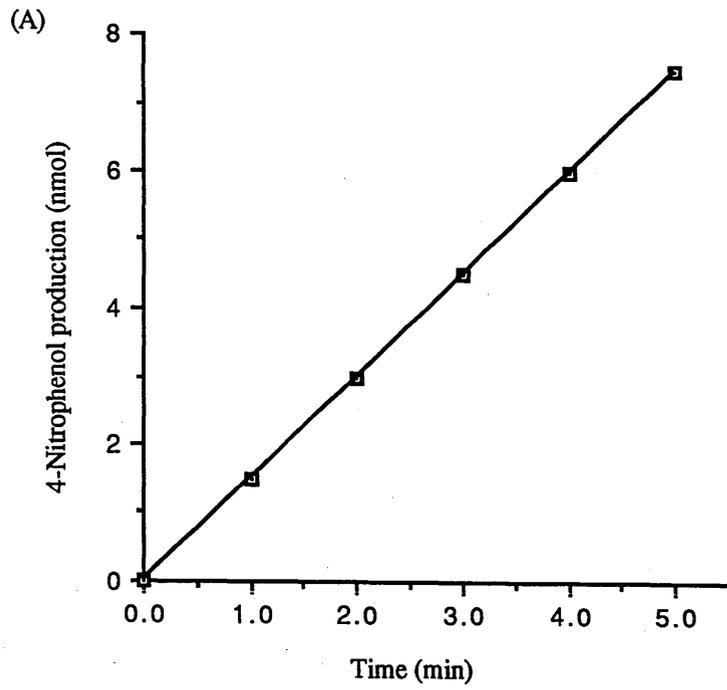


Figure 3.5 The effect of time and protein concentration on esterase activity.

The supernate used was prepared as described in the legend to Figure 3.3. The esterase activity was assayed as described in Methods 2.11.1. The liberation of 4-nitrophenol was followed for 5 min (A). The esterase activity was determined with different amounts of protein (B).



3.5.1.3 The effect of pH on esterase activity.

The pH of the assay was varied over the range of 5.0-9.5 by using 50 mM buffers of different pH values (see legend to Figure 3.6). The maximum esterase activity was obtained at about pH 8.5 in 50 mM Tris HCl buffer (see Figure 3.6).

3.5.1.4 The effect of temperature on esterase activity.

The assay temperature was varied from 25 °C to 50 °C (see Figure 3.7) to determine the temperature that gave the maximum esterase activity. The maximum esterase activity was observed at about 37 °C, therefore, the assay was always carried out at 37 °C.

3.5.1.5 The effect of detergents on esterase activity.

The effect of different concentrations of Triton X-100 and SDS on esterase activity in supernate is shown in Figure 3.8 A and B. It can be seen that the esterase activity decreased by 75% as the Triton X-100 concentration increased 0-1.2%. The esterase activity was also inhibited by 80% with SDS concentrations above about 0.1%.

Figure 3.6 The effect of pH on esterase activity.

The supernate used was prepared as described in the legend to Figure 3.3. The esterase activity was determined as described in Methods 2.11.1 using the 50 mM buffers listed below. The enzyme activity is expressed as a percentage of the activity at pH 8.5, which was $80.5 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$.

Buffer	Concentration (mM)	pH
Acetate-NaOH	50	5.0
Mes-NaOH	50	6.0
Phosphate-NaOH	50	7.0
Tris-HCl	50	7.5, 8.0, 8.5, 9.0
Glycine-NaOH	50	9.5, 10, 10.5

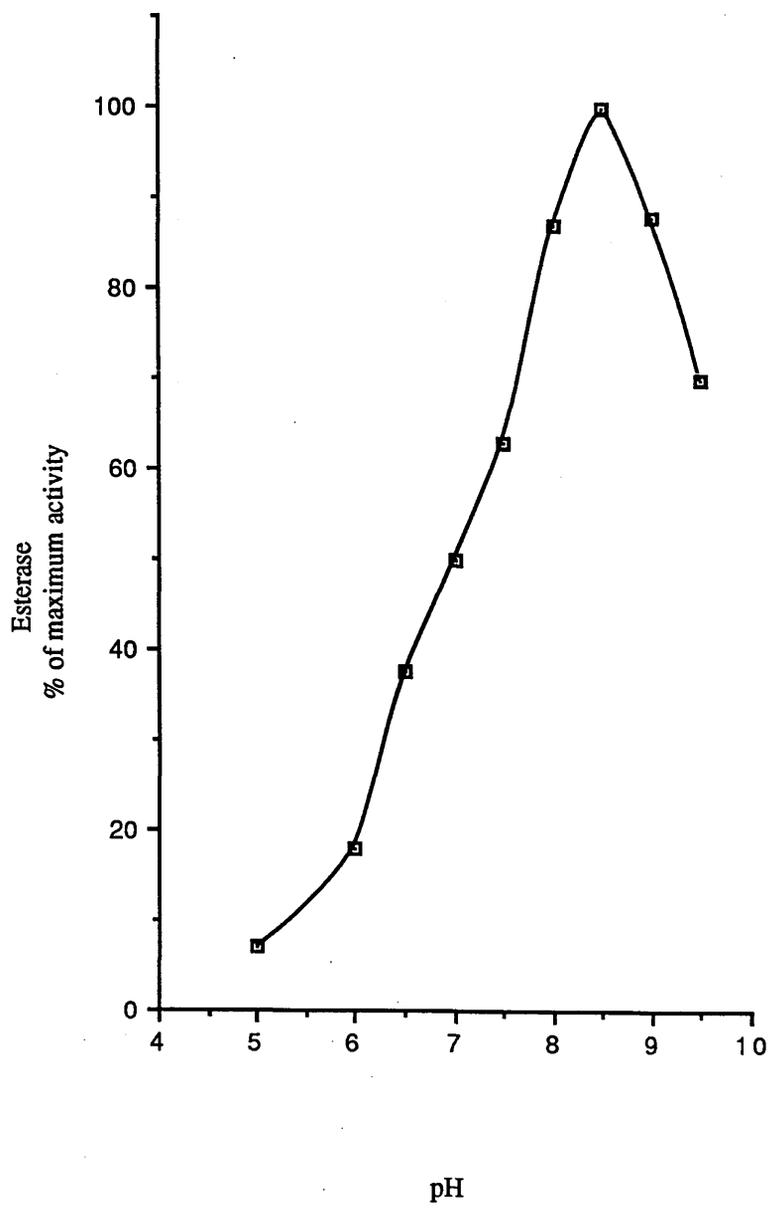


Figure 3.7 The effect of assay temperature on esterase activity.

The supernate used was prepared as described in the legend to Figure 3.3. The esterase activity was determined as described in Methods 2.11.1 except that different assay temperatures were used. At 37 °C the activity of this extract was 85.3 nmol min⁻¹ (mg protein)⁻¹.

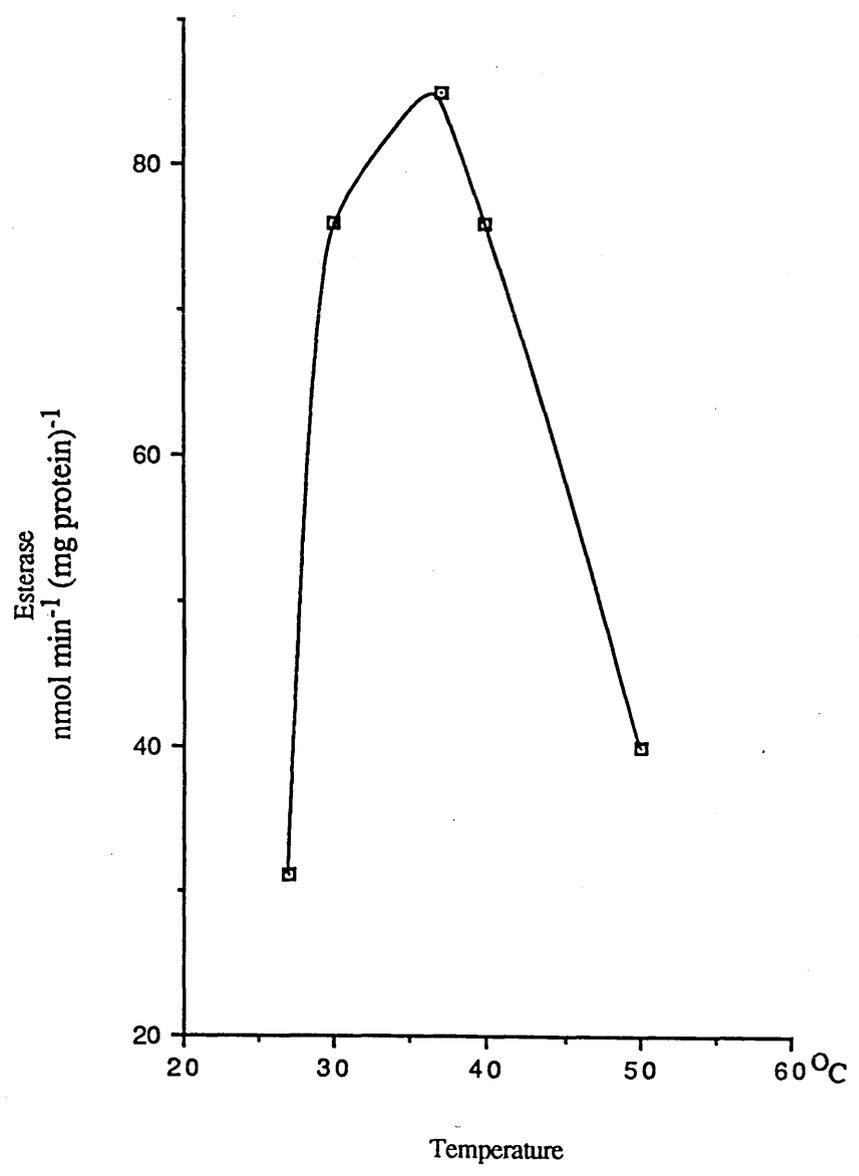
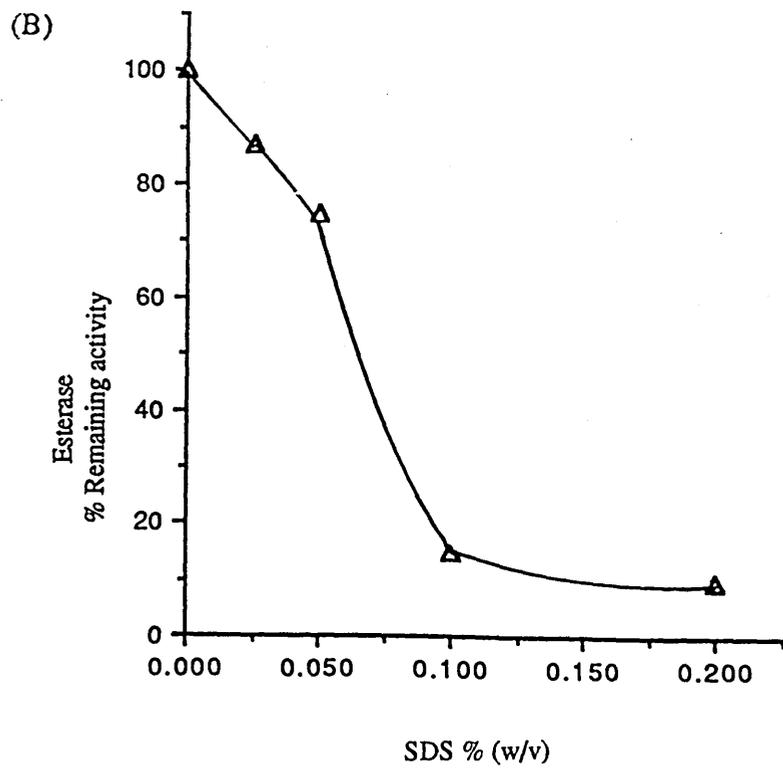
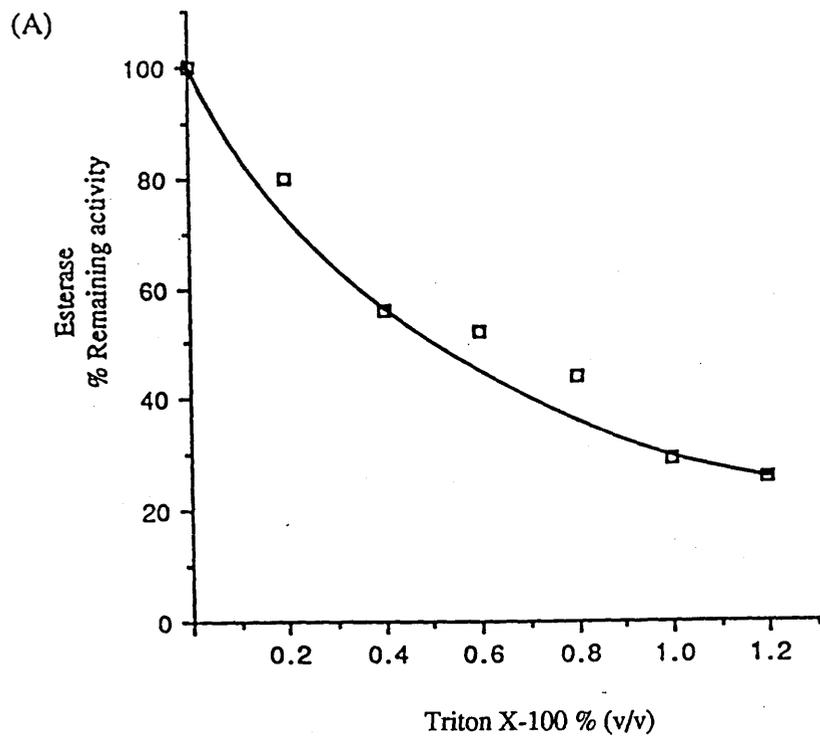


Figure 3.8 The effect of Triton X-100 and SDS on esterase activity.

The supernate used was prepared as described in the legend to Figure 3.3. The esterase activity was determined as described in Methods 2.11.1 except that different volumes of 1.2% (v/v) Triton X-100 in 50 mM Tris HCl, pH 8.5 or SDS in 50 mM Tris HCl, pH 8.5 were added to the assay buffer to give an increasing detergent concentration in the assay. The 100% value was 65.4 nmol min⁻¹ (mg protein)⁻¹.

(A) Triton X-100

(B) SDS



3.5.1.6 The effect of reagents which react with an active site serine on esterase activity.

The supernate was treated with three serine reactive inhibitors and the effect of these inhibitors on the esterase activity was determined for 2 h (see Figure 3.9). All three reagents inhibited the esterase activity irreversibly but to different extents. The esterase activity was inhibited completely and rapidly (within 15 min) by 5 mM bis-*p*-nitrophenyl phosphate. PMSF (0.1 mM) also inhibited the enzyme rapidly reaching maximal inhibition in about 15 min but the inhibition was only about 80-90%. Similar effects were observed at higher concentrations of PMSF (0.5 and 1.0 mM). The inhibition with eserine was slower and at a concentration of 4.0 mM was about 75% after 1 h incubation. The lower concentrations of eserine (0.5 mM) did not inhibit the esterase activity.

3.5.1.7 The effect of sulphydryl reagents on esterase activity.

The supernate was also treated with sulphydryl reagents and the effect of these reagents on esterase activity is shown in Figure 3.10. These inhibitors inhibited the esterase activity irreversibly but to different extents. There was no significant inhibition with sulphydryl reagents when the supernates were incubated on ice but these reagents were effective when incubated with the supernates at 37 °C. Both iodoacetate (10 mM) and iodoacetamide (10 mM) had only inhibited the enzyme by about 45-55% after 1 h. The esterase activity was inhibited by about 100% in 20 min with mercuric chloride (10 mM). The lower concentration of iodoacetate (1 mM), iodoacetamide (1 mM) and mercuric chloride (1 mM), had no significant effect on esterase activity.

Figure 3.9 The effect of serine-reactive inhibitors on esterase activity.

The supernate used was prepared as described in the legend to Figure 3.3. The supernate (0.5 ml) was treated with bis-*p*-nitrophenyl phosphate (5, 10 mM, A), PMSF (0.1, 0.5, 1.0 mM, B) or eserine (0.05, 0.5, 4.0 mM, C) as described in Methods 2.13. The esterase activity was determined as described in Methods 2.11.1 at various time intervals. The values shown are averages of duplicate assays. The 100% value was $80.4 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$.

(A) Control \blacklozenge , Bis-*p*-nitrophenyl phosphate 5 mM \square , 10 mM \blacksquare

(B) Control \blacklozenge , PMSF 0.1 mM \blacksquare , 0.5 mM \square , 1.0 mM \blacktriangle

(C) Control \square , Eserine 0.05 mM \blacklozenge , 0.5 mM \blacktriangle , 4.0 mM \blacktriangle

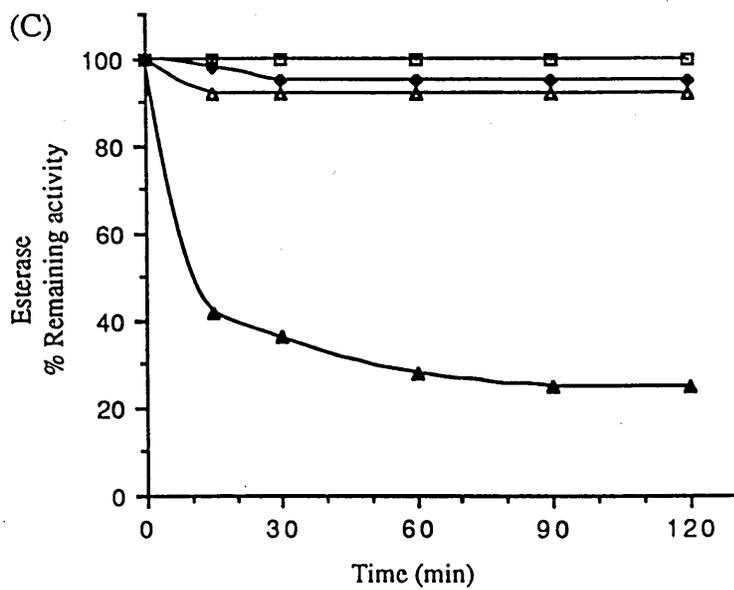
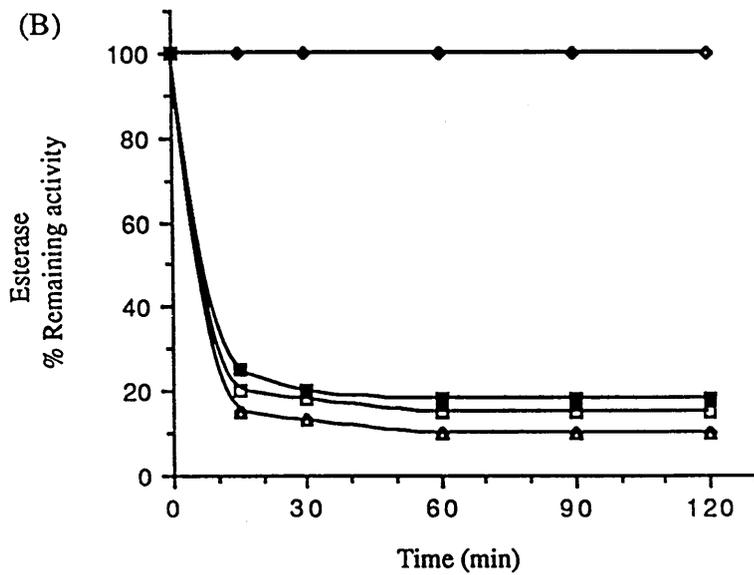
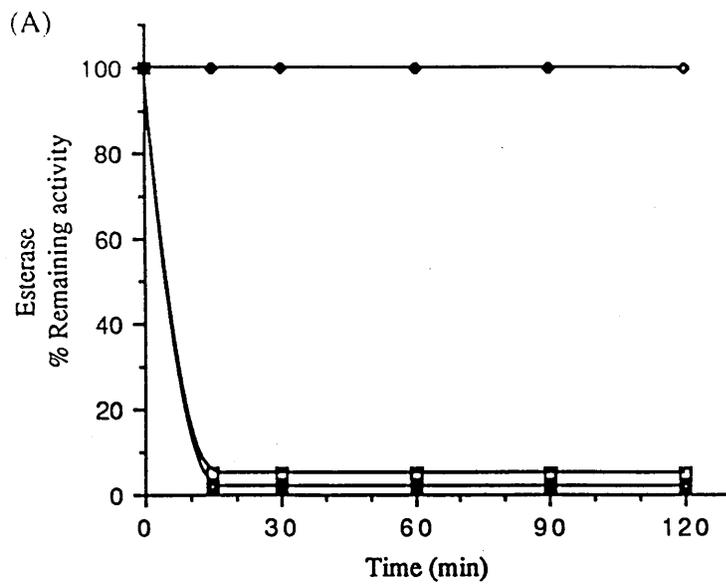
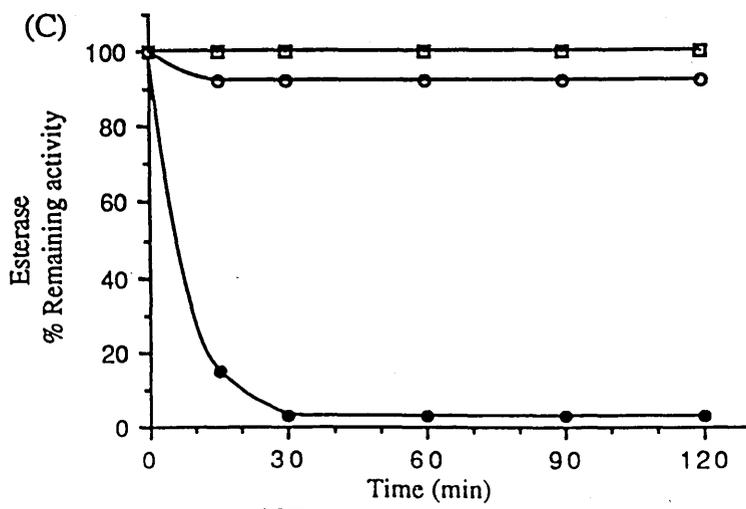
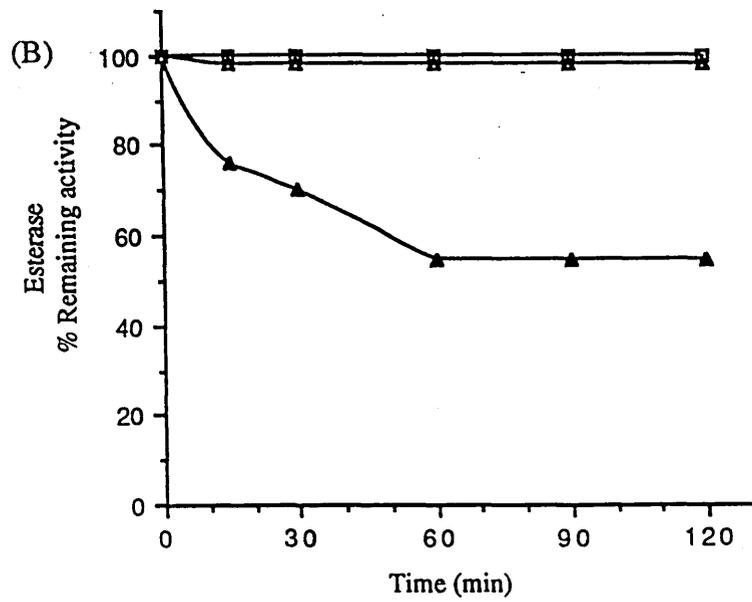
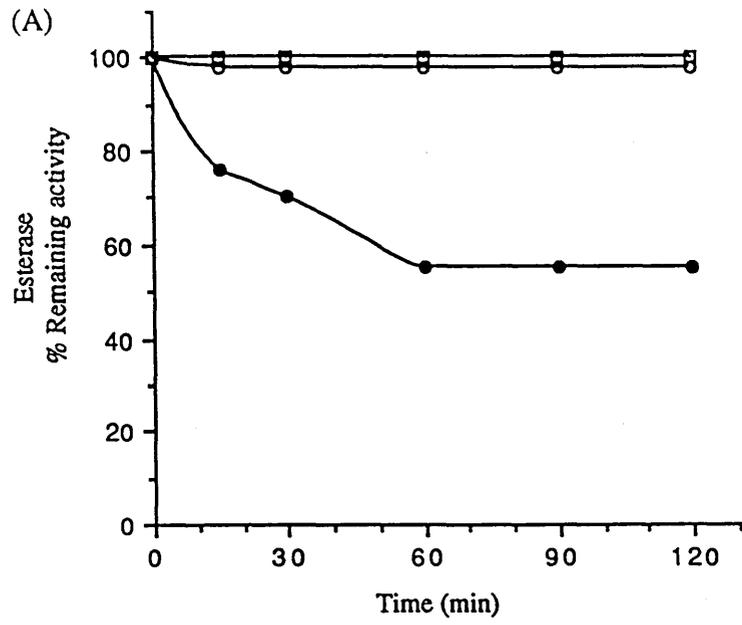


Figure 3.10 The effect of sulphhydryl reagents on esterase activity.

The supernate used was prepared as described in the legend to Figure 3.3 except 50 mM-potassium phosphate buffer, pH 7.5 was used. The supernate (0.5 ml) was treated with iodoacetate (1, 10 mM, A), iodoacetamide (1, 10 mM, B) or mercuric chloride (1, 10 mM, C) as described in Methods 2.13. The esterase activity was determined as described in Methods 2.11.1 at various time intervals. The values shown are averages of duplicate assays. The 100% value was $80.5 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$.

- (A) Control \square , Iodoacetate 1 mM \circ , 10 mM \bullet
- (B) Control \square , Iodoacetamide 1 mM Δ , 10 mM \blacktriangle
- (C) Control \square , Mercuric chloride 1 mM \circ , 10 mM \bullet



3.5.1.8 The effect of tryptophan modification on esterase activity.

The inhibition of esterase activity with tryptophan modifying reagents, *N*-bromosuccinamide (NBS) and 2-hydroxy-5-nitrobenzyl bromide (see Figure 3.11) was slower than with bis-*p*-nitrophenyl phosphate (see Figure 3.9). NBS (10 mM) inhibited the esterase activity by 70% and 2-hydroxy-5-nitrobenzyl bromide (20 mM) inhibited the activity by 80%. The lower concentrations of NBS (5 mM) and 2-hydroxy-5-nitrobenzyl bromide (10 mM) were half as effective, as the high concentrations.

3.5.1.9 The effect of tetrahydrolipstatin on esterase activity.

The effect of different concentrations of tetrahydrolipstatin (THL) on esterase activity in the supernate fraction was also studied (see Figure 3.12). Three different concentrations of THL (0.1, 0.4 and 0.8 mM) were used. The esterase activity was inhibited by about 70% with lower concentrations of THL (0.1 and 0.4 mM), while the higher concentration of THL (0.8 mM) increased the inhibition by only 10%. Incubating the supernate with THL at either at 0 °C or at 37 °C did not effect the extent of inhibition.

Figure 3.11 The effect of tryptophan modifying reagents on esterase activity.

The supernate used was prepared as described in the legend to Figure 3.3 except 50 mM-potassium phosphate buffer, pH 7.5 was used. The supernate (0.5 ml) was treated with NBS (5, 10 mM, A) or 2-hydroxy-5-nitrobenzyl bromide (10, 20 mM, B) as described in Methods 2.13. The esterase activity was determined as described in Methods 2.11.1 at various time intervals. The values shown are averages of duplicate assays. The 100% value was 65.3 nmol min⁻¹ (mg protein)⁻¹.

(A) Control.□, NBS 5 mM▲, 10 mM.▲

(B) Control.□, 2-hydroxy-5-nitrobenzyl bromide 10 mM◐, 20 mM.●

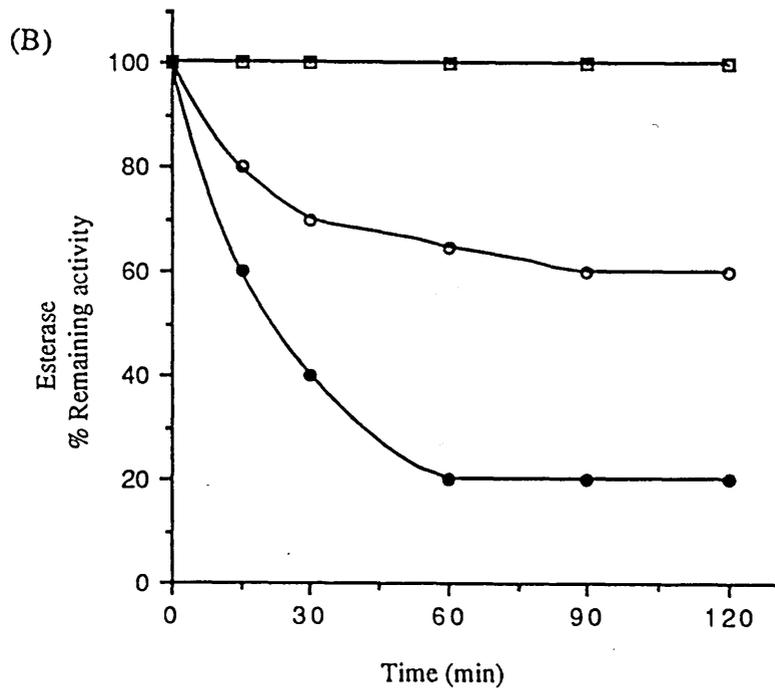
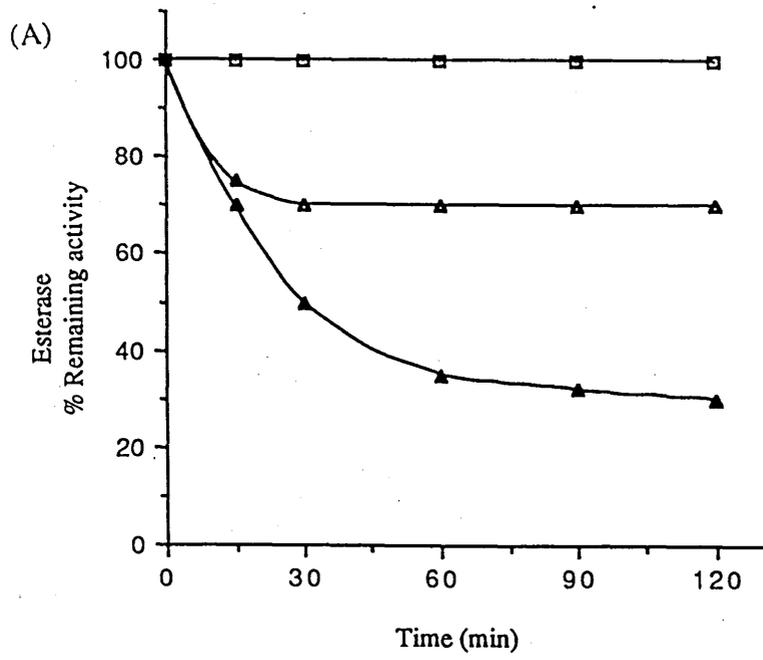
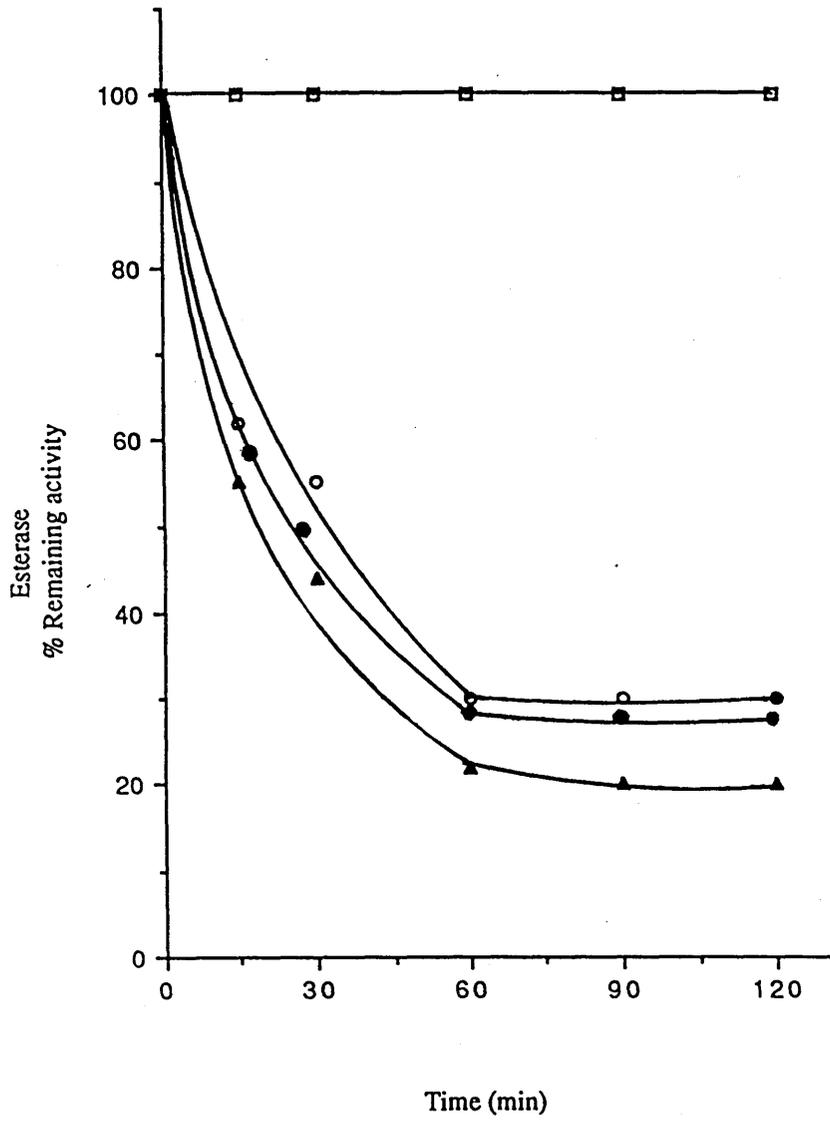


Figure 3.12 The effect of tetrahydrolipstatin on esterase activity.

The supernate used was prepared as described in the legend to Figure 3.3 . The supernate (0.5 ml) was treated with THL (0.1, 0.4, 0.8 mM) as described in Methods 2.13. The esterase activity was determined as described in Methods 2.11.1 at various time intervals. The values shown are averages of duplicate assays. The 100% value was $85.5 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$.

Control \square , THL 0.1 \circ , 0.4 mM \bullet , 0.8 mM \blacktriangle



3.5.1.10 Molecular mass of esterase activity.

The supernate protein was eluted from Superose-6 gel filtration column using f.p.l.c. The elution pattern is shown in Figure 3.13. The esterase activity was eluted into two different molecular mass forms. Most of the esterase activity (85%) was present in a high molecular mass peak near the excluded volume of the column and a small amount (15%) had a lower molecular mass. The relative molecular mass of the eluted peaks was estimated by running proteins of known molecular mass. The estimated relative molecular mass (M_r) of two forms were 1,000,000 and 600,000 respectively

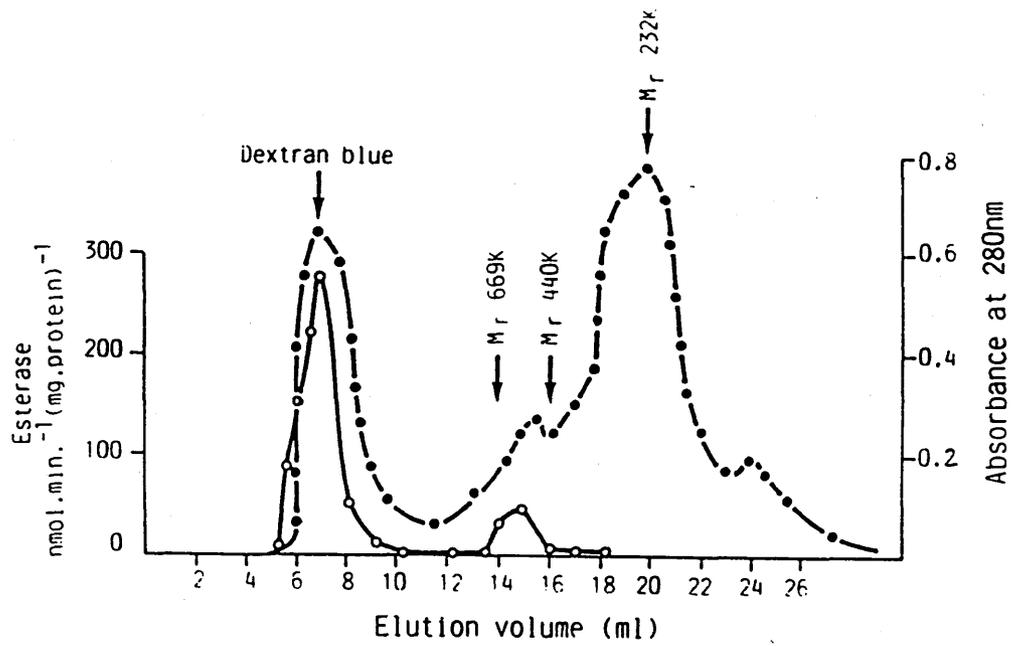
Figure 3.13 Molecular mass determination of the esterase activity by gel filtration chromatography.

The supernate used was prepared as described in the legend to Figure 3.3. The supernate 0.5 ml (5 mg protein) was applied to a 30 cm Superose-6 f.p.l.c. column as described in Methods 2.12. The esterase activity was determined in eluted fractions as described in Methods 2.11.1. The column was calibrated using thyroglobulin (M_r 669,000), ferritin (M_r 440,000) and catalase (M_r 232,000). The elution volume of Dextran blue (approx. M_r 2,000,000) was taken to be the void volume.

Esterase activity $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ —○—

A_{280} —●—

Elution position of standard proteins ↓



3.5.1.11 The effect of culture conditions on esterase activities in *A. calcoaceticus* NCIB 8250.

The esterase activity was assayed in the broken cells, supernate and membrane fractions of bacteria harvested from exponential phase and of bacteria harvested from stationary phase in carbon or nitrogen limited cultures (see Table 3.7). Approximately the same total esterase activity [$120 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] was present in broken cells from all growth conditions. The supernate and membrane fractions of exponentially growing, carbon and nitrogen limited bacteria showed significant difference in activity. Total esterase activity was higher (about 70% of the total activity) in the supernatant fractions of exponentially growing and nitrogen limited bacteria than the supernatant fractions of carbon limited bacteria (about 30% of the total activity). The specific activities of supernate fractions of exponentially growing and nitrogen limited bacteria were significantly higher than carbon limited bacteria. The crude membranes (i.e. inner + outer membranes) from carbon limited bacteria showed higher total activity (about 70%) than in exponentially growing (total activity about 30%) and nitrogen limited bacteria (total activity about 30%). A significantly higher specific activity of esterase was detected in crude membranes of carbon limited bacteria than in exponentially growing bacteria and nitrogen limited bacteria. There was no significant effect observed on the specific activities of esterase in the supernatant by interchanging the culture conditions from carbon to nitrogen or nitrogen to carbon limitation.

**Table 3.7 Distribution of esterase activities in *A. calcoaceticus*
NCIB 8250 grown in different culture conditions**

The bacteria used were exponentially growing, carbon limited or nitrogen limited as described in Methods 2.2.3.1 and 2.2.3.2 respectively. Supernates were prepared as described in the legend to Figure 3.3. The esterase activity was assayed in broken cells, supernates and membrane fractions as described in Methods 2.11.1. Using the Student's 't' test, the value for the specific activity in supernates of carbon limited bacteria is significantly different from nitrogen limited bacteria ($P = < 0.01$, $n = 4$). The value of specific activity in membranes of carbon limited bacteria is also significantly different from nitrogen limited bacteria ($P = < 0.05$, $n = 4$). The total activity in supernate and membrane fractions of exponentially growing bacteria and nitrogen limited bacteria were significantly different from carbon limited bacteria ($P = < 0.01$, $n = 4$). No other significant differences were revealed by this test.

	Esterase	
	Total % activity	Specific activity nmol min ⁻¹ (mg protein) ⁻¹
Exponentially growing		
Broken cell	100	102.5 ± 42.7
Supernatant	68.3 ± 3.5	90.4 ± 32.4
Membranes	29.3 ± 1.8	65.5 ± 48.2
Carbon limited		
Broken cells	100	131.2 ± 33.4
Supernatant	32.5 ± 4.1	35.4 ± 10.1
Membranes	68.0 ± 3.3	117.0 ± 42.3
Nitrogen limited		
Broken cells	100	135.1 ± 18.2
Supernatant	65.5 ± 5.2	108.2 ± 26.0
Membranes	35.3 ± 3.0	83.4 ± 26.4

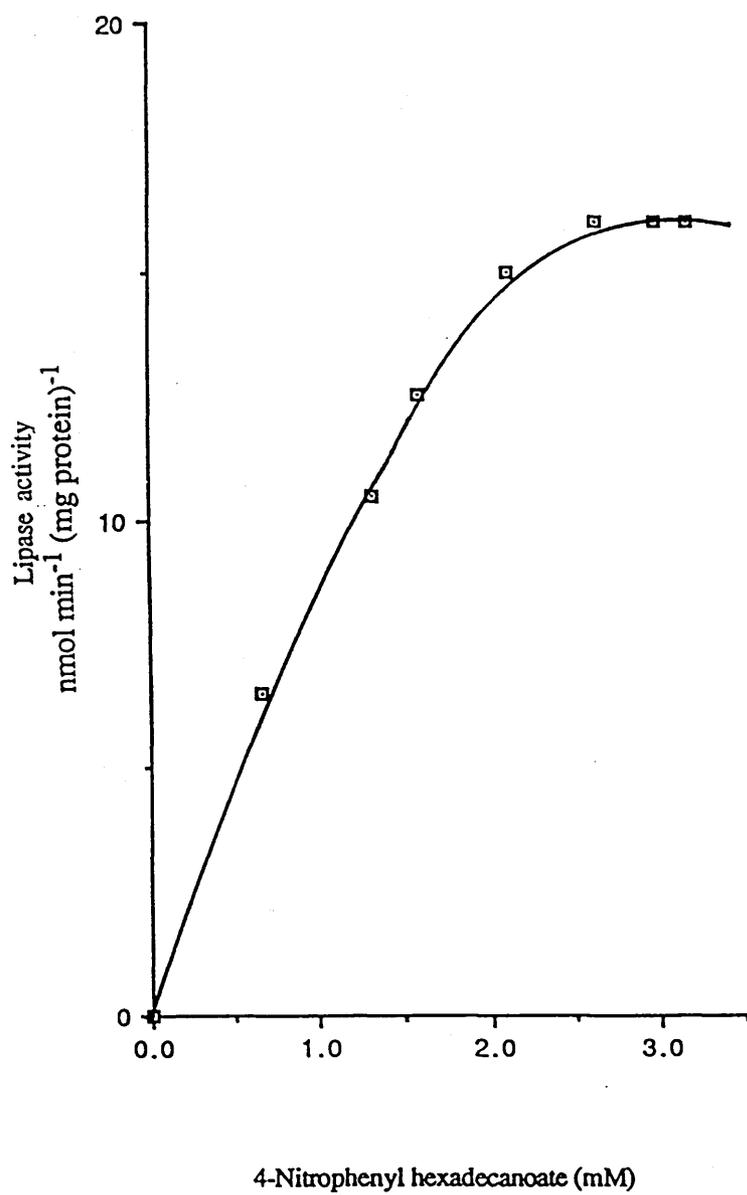
3.5.2 Development of an assay for lipase activity.

3.5.2.1 The effect of concentration of 4-nitrophenyl hexadecanoate on lipase activity.

4-nitrophenyl hexadecanoate was used as substrate for the lipase activity. Two approaches were tried to emulsify the substrate. Firstly, 4-nitrophenyl hexadecanoate (30 mg) was dissolved in 1 ml of isopropanol and this solution was then dispersed in 50 mM Tris HCl buffer, pH 8.5 containing 0.1% (w/v) deoxycholic acid and 0.05% (w/v) gum arabic. Secondly, 4-nitrophenyl hexadecanoate was dissolved in dimethyl formamide and then dispersed in 50 mM Tris HCl buffer, pH 8.5 containing 1% (v/v) Triton X-100. It was found that the activity was approximately twice as high with the second method i.e. 15-20 nmol min⁻¹ (mg protein)⁻¹. Therefore, Triton X-100 was used to emulsify the substrate for lipase activity throughout this work. Different concentrations of 4-nitrophenyl hexadecanoate were used to determine the maximum lipase activity in the supernate. At a concentration of 2.65 mM or higher 4-nitrophenyl hexadecanoate showed the maximum activity (see Figure 3.14), therefore, a higher concentration of 4-nitrophenyl hexadecanoate (3 mM) was always used for lipase activity. Attempts were made to determine the K_m of the lipase activity with both types of emulsified substrate. The values obtained with substrate emulsified with deoxycholic acid and gum-arabic were not consistent and the results were very variable, values of -5.2 or 0.6 mM for the K_m being obtained in different experiments. However, experiments with substrate emulsified with Triton X-100 gave values which always fitted Lineweaver-Burk plots in the expected manner and the calculated K_m was approximately 1.74 mM.

Figure 3.14 The effect of 4-nitrophenyl hexadecanoate concentration on lipase activity.

The supernate used was prepared as described in the legend to Figure 3.3. The substrate 4-nitrophenyl hexadecanoate was prepared using Triton X-100 and the assay was done as described in Methods 2.11.4. The lipase activity is plotted against substrate concentration.



3.5.2.2 The effect of time and protein concentration on lipase activity.

The production of nitrophenol from 4-nitrophenyl hexadecanoate by the action of the enzyme was linear from 0-5 min using 3 mM 4-nitrophenyl hexadecanoate at pH 8.5. Therefore, the enzyme activity was always calculated within this period (see Figure 3.15 A). The rate of production of nitrophenol was proportional to the amount of bacterial protein between 0.1-0.25 mg protein per assay (see Figure 3.15 B), therefore, protein concentrations within this range were used for the lipase activity.

3.5.2.3 The effect of pH on lipase activity.

The pH of the assay was varied over the range of about 5 - 9.5 by using 50 mM buffers of different pH values (see legend to Figure 3.6). The maximum lipase activity was at pH 8.5 in 50 mM Tris HCl buffer (see Figure 3.16).

3.5.2.4 The effect of temperature on lipase activity.

The assay temperature was varied from 27 °C to 50 °C (see Figure 3.17) to determine the optimum lipase activity. The maximum lipase activity was observed at about 37 °C, therefore, the assay was always carried out at 37 °C.

Figure 3.15 The effect of time and protein concentration on lipase activity.

The supernate used was prepared as described in the legend to Figure 3.3. The lipase activity was assayed as described in Methods 2.11.4. The liberation of 4-nitrophenol was followed for 5 min (A). The effect of protein concentration on lipase activity was determined (B).

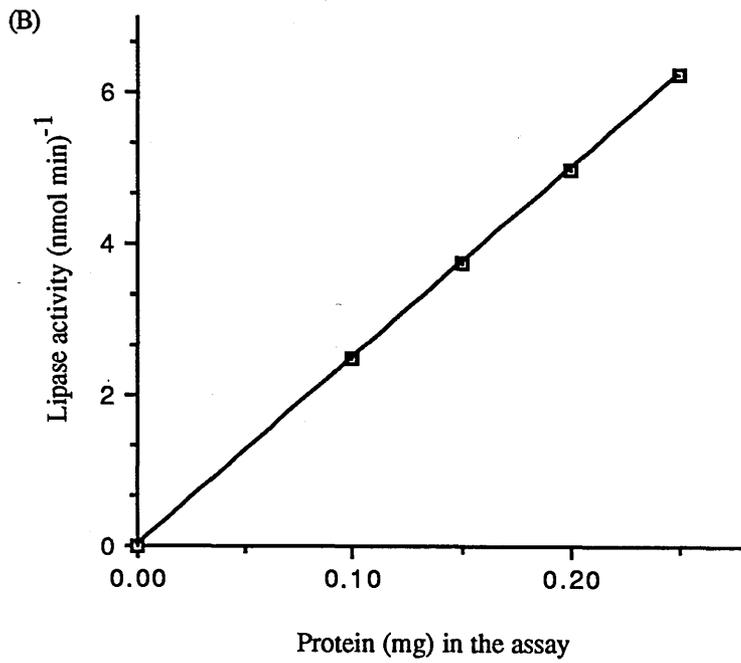
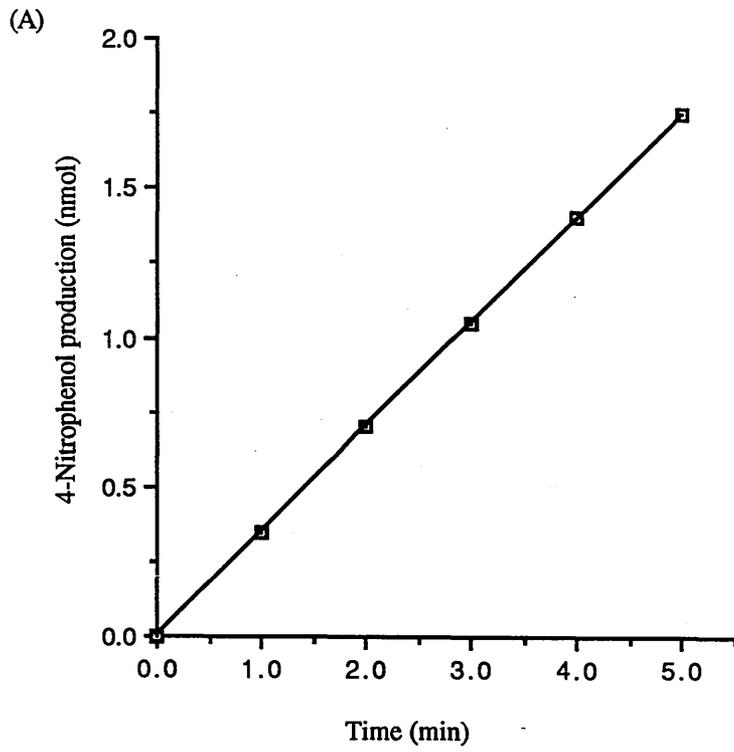


Figure 3.16 The effect of pH on lipase activity.

The supernate used was prepared as described in the legend to Figure 3.3. The lipase activity was determined using the 50 mM buffers listed in the legend to Figure 3.6 and the assay was carried out as described in Methods 2.11.4. The enzyme activity is expressed as a percentage of the activity at pH 8.5, which was $18.5 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$.

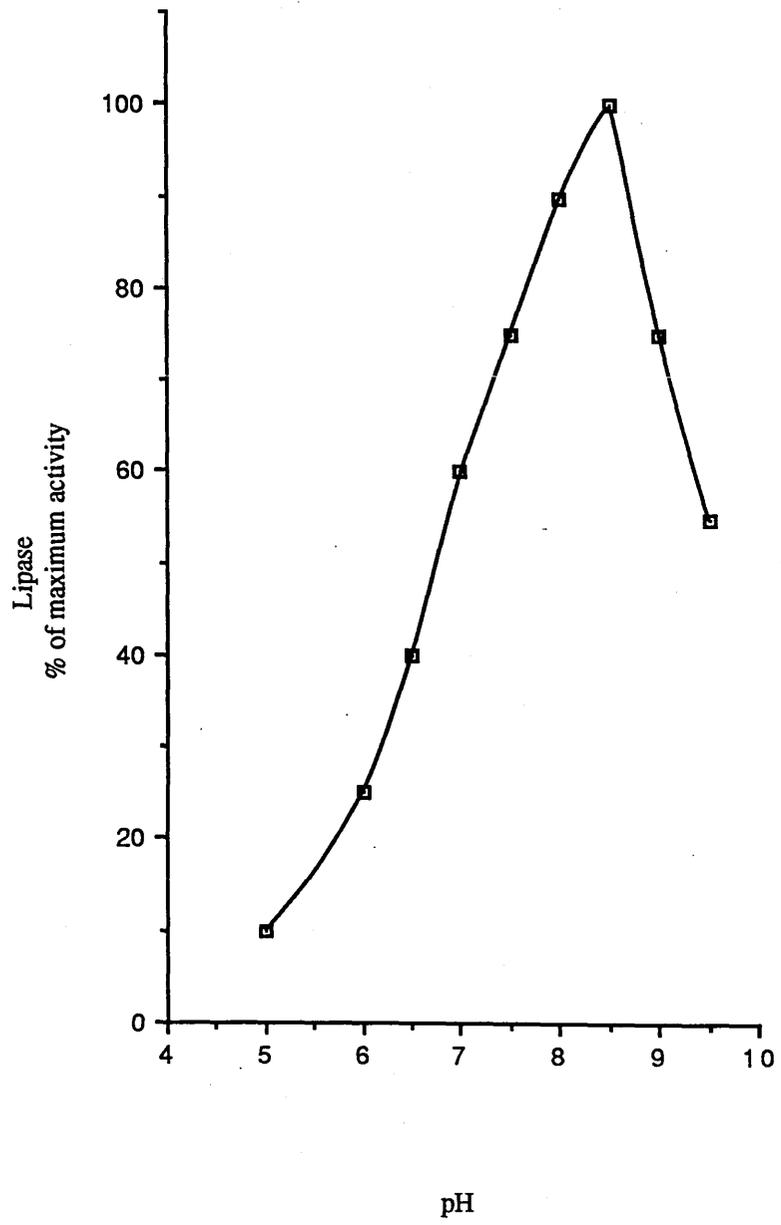
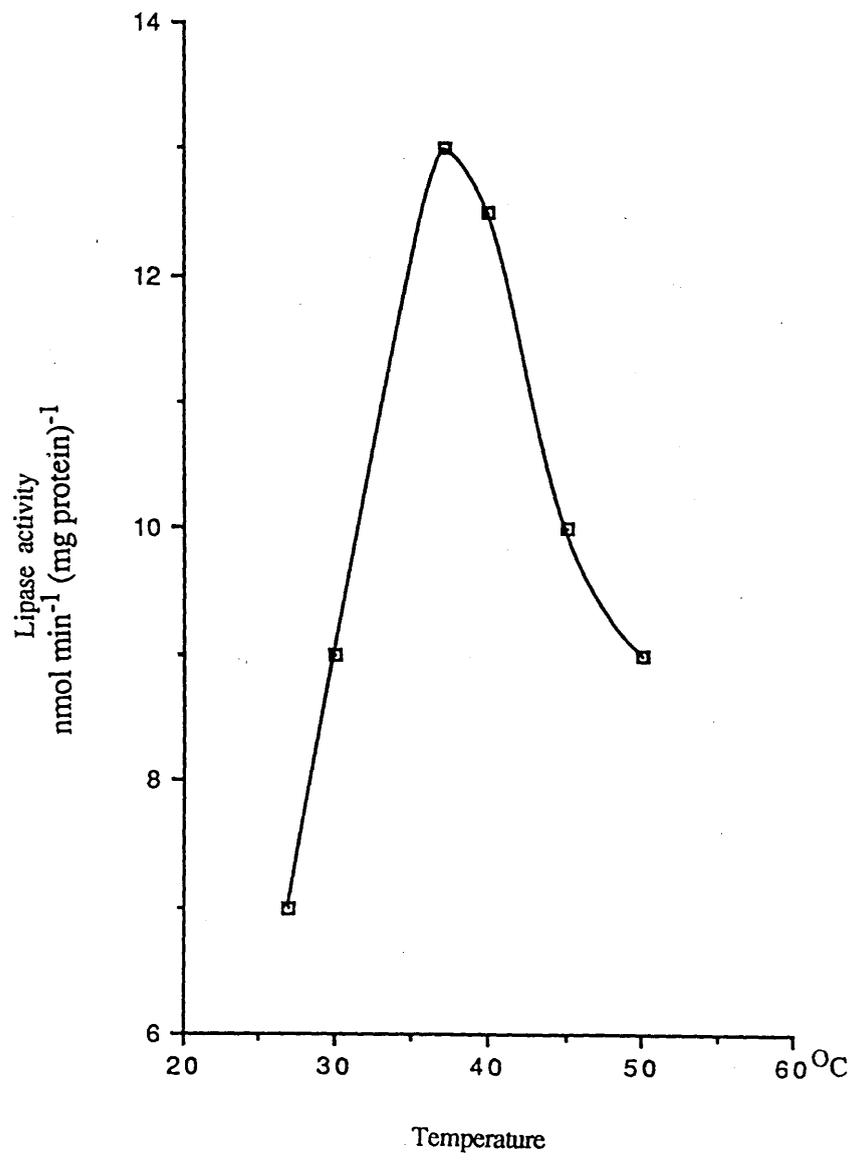


Figure 3.17 The effect of assay temperature on lipase activity.

The supernate used was prepared as described in the legend to Figure 3.3. The lipase activity was determined as described in Methods 2.11.4 except that different assay temperatures were used. The maximum enzyme activity was obtained at 37 °C, which was 13.5 nmol min⁻¹ (mg protein)⁻¹.



3.5.2.5 The effect of detergents on lipase activity.

The effect of different concentrations of Triton X-100 and SDS was determined on lipase activity using supernate protein. The lipase activity initially increased with an increasing concentration of Triton X-100 to a maximum activity with 1% (v/v) Triton X-100 in the assay. Therefore, 1% (v/v) Triton X-100 was used (see Figure 3.18 A). In contrast, the lipase activity decreased with an increasing concentration of SDS being reduced by about 50% at concentration greater than 0.1% (see Figure 3.18 B).

3.5.2.6 The effect of reagents which react with an active site serine on lipase activity.

The effect of the inhibitors, bis-*p*-nitrophenyl phosphate, PMSF and eserine on lipase activity in supernate is shown in Figure 3.19. Lower concentration of bis-*p*-nitrophenyl phosphate (5 mM) had no effect on lipase activity, however, it was slowly inhibited by 10-20% with higher concentration of bis-*p*-nitrophenyl phosphate (10 mM). There was no effect observed due to PMSF (0.5, 1.0 mM) or eserine (0.05, 0.5 mM). Even at higher concentrations of PMSF (10 mM) and eserine (4 mM) the lipase activity was not inhibited.

3.5.2.7 The effect of sulphydryl reagents on lipase activity.

The supernate was treated with sulphydryl reagents and the effect of these on the lipase activity is shown in Figure 3.20. The sulphydryl reagents had no significant effect on the lipase activity when incubated with the supernate on ice. While all reagents used i.e. iodoacetate (10 mM), iodoacetamide

(10 mM) and mercuric chloride (10 mM) slowly inhibited the lipase activity by about 30 % when incubated with the supernate at 37 °C. The lower concentrations of these reagents, iodoacetate (5 mM), iodoacetamide (5 mM) and mercuric chloride (5 mM) did not show any significant inhibition of the lipase activity.

3.5.2.8 The effect of tryptophan modification on lipase activity.

The supernate was treated with reagents which modify tryptophan residues. Both NBS (10 mM) and 2-hydroxy-5-nitrobenzyl bromide (20 mM) inhibited the lipase activity approximately by 45-50% (Figure 3.21). The lower concentrations of NBS (5 mM) and 2-hydroxy nitrobenzyl bromide (10 mM) inhibited the activity by 30%.

Figure 3.18 The effect of Triton X-100 and SDS on lipase activity.

The supernate used was prepared as described in the legend to Figure 3.3. Different amounts of Triton X-100 in 50 mM Tris HCl, pH 8.5 were used to prepare the substrate. The lipase activity was determined as described in Methods 2.11.4. The maximum activity was $18.5 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ (A). The effect of SDS was determined by adding different amounts of 2% SDS in 50 mM Tris HCl, pH 8.5 to the assay (B). The 100% activity was $18.5 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$. The results plotted are the average of two experiments.

(A) Triton X-100

(B) SDS

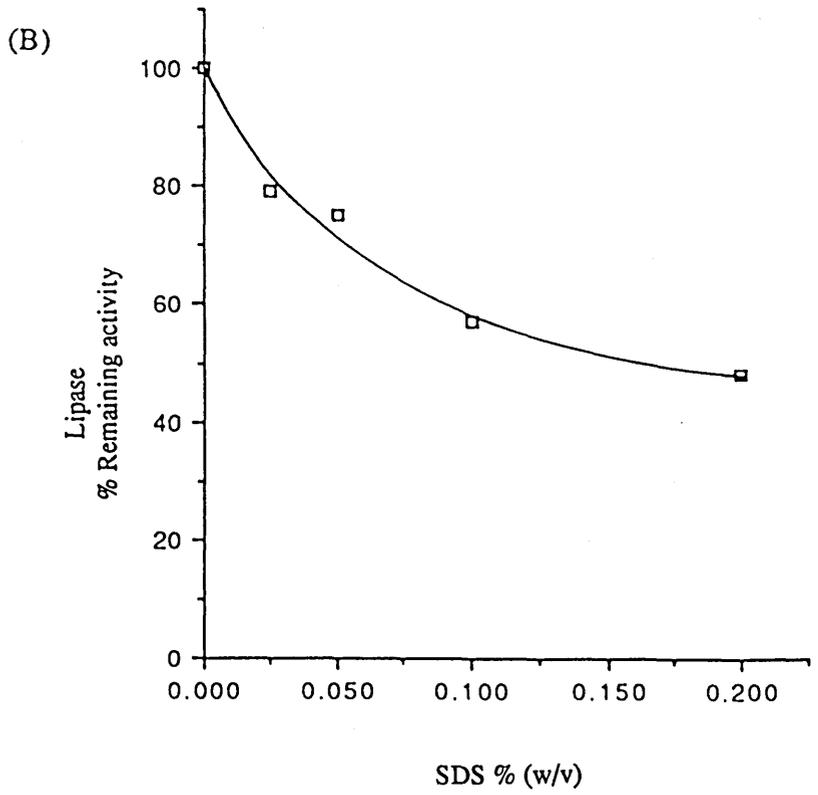
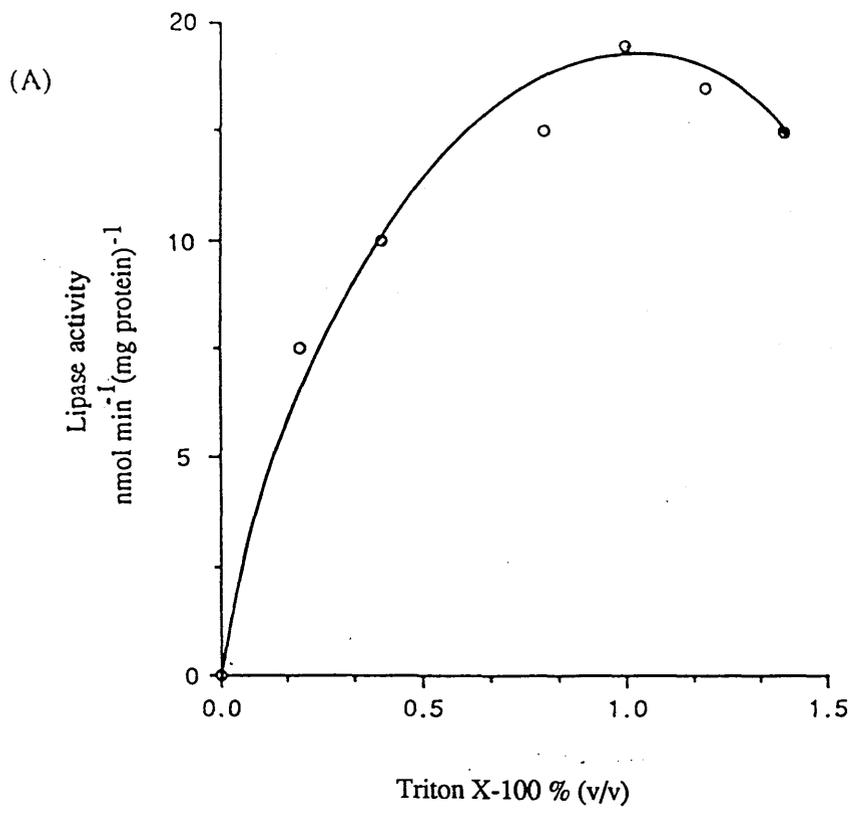


Figure 3.19 The effect of serine-reactive inhibitors on lipase activity.

The supernate used was prepared as described in the legend to Figure 3.3. The supernate (0.5 ml) was treated with bis-*p*-nitrophenyl phosphate (5, 10 mM A), PMSF (0.5, 1.0, 10 mM, B) or eserine (0.5, 4.0 mM, C) as described in Methods 2.13. The lipase activity was determined as described in Methods 2.11.4 at various time intervals. The values shown are averages of duplicate assays. The 100% value was $18.5 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$.

- (A) Control □, Bis-*p*-nitrophenyl phosphate 5 mM ●, 10 mM ▲
(B) Control □, PMSF 0.5 mM ▲, 1.0 mM ■, 10.0 mM ○
(C) Control □, Eserine 0.5 mM ■, 4.0 mM ▲

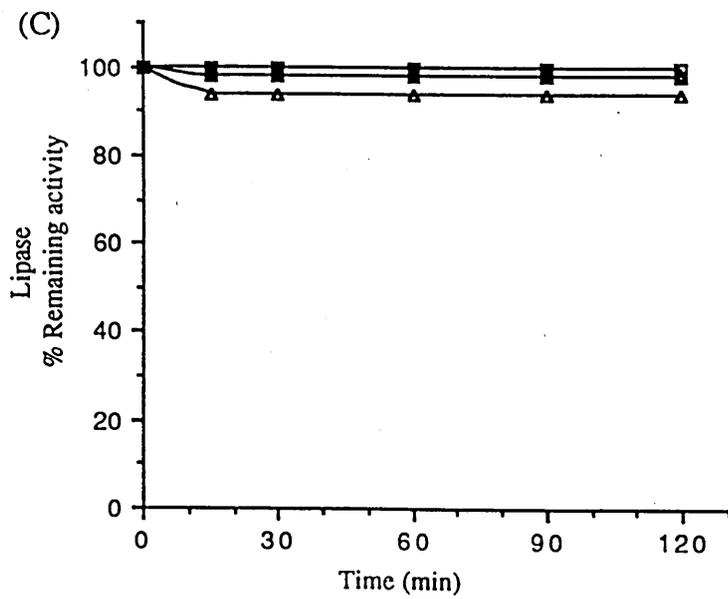
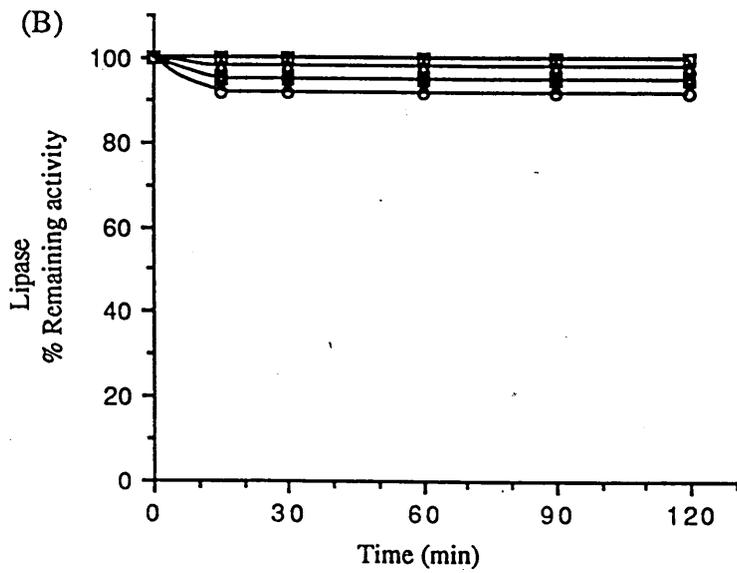
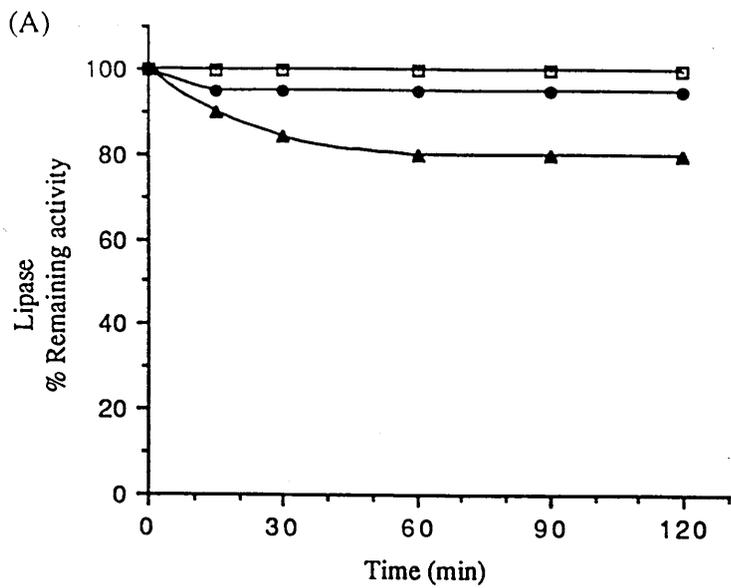


Figure 3.20 The effect of sulphhydryl reagents on lipase activity.

The supernate used was prepared as described in the legend to Figure 3.3 except that 50 mM-potassium phosphate buffer, pH 7.5 was used. The supernate (0.5 ml) was treated with iodoacetate (1, 10 mM, A), iodoacetamide (1, 10 mM, B) or mercuric chloride (1, 10 mM, C) as described in Methods 2.13. The lipase activity was determined as described in Methods 2.11.4 at time various intervals. The values shown are averages of duplicate assays. The 100% value was $17.5 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$.

- (A) Control \square , Iodoacetate 1 mM \circ , 10 mM \bullet ...
- (B) Control \square , Iodoacetamide 1 mM Δ , 10 mM \blacktriangle ...
- (C) Control \square , Mercuric chloride 1 mM \circ , 10 mM \bullet ...

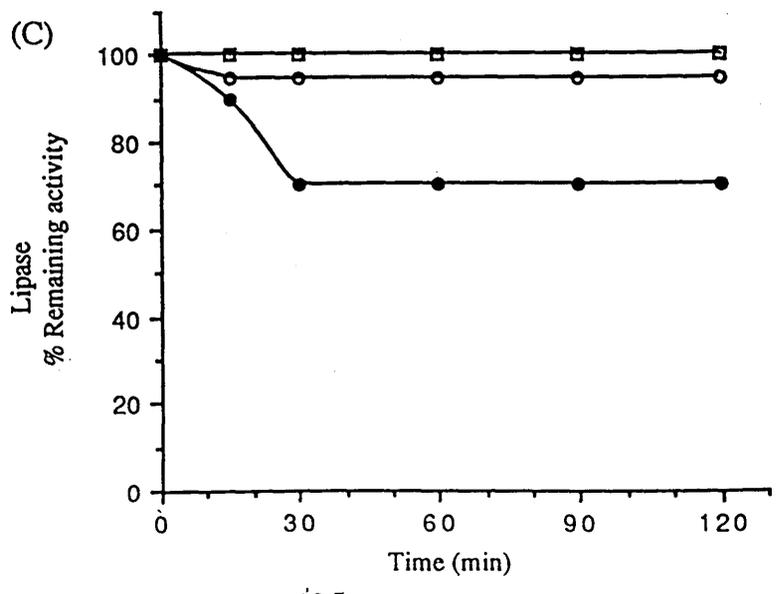
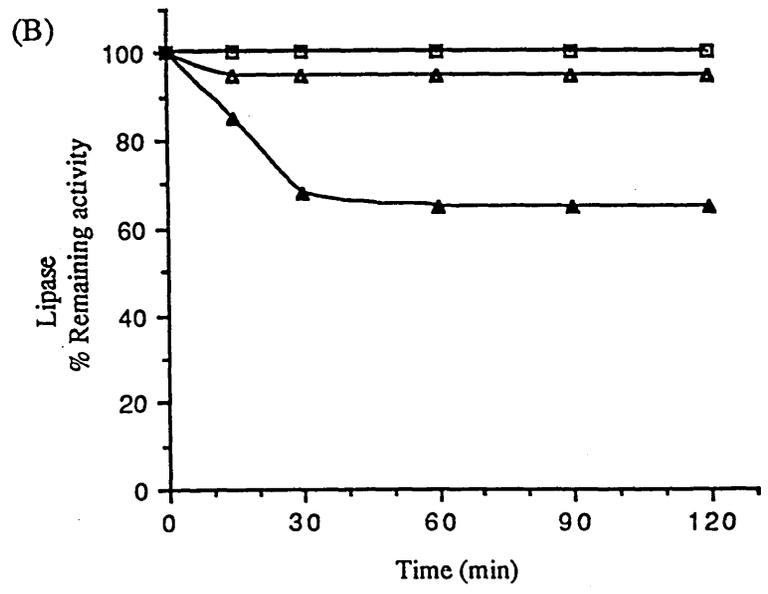
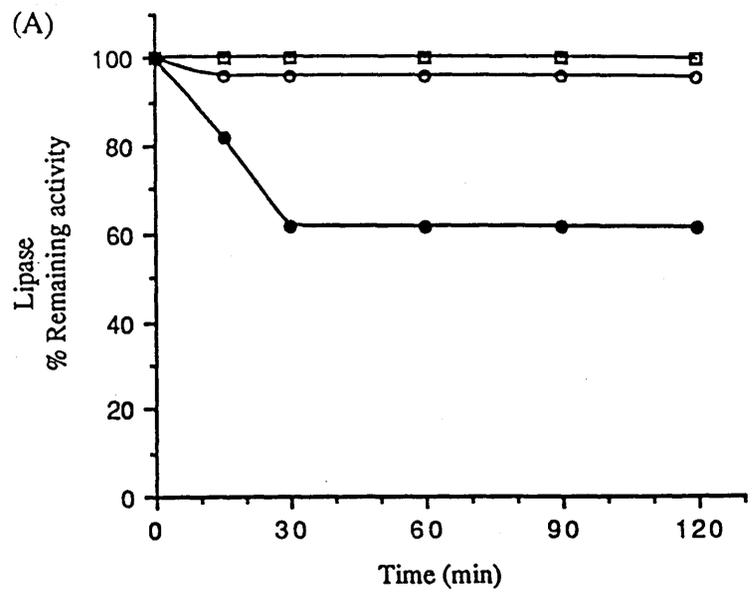
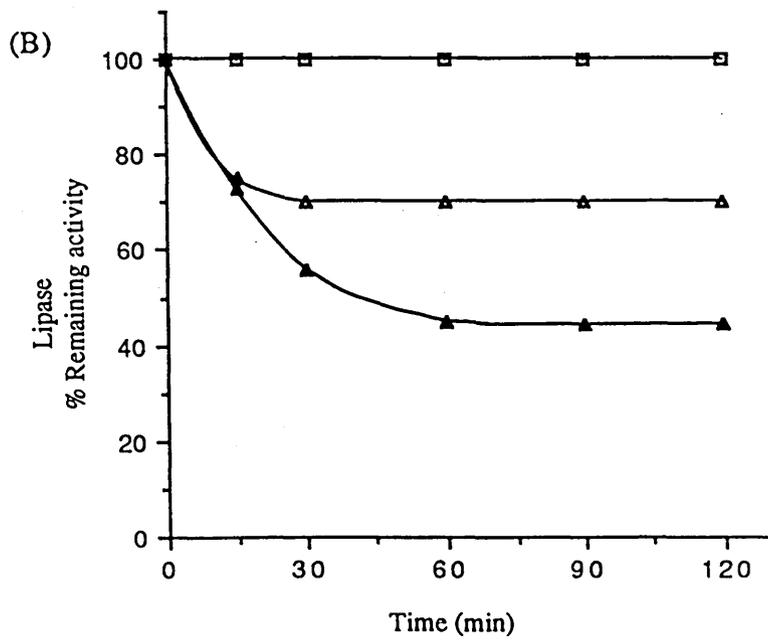
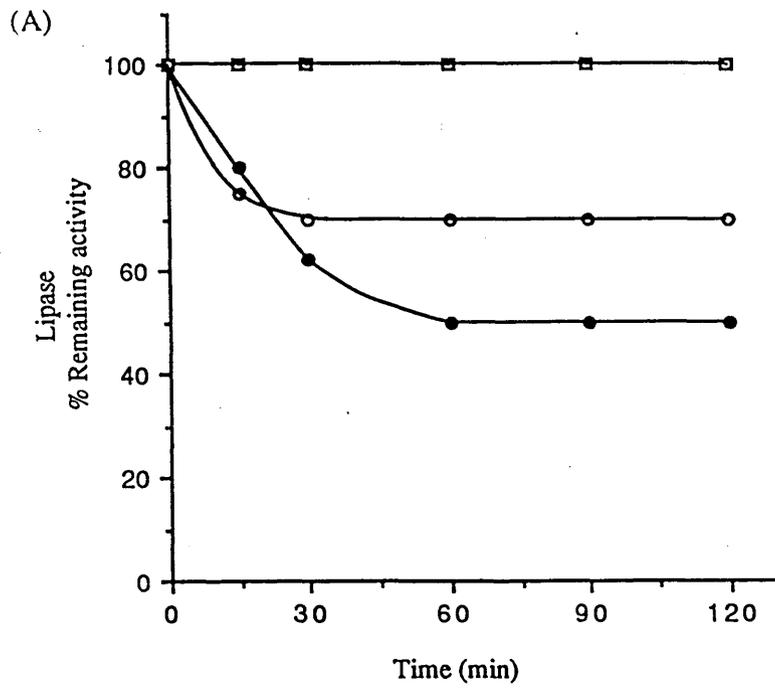


Figure 3.21 The effect of tryptophan modifying reagents on lipase activity.

The supernate used was prepared as described in the legend to Figure 3.3 except that 50 mM-potassium phosphate buffer, pH 7.5 was used. The supernate (0.5 ml) was treated with NBS (5, 10 mM A) or 2-hydroxy-5-nitrobenzyl bromide (10, 20 mM B) as described in Methods 2.13. The lipase activity was determined as described in Methods 2.11.4 at time various intervals. The values shown are averages of duplicate assays. The 100% value was $16.5 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$.

(A) Control \square , NBS 5 mM \circ , 10 mM \bullet

(B) Control \square , 2-hydroxy-5-nitrobenzyl bromide 10 mM Δ , 20 mM \blacktriangle .



3.5.2.9 The effect of tetrahydrolipstatin on lipase activity.

The effect of different concentrations of THL on lipase activity in the supernate is shown in Figure 3.22. It can be seen that the lipase activity was inhibited by about 25-30% with THL (0.1-0.4 mM) in 30 min. Increasing the concentration of THL to 0.8 mM increased the inhibition to about 40% in 30 min. The extent of incubation did not increase the inhibition after 30 min incubation. Incubation of the supernate with THL at 0 °C or at 37 °C did not effect the extent of inhibition.

3.5.2.10 Molecular mass of lipase activity.

The supernate protein was eluted on Superose-6 gel filtration column using f.p.l.c. The eluted peaks were assayed for lipase activity which was shown to be present in one very high molecular mass form (see Figure 3.23). Proteins of known molecular mass were used to determine the relative molecular mass of the eluted peak. The estimated relative molecular mass of the native enzyme was 1040,000.

Figure 3.22 The effect of tetrahydrolipstatin on lipase activity.

The supernate used was prepared as described in the legend to Figure 3.3. The supernate (0.5 ml) was treated with THL (0.1, 0.4, 0.8 mM) as described in Methods 2.13. The lipase activity was determined at various time intervals described in Methods 2.11.4. The values shown are averages of duplicate assays. The 100% value was $16.5 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$.

Control \square , THL 0.1 Δ , 0.4 mM \circ , 0.8 mM \bullet .

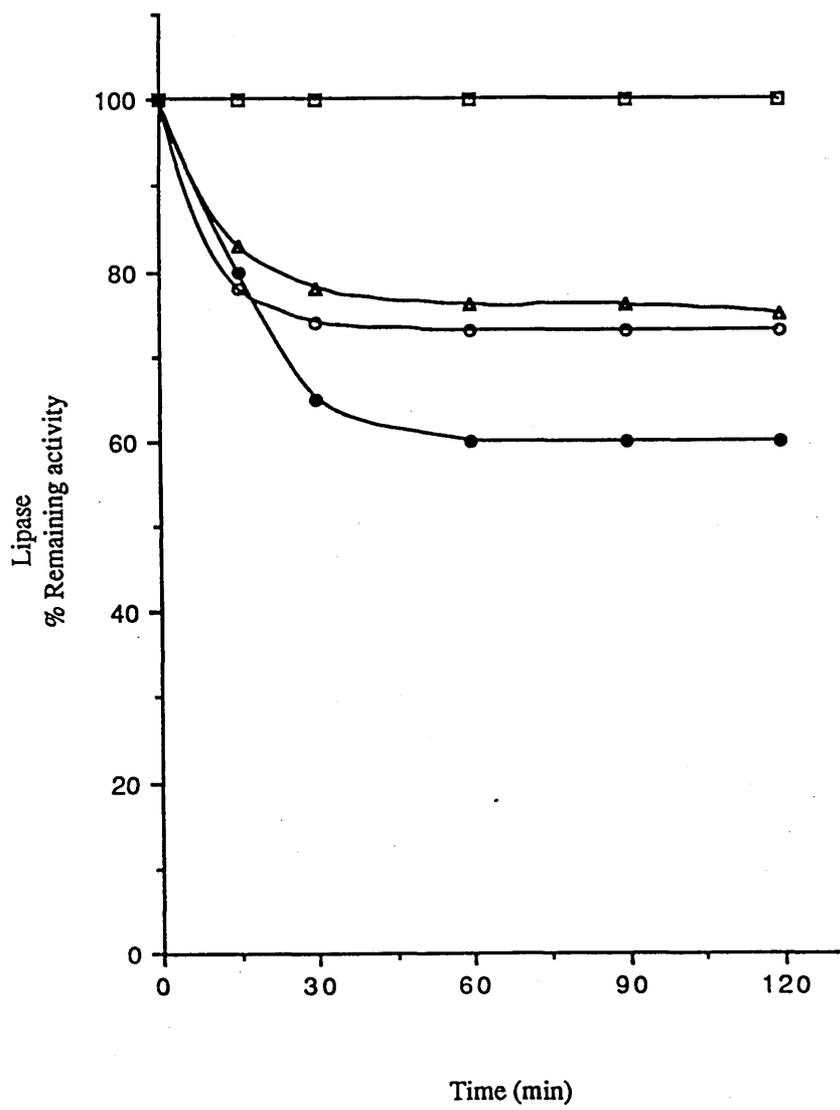


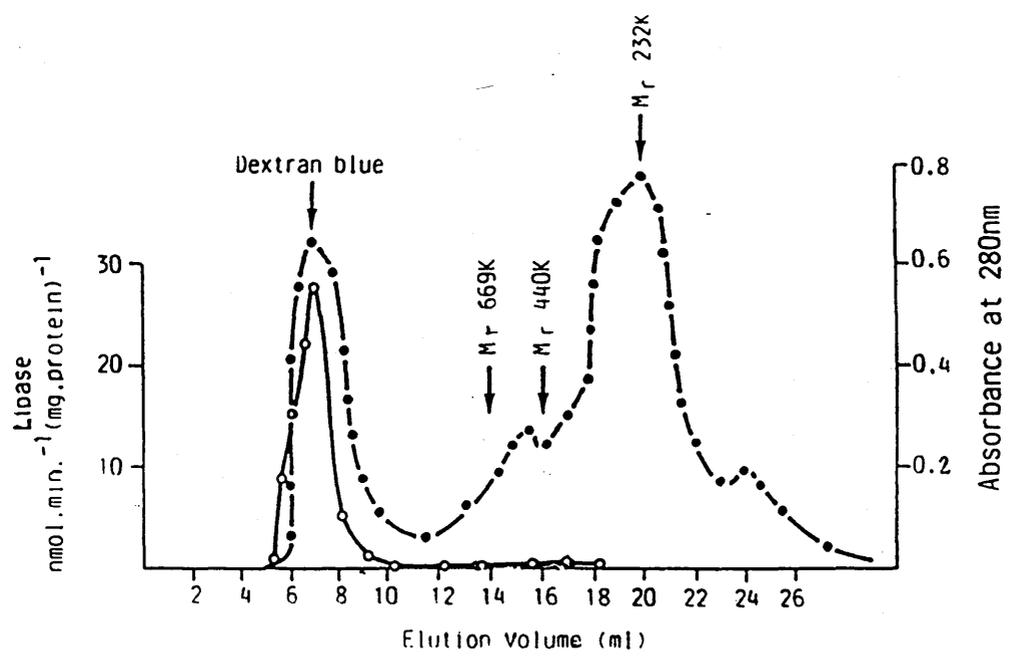
Figure 3.23 Molecular mass determination of lipase activity by gel filtration chromatography.

The supernate used was prepared as described in the legend to Figure 3.3. The supernate 0.5 ml (5 mg protein) was applied to a 30 cm Superose-6 f.p.l.c.column as described in Methods 2.12. The lipase activity was determined in eluted fractions as described in Methods 2.11.4. The column was calibrated using thyroglobulin (M_r 669,000), ferritin (M_r 440,000) and catalase (M_r 232,000). The elution volume of Dextran blue (approx. M_r 2,000,000) was taken to be the void volume

Lipase activity $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ —○—

A_{280} —●—

Elution position of standard proteins ↓



3.5.2.11 The effect of culture conditions on lipase activities in *A. calcoaceticus* NCIB 8250 .

The lipase activity was assayed in the broken cells, supernate and membrane fractions of bacteria harvested from exponential phase and of bacteria harvested from stationary phase in carbon or nitrogen limited cultures (see Table 3.8). Approximately the same total lipase activity [$25 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] was present in broken cells from all culture conditions. The total lipase activity was higher (total activity about 60%) in the supernate fraction of nitrogen limited bacteria than the supernate fraction from exponentially growing bacteria and from carbon limited bacteria (total activity about 40%). The specific activity of lipase in the supernate fraction of nitrogen limited bacteria is significantly higher than the exponentially growing and carbon limited bacteria. The total lipase activity was higher in crude membrane fractions of exponentially growing and carbon limited bacteria (total activity about 60%) than nitrogen limited bacteria (total activity about 40%). There was no significant change in the specific activities of the lipase in membrane fractions or in the supernates from the bacteria caused by changing the culture conditions from carbon to nitrogen or nitrogen to carbon limitation.

Table 3.8 Distribution of lipase activities in *A. calcoaceticus* NCIB 8250 grown in different culture conditions

The bacteria used were exponentially growing, carbon limited or nitrogen limited as described in Methods 2.2.3.1 and 2.2.3.2 respectively. Supernates were prepared as described in the legend to Figure 3.3. The lipase activity was assayed in broken cells, supernates and membrane fractions as described in Methods 2.11.4. Using the Student's 't' test, the value for the total and specific activity in supernates of nitrogen limited bacteria is significantly different from carbon limited and exponentially growing bacteria ($P = < 0.01$, $n = 4$). Total activity in membrane fraction of nitrogen limited bacteria is significantly different from exponentially growing bacteria and carbon limited bacteria ($P = < 0.01$, $n = 4$). No other significant differences were revealed by this test.

	Lipase	
	Total % activity	Specific activity nmol min ⁻¹ (mg protein) ⁻¹
Exponentially growing		
Broken cell	100	25.3 ± 4.8
Supernatant	39.5 ± 2.4	15.5 ± 1.9
Membranes	61.2 ± 5.5	35.3 ± 3.4
Carbon limited		
Broken cells	100	25.6 ± 3.7
Supernatant	41.5 ± 1.9	11.6 ± 1.5
Membranes	61.4 ± 2.3	32.1 ± 5.3
Nitrogen limited		
Broken cells	100	30.3 ± 2.6
Supernatant	62.2 ± 2.5	21.2 ± 2.3
Membranes	37.4 ± 1.5	33.0 ± 2.2

3.6 The separation of inner and outer membranes from *A. calcoaceticus* NCIB 8250 grown in carbon or nitrogen limited conditions.

The membranes from the bacteria grown in carbon or nitrogen limited conditions were separated on a sucrose density gradient into four and six distinct bands respectively (see Figure 3.24 A and B). Bands are numbered from top to bottom. All bands were assayed for NADH oxidase activity (a marker of the inner membrane) and the amount of 2-keto-3-deoxy octonic acid (KDO) (a marker of the outer membrane). In carbon limited bacteria band-1 and band-4 had similar amounts of NADH oxidase activity and KDO (see Table 3.9). However, the protein profile on SDS-PAGE gel showed more contamination of outer membranes in band-4 than in band-1 (see Figure 3.25), therefore band-1 was selected as a typical inner membrane fraction in carbon limited bacteria. Band-1, which contained high NADH oxidase activity and low KDO was designated as the inner membrane fraction from nitrogen limited bacteria (see Table 3.10). Band-3 in the membrane fractions from the carbon limited bacteria and band-4 in the membrane fractions from the nitrogen limited bacteria were selected as outer membranes, as these bands contained a high content of KDO and a low NADH oxidase activity (see Tables 3.9 and 3.10). The other fractions appeared to be mixtures of different proportions of both membranes. The protein composition of the bands from the sucrose density gradient were analysed by SDS-PAGE (see Figure 3.25). Both inner and outer membrane fractions show very different protein banding patterns on SDS-PAGE as expected. The outer membranes contain seven major components proteins of M_r 22,000; 28,000; 30,000; 40,000; 43,000; 67,000 and 70,000; while the inner membranes contain many proteins M_r 's ranging from 20,000 - 94,000.

3.6.1 Distribution of the esterase and the lipase activities in membrane fractions of the bacteria grown in carbon or nitrogen limited conditions.

The esterase and lipase activities were assayed in the separated fractions of inner or outer membranes from the bacteria grown in carbon and nitrogen limited conditions (see Tables 3.9 and 3.10). The esterase and lipase activity were present in both inner and outer membranes. The esterase activity in the inner membranes was about five times higher than the activity in outer membranes from the nitrogen limited bacteria, while in carbon limited bacteria the esterase activity in the inner membranes was about eight times higher than that of outer membranes. The distribution of lipase activity was different from esterase in that the lipase activity was present in about equal amounts in both the inner and the outer membranes of carbon limited bacteria and that the lipase activity was about double in the inner membranes of nitrogen limited bacteria than in the outer membranes.

Figure 3.24 Separation of inner and outer membranes on sucrose density gradients.

The bacteria were grown in carbon or nitrogen limited conditions and harvested as described in Methods 2.2.3.2 and 2.2.7 respectively. The membrane fractions were prepared as described in Methods 2.15. The membranes from the bacteria grown in carbon or nitrogen limited conditions were separated on a sucrose density gradient into four and six bands respectively and the separated bands were numbered from top to bottom.

- (A) Membranes fractions from carbon limited bacteria
- (B) Membranes fractions from nitrogen limited bacteria

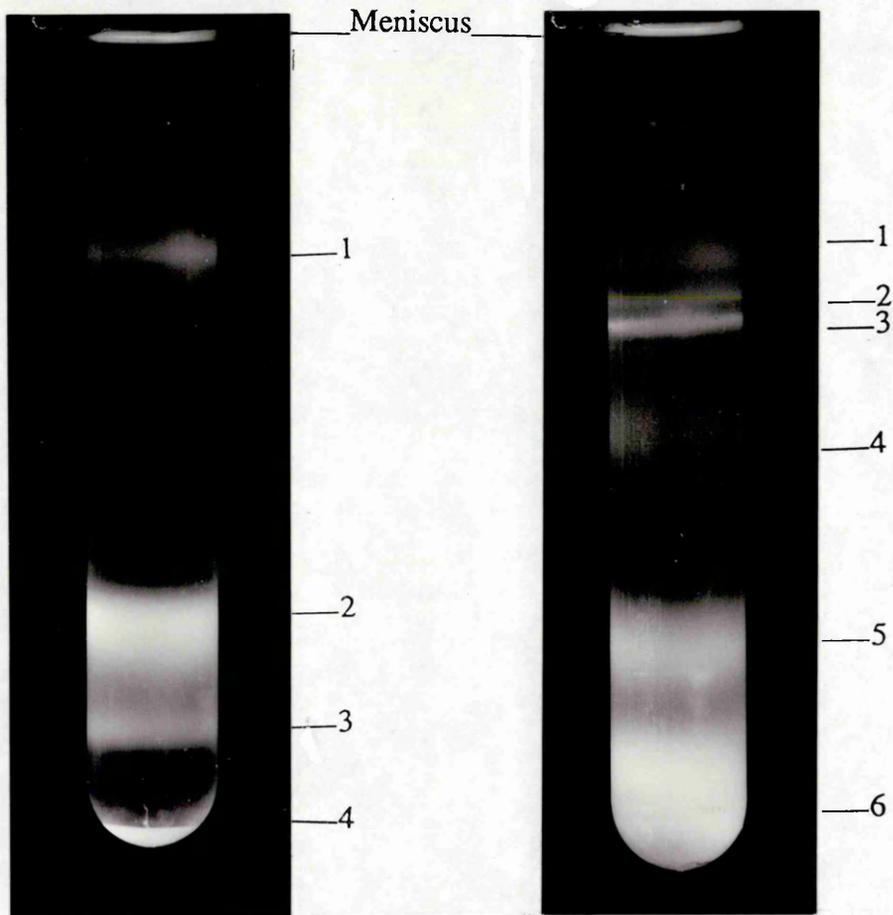


Table 3.9 Distribution of esterase and lipase activities in the membrane fractions of *A. calcoaceticus* NCIB 8250 grown in carbon limited conditions.

The bacteria were grown in carbon limited conditions and harvested as described in Methods 2.2.3.2 and 2.2.7 respectively. The membrane fractions were prepared as described in Methods 2.15. The membranes from the bacteria grown in carbon limited conditions were separated on a sucrose density gradient into four bands. NADH oxidase activity and KDO were determined as described in Methods 2.15.1 and 2.15.2 respectively. The esterase and lipase activities were assayed in all the separated bands as described in Methods 2.11.1 and 2.11.4 respectively. The results are the means of determinations from two separate bacterial cultures.

	KDO nmol (mg protein) ⁻¹	NADH oxidase nmol min ⁻¹ (mg protein) ⁻¹	Esterase nmol min ⁻¹ (mg protein) ⁻¹	Lipase nmol min ⁻¹ (mg protein) ⁻¹
Carbon limited				
Band 1 (Inner membranes)	50.4	48.4	40.7	86.1
Band 2	197.2	6.1	16.0	80.0
Band 3 (Outer membranes)	235.4	3.4	5.6	80.1
Band 4	78.3	109.1	53.3	36.9

Table 3.10 Distribution of esterase and lipase activities in the membrane fractions of *A. calcoaceticus* NCIB 8250 grown in nitrogen limited conditions

The bacteria were grown in nitrogen limited conditions and harvested as described in Methods 2.2.3.2 and 2.2.7 respectively. The membrane fractions were prepared as described in Methods 2.15. The membranes from the bacteria grown in nitrogen limited conditions were separated on a sucrose density gradient into six bands. NADH oxidase activity and KDO were determined as described in Methods 2.15.1 and 2.15.2 respectively. The esterase and lipase activities were assayed in all the separated bands as described in Methods 2.11.1 and 2.11.4 respectively. The results are the means of determinations from two separate bacterial cultures.

	KDO nmol (mg protein) ⁻¹	NADH oxidase nmol min ⁻¹ (mg protein) ⁻¹	Esterase nmol min ⁻¹ (mg protein) ⁻¹	Lipase nmol min ⁻¹ (mg protein) ⁻¹
Nitrogen limited				
Band 1 (Inner membranes)	62.1	70.4	63.8	91.3
Band 2	98.0	31.5	61.11	65.2
Band 3	127.1	28.5	38.4	50.0
Band 4 (Outer membranes)	163.3	12.1	14.4	46.4
Band 5	101.0	44.0	58.2	23.5
Band 6	344.2	69.4	91.75	19.4

Figure 3.25 SDS-PAGE of membrane fractions.

The membrane fractions were prepared as described in legend to Figure 3.24. The membranes from the bacteria grown in carbon or nitrogen limited conditions were separated on sucrose density gradients into four and six bands respectively. The membrane fractions (45 μg protein) were separated on a 12.5% acrylamide gel as described in Methods 2.19.1 and stained with Coomassie Blue as described in Methods 2.20.1. Standard proteins of known relative molecular mass were used to calibrate the gel: phosphorylase b (M_r 94,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,000), lactalbumin (M_r 14,400).

(A) Membranes fractions from carbon limited bacteria, bands 1-4

(B) Membranes fractions from nitrogen limited bacteria, bands 1-6

n = number of the band

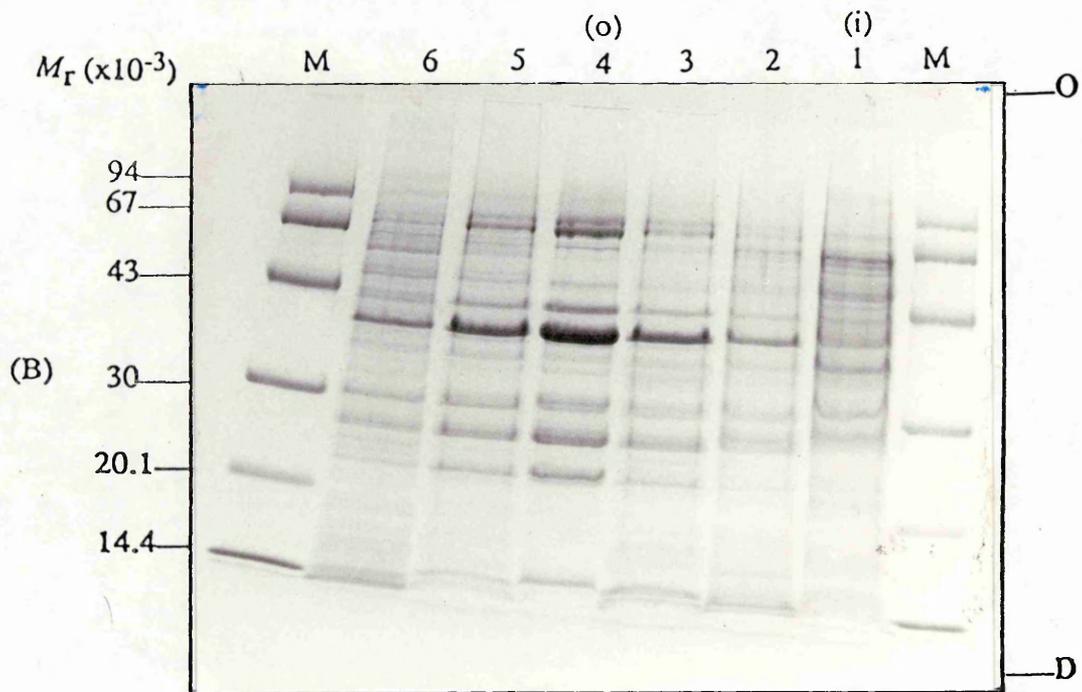
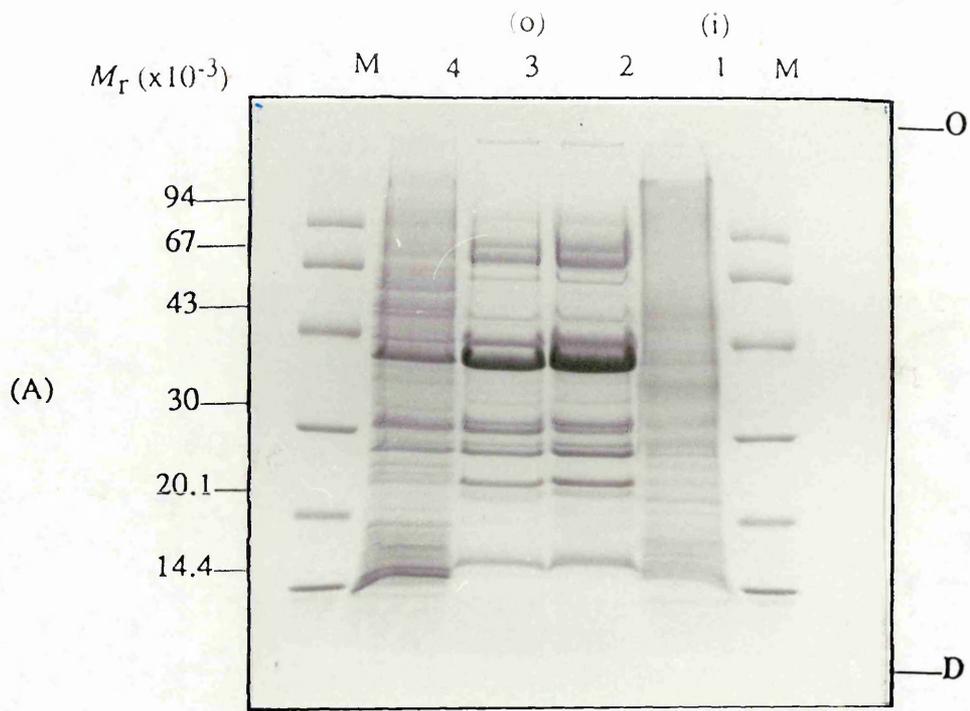
i = inner membranes

o = outer membranes

M = molecular mass markers

O = origin

D = dye front



3.7 Distribution of the esterase and the lipase activities in strains NCIB 8250, HO1-N and mutants from different growth conditions.

A. calcoaceticus NCIB 8250, HO1-N and mutant strains derived from HO1-N were grown on nutrient broth, their esterase and lipase activities were compared (see Table 3.11 a). The esterase activities in the strain HO-1 N and the mutants derived from it were approximately the same in the broken cells, supernate and membrane fractions except CP 179-22 which had less activity in supernate and membrane fractions and CP 4-18 which had higher activity in membrane fractions. The esterase activities in the broken cells, supernate and membrane fractions of NCIB 8250 were nearly twice as high as that of HO1-N and mutants grown in nutrient broth. NCIB 8250, HO1-N and CP 179-22 were grown on acetate as sole carbon source in minimal salts medium instead of succinate because the CP 179-22 does not grow well in succinate minimal salts medium. The esterase activity in the supernate of NCIB 8250 was nearly ten times that in the supernate of HO1-N grown in acetate (see Table 3.11 b) or succinate (see Table 3.6). The esterase activities of the membranes and broken cells are about three times greater in NCIB 8250 than in those that of HO1-N when grown in acetate. The esterase activities in broken cells, the supernate and the membrane fractions of HO1-N are about twice than that of the CP179-22 when grown in acetate (see Table 3.11 b).

The lipase activity was higher in the broken cells, the supernate and the membrane fractions of NCIB 8250 than HO1-N or the mutants derived from it when grown in nutrient broth (see Table 3.11 a). The lipase activity in the broken cells and the supernate fractions was also higher in NCIB 8250 than that of HO1-N and CP179-22 when grown in acetate (see Table 3.11 b).

The esterase activities in the supernate and the membrane fractions of HO1-N and its mutant CP 179-22, were treated with PMSF and were found to be inhibited by 90%. The lipase activities were insensitive to this compound in both the supernate and membrane fractions. However, the esterase and lipase activities in HO1-N and CP 179-22 were inhibited by 70% in both supernate and membrane fractions when treated with NBS.

Table 3.11-a Distribution of esterase and lipase activities in different strains of *A. calcoaceticus* grown on nutrient broth.

The strains NCIB 8250, HO1-N, CP 179-22, CP 173-26, CP 3-4, CP 4-18 and CP 3-24 were grown on nutrient broth as described in Methods 2.2.3.4. The bacteria ($0.5 \text{ g wet wt ml}^{-1}$) were resuspended in 1 ml of 50 mM Tris HCl pH 8.5 and broken by sonication as described in Methods 2.2.8.1. The esterase and lipase activities were assayed as described in Methods 2.11.1 and 2.11.4 respectively. The results are the means of determinations from two separate bacterial cultures.

	Esterase Specific activity nmol min ⁻¹ (mg protein) ⁻¹	Lipase Specific activity nmol min ⁻¹ (mg protein) ⁻¹
<i>A. calcoaceticus</i> NCIB 8250		
Broken cells	70.4	15.5
Supernatant	40.5	10.3
Membranes	50.3	8.5
<i>A. calcoaceticus</i> HO1-N		
Broken cells	48.2	8.6
Supernatant	16.5	7.0
Membranes	28.5	6.3
<i>A. calcoaceticus</i> HO1-N mutant strain CP 179-22		
Broken cells	30.0	7.5
Supernatant	7.5	7.5
Membranes	20.3	6.4
<i>A. calcoaceticus</i> HO1-N mutant strain CP 173-26		
Broken cells	36.6	7.5
Supernatant	14.1	6.5
Membranes	31.3	7.9
<i>A. calcoaceticus</i> HO1-N mutant strain CP 3-4		
Broken cells	40.0	6.5
Supernatant	14.3	6.6
Membranes	26.3	6.4
<i>A. calcoaceticus</i> HO1-N mutant strain CP 4-18		
Broken cells	27.0	5.8
Supernatant	15.8	6.3
Membranes	45.0	6.7
<i>A. calcoaceticus</i> HO1-N mutant strain CP 3-24		
Broken cells	40.0	5.5
Supernatant	16.4	7.0
Membranes	34.0	4.5

Table 3.11-b Distribution of esterase and lipase activities in different strains of *A. calcoaceticus* grown on acetate.

The strains NCIB 8250, HO1-N and CP 179-22 were grown on acetate as carbon source as described in Methods 2.2.3.1. The bacteria (0.5 g wet wt ml⁻¹) were resuspended in 1 ml of 50 mM Tris HCl pH 8.5 and broken by sonication as described in Methods 2.2.8.1. The esterase and lipase activities were assayed as described in Methods 2.11.1 and 2.11.4 respectively. The results are the means of determinations from two separate bacterial cultures.

	Esterase Specific activity nmol min ⁻¹ (mg protein) ⁻¹	Lipase Specific activity nmol min ⁻¹ (mg protein) ⁻¹
<i>A. calcoaceticus</i> NCIB 8250		
Broken cells	161.1	20.6
Supernatant	110.3	17.4
Membranes	180.4	9.6
<i>A. calcoaceticus</i> HO1-N		
Broken cells	56.6	13.4
Supernatant	13.5	8.8
Membranes	52.3	9.5
<i>A. calcoaceticus</i> HO1-N mutant strain CP 179-22		
Broken cells	30.4	7.5
Supernatant	8.5	6.8
Membranes	35.6	6.8

3.8 Gel electrophoresis.

3.8.1 Esterase activity on native PAGE.

The supernate protein was separated using PAGE in non denaturing conditions to resolve esterase activity (see Figure 3.26) It can be seen that the esterase activity was not resolved into distinct bands, however, there was a large band in the gel and a smear appeared on the top of the gel. Different concentrations of acrylamide 3% to 10% were used in the gels, but these conditions did not solve this problem.

3.8.2 Esterase activity on SDS-PAGE.

3.8.2.1 Stained with 1-naphthyl acetate and 2-naphthyl acetate.

The esterase activity in the supernate was resolved into separate components which after activity staining appeared as three brown bands with 1-naphthyl acetate and two red bands with 2-naphthyl acetate (see Figure 3.27 A and B). The relative molecular mass of these bands was estimated by running molecular mass marker proteins besides the supernate tracks. The brown bands were of M_r 38,000, 40,000 and 43,000 and the red bands of M_r 38,000 and 43,000. The molecular mass of these bands always varied over a range $\pm 2,000$.

Figure 3.26 The separation of esterase activity on native-PAGE.

The supernate used was prepared as described in the legend to Figure 3.3. The supernate proteins (250 μg) were separated using 5% acrylamide gel as described in Methods 2.19.2. The gel was stained for esterase activity with 1-naphthyl acetate as described in Methods 2.20.2.

O = origin

D = dye front

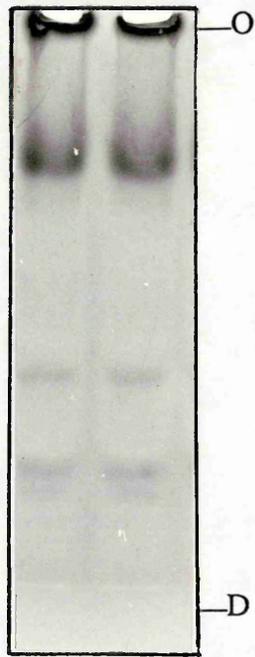


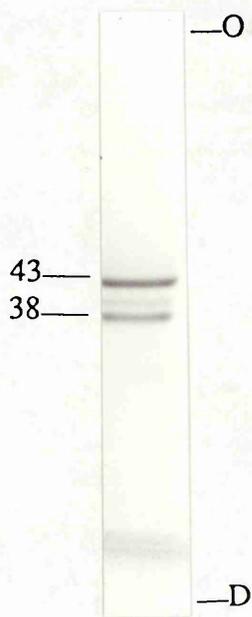
Figure 3.27 The separation of esterase activity on SDS-PAGE.

The supernate used was prepared as described in the legend to Figure 3.3. The supernate proteins (250 μg protein) were separated using 12.5% acrylamide gel as described in Methods 2.19.1. The gel was stained for esterase activity with 1-naphthyl acetate (A) or 2-naphthyl acetate (B) as described in Methods 2.20.2.

O = origin

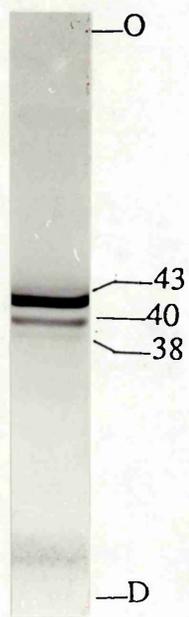
D = dye front

M_r ($\times 10^{-3}$)



(B)

M_r ($\times 10^{-3}$)



(A)

3.8.2.2 The effect of protein concentration and incubation time of the gel in the staining solution on esterase activity.

The esterase activity separated on SDS-PAGE gels was proportional to the amount of supernate protein loaded on to the gel (see Figure 3.28 A) and proportional to the time of incubation of the gel in the substrate (see Figure 3.28 B). It can be seen from the figure that increasing the incubation time of the gel in the substrate increased the staining of esterase bands. The M_r 43,000 component was used in this experiment because this component stained more intensely than the others.

3.8.2.3 The effect of culture conditions on the esterase activity on SDS-PAGE gel.

A different banding pattern of esterase activities was detected when the bacteria were harvested from various culture conditions and when stained using 1- or 2-naphthyl acetates (see Figure 3.29 A and B). In the exponentially growing and nitrogen limited stationary phase harvested bacteria, the percentage distribution of esterase activity was approximately similar with regard to the M_r 38,000, 40,000 and 43,000 components with 1-naphthyl acetate and in the M_r 38,000 and 43,000 components with 2-naphthyl acetate. In carbon limited bacteria, the M_r 43,000 component showed lower activity and the M_r 38,000 component a higher activity than with exponentially growing and nitrogen limited bacteria when 1-naphthyl acetate was used (see Table 3.12 A). Furthermore, the M_r 43,000 component showed about a 50% lower activity and the M_r 38,000 component had a 20% higher activity in carbon limited bacteria with 2-naphthyl acetate as substrate compared to exponentially growing and nitrogen

limited bacteria (see Table 3.12 B). There was no effect observed on the intensities of bands of esterase activity on the gel on interchanging the culture conditions from carbon to nitrogen or nitrogen to carbon limitation.

Figure 3.28 The effect of the amount of protein loaded on the gel and incubation time of the gel in the staining solution on the staining of esterase activity.

The supernate used was prepared as described in the legend to Figure 3.3. Different amounts of supernate proteins (150, 200, 250, 300 μg protein) were separated using 12.5% acrylamide gel as described in Methods 2.19.1. The gel was stained for esterase activity with 1-naphthyl acetate as described in Methods 2.20.2 and the bands of stained esterase activity were scanned as described in Methods 2.22 and the results are reported as the area of the peak determined by the Densitometer. The results shown are for the M_r 43,000 component and are the means of two separate experiments (A).

The effect of incubation time of gels in the staining solution on esterase activity was determined by separating the same amount of supernate proteins (250 μg) using 12.5% acrylamide gel as described in Methods 2.19.1. The gel was cut into 4 pieces and stained the pieces for esterase activity with 1-naphthyl acetate for 0, 20, 40, and 60 min as described in Methods 2.20.2. The bands of esterase activity were scanned as described in Methods 2.22. The results shown are for the M_r 43,000 component and are the means of two separate experiments (B)

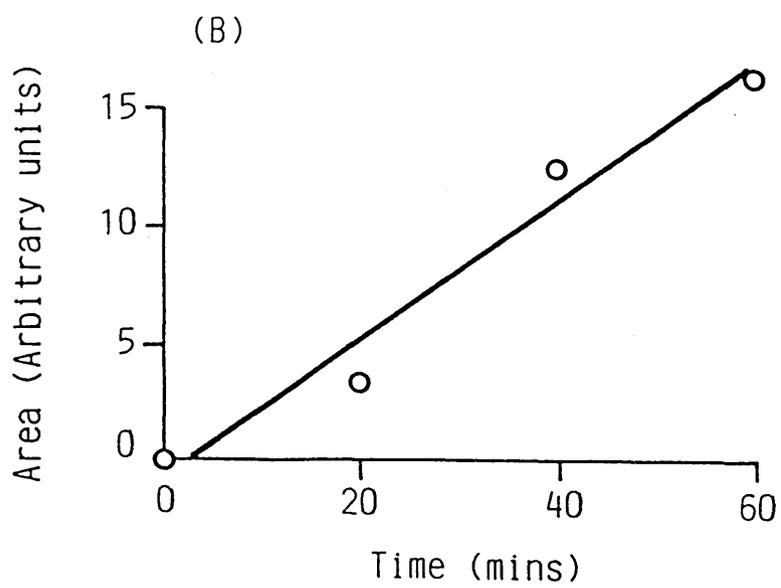
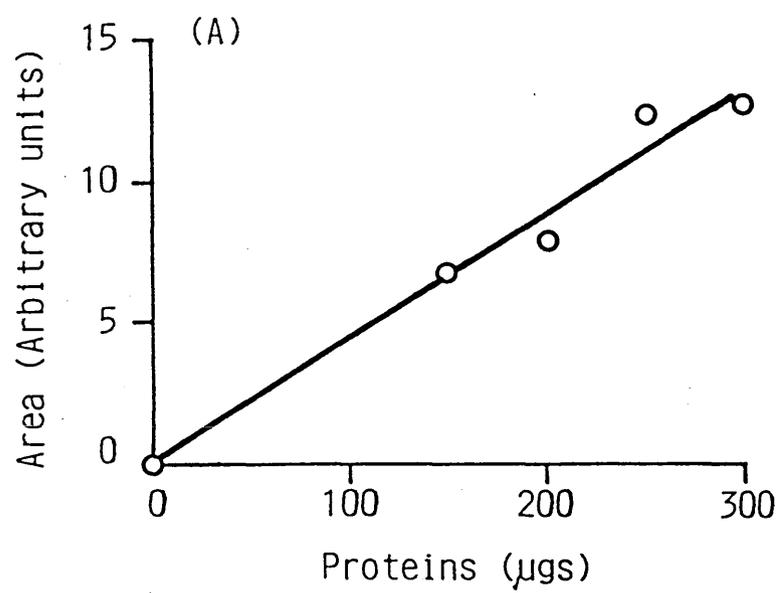


Figure 3.29 The separation of esterase activity on SDS-PAGE of *A. calcoaceticus* NCIB 8250 grown in different culture conditions.

The supernate proteins were obtained from *A. calcoaceticus* NCIB 8250 grown in different culture conditions and harvested as described in the legend to Table 3.7. The supernate were prepared as described in the legend to Figure 3.3. The supernate proteins (250 µg protein) were separated using 12.5% acrylamide gel as described in Methods 2.19.1. The gel was stained with 1-naphthyl acetate (A) or 2-naphthyl acetate (B) as described in Methods 2.20.2.

O = origin

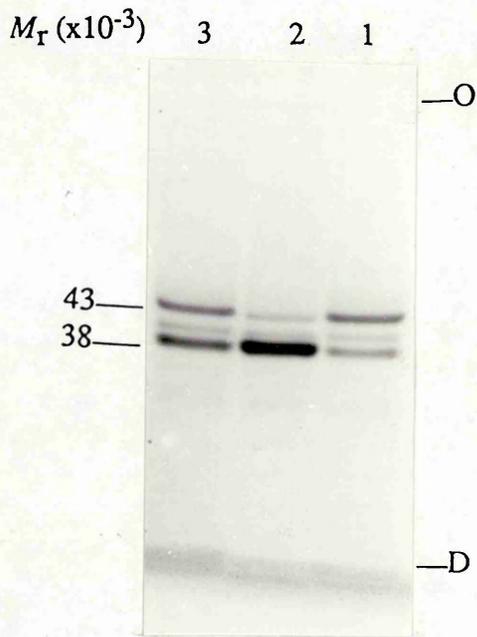
D = dye front

(A)

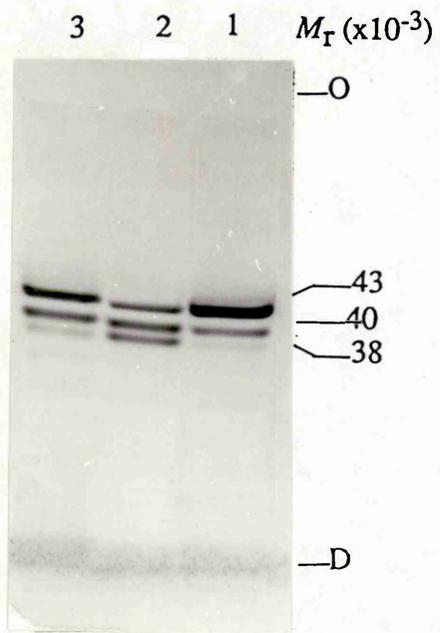
Nitrogen limited bacteria, lane 1; Carbon limited bacteria, lane 2; Exponentially growing bacteria, lane 3

(B)

Nitrogen limited bacteria, lane 1; Carbon limited bacteria, lane 2; Exponentially growing bacteria, lane 3



(B)



(A)

Table 3.12 The effect of culture conditions on the distribution of esterase activity between the different components on SDS-PAGE gels.

The esterase activity on SDS-PAGE gel was determined as described in the legend to Figure 3.29. The esterase activity was stained with 1-naphthyl acetate (A) or with 2-naphthyl acetate (B). The activity bands were scanned as described in Methods 2.22. The 100% value is the total activity in all three components with 1-naphthyl acetate (A) and 100% value is the total activity with 2-naphthyl acetate in both components (B). The results are averages of two separate experiments.

(A)

	Mr 43 % activity	Mr 40 K % activity	Mr 38 K % activity
Exponentially growing bacteria	60	25	15
Carbon limited bacteria	30	35	35
Nitrogen limited bacteria	65	25	10

(B)

	Mr 43 % activity	Mr 38 K % activity
Exponentially growing bacteria	34	62
Carbon limited bacteria	15	85
Nitrogen limited bacteria	32	68

3.8.3 Esterase activity with ethyl butyrate as substrate.

Ethyl butyrate esterase activity could also be detected on SDS-PAGE gels. A yellow coloured band of esterase activity appeared on the blue background as thymol blue indicator changed colour due to liberation of acid (see Figure 3.30). The relative molecular mass of the activity band was determined by running molecular mass marker on the same gel. The approximate molecular mass of the activity band was M_r 43,000.

3.8.4 Comparison of esterase activities from *A. calcoaceticus* NCIB 8250 with other strains of *Acinetobacter* by SDS-PAGE.

The esterase activity on SDS-PAGE gel was also tested in five other stains of *A. calcoaceticus*. The esterase banding pattern of EBF 65/65, ATCC 23055 and RAG-1 was compared with strain NCIB 8250 (see Figure 3.31 A and B). After staining with 1- or 2-naphthyl acetates, strains NCIB 8250 and EBF 65/65 showed similar banding pattern with both substrates. While the strains RAG-1 and ATCC 23055 showed a different banding patterns from the strains NCIB 8250 with both substrates. The strains RAG-1 did not show activity in the component of M_r 38,000 and ATCC 23055 the components of M_r 38,000 and 43,000 did not appear.

Similarly the strain HO1-N and its mutant showed a different banding pattern from strain NCIB 8250 with 1- or 2-naphthyl acetate (see Figure 3.31 A and B). The strain HO1-N showed two new components of M_r 20,000 and 50,000 in addition to M_r 43,000 component with 1-naphthyl acetate, while the

components M_r 38,000 and 40,000 did not appear with this substrate. The component of M_r 50,000 and M_r 38,000 appeared in addition to the component of M_r 43,000 with 2-naphthyl acetate but the component of M_r 20,000 was missing. The banding pattern of the mutant was similar to HO1-N except the component of M_r 20,000 which did not appear with 1-naphthyl acetate.

3.8.5 The effect of inhibitors on esterase activity.

Esterase activity on SDS-PAGE gel was treated with different inhibitors. PMSF, bis-*p*-nitrophenyl phosphate and eserine showed inhibition to different extents when 1- or 2-naphthyl acetate were the substrates (see Figure 3.32 A and B). When 1-naphthyl acetate was used as a substrate PMSF reduced the activity in components of M_r 40,000 and 43,000, bis-*p*-nitrophenyl phosphate inhibited totally the activity in the components of M_r 43,000 and 38,000 and eserine inhibited the most of the activity in components of M_r 40,000 and 43,000. When 2-naphthyl acetate was used as substrate, PMSF decreased the activity in component of M_r 43,000, bis-*p*-nitrophenyl phosphate inhibited all the activity in component of M_r 43,000 but component of M_r 38,000 and M_r 40,000 appeared to have higher activity than the control and eserine inhibited the activity in component of M_r 43,000 only.

3.8.6 Lipase activity on PAGE gels.

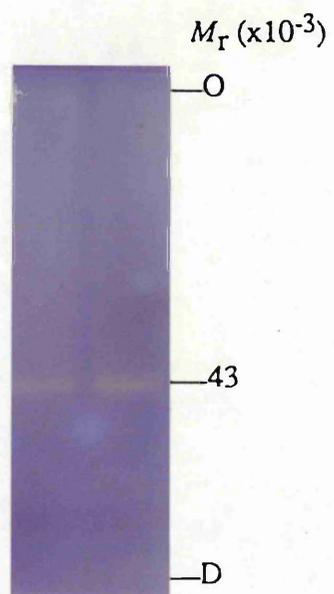
Efforts to stain lipase activity on native or SDS-PAGE gels of acrylamide concentration 3% to 12.5%, with 2-naphthyl esters of fatty acids of chain length 8-18 carbon atoms were not fruitful.

Figure 3.30 The detection of esterase activity using ethyl butyrate as substrate.

The supernate used was prepared as described in the legend to Figure 3.3. The supernate proteins (250 μg protein) were separated using a 10% acrylamide gel as described in Methods 2.19.1. The gel was stained with ethyl butyrate as described in Methods 2.20.3

O = origin

D = dye front



**Figure 3.31 The separation of esterase activity of
A. calcoaceticus strains by SDS-PAGE.**

Different strains of *A. calcoaceticus* were grown on nutrient broth as described in Methods 2.2.3.4. The supernate were obtained from bacteria (0.5 g wet wt/ml) broken by sonication as described in Methods 2.2.8.1. The supernates (250 µg protein) were separated using 12.5% acrylamide gel as described in Methods 2.19.1. The gel was stained with 1-naphthyl acetate (A) or 2-naphthyl acetate (B) as described in Methods 2.20.2. The supernates were from strains NCIB 8250, RAG-1, ATCC 23055, EBF 65/65, HO1-N, CP 179-22.

O = origin

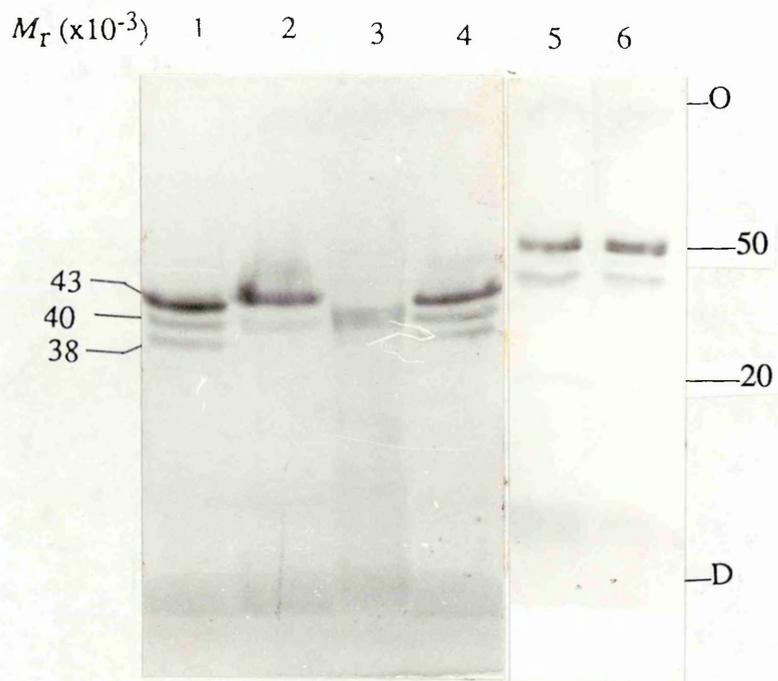
D = dye front

(A)

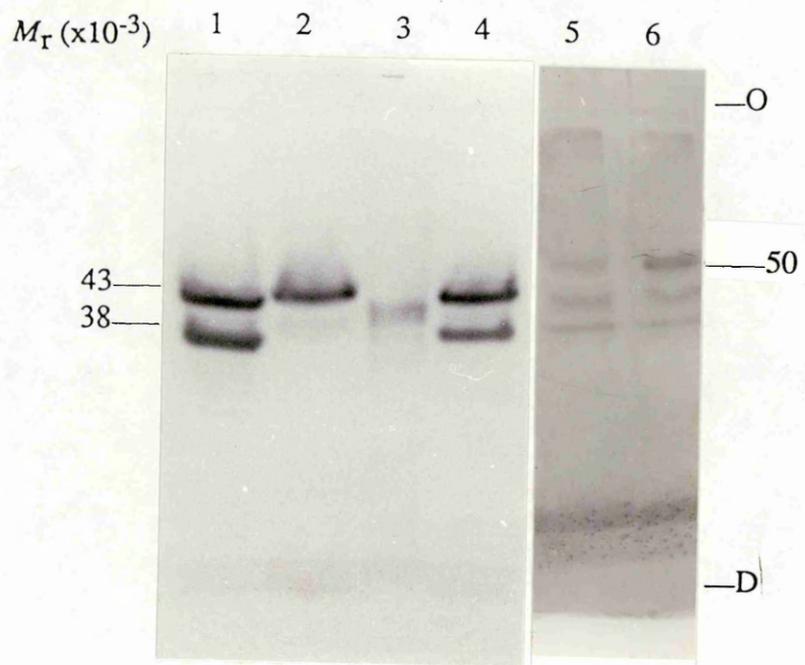
NCIB 8250, lane 1; RAG-1, lane 2; ATCC 23055, lane 3; EBF 65/65, lane 4;
HO1-N, lane 5; CP 179-22, lane 6

(B)

NCIB 8250, lane 1; RAG-1, lane 2; ATCC 23055, lane 3; EBF 65/65, lane 4;
HO1-N, lane 5; CP 179-22, lane 6



(A)



(B)

Figure 3.32 The effect of inhibitors on esterase activity on SDS-PAGE gel.

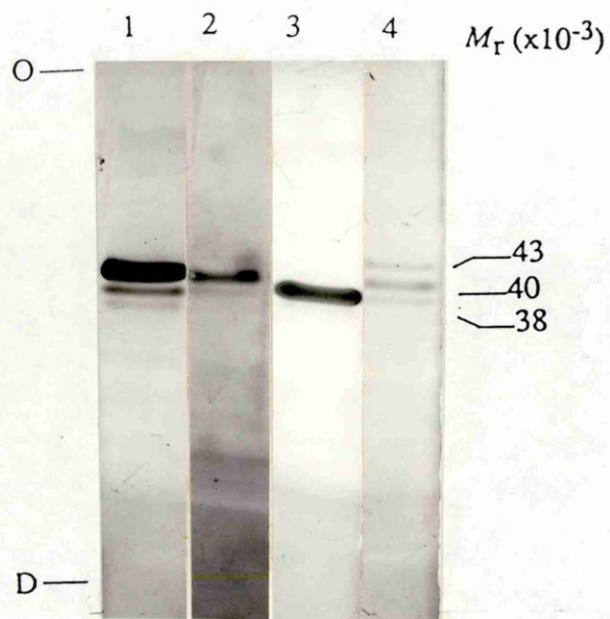
The supernate used was prepared as described in the legend to Figure 3.3. The samples of supernate proteins (250 µg protein) were separated using a 12.5% acrylamide gel as described in Methods 2.19.1. The gels were treated with different inhibitors as described in Methods 2.21. The gels were stained with 1-naphthyl acetate (A) and 2-naphthyl acetate (B) as described in Methods 2.20.2. The inhibitors were tested on esterase activity separated by SDS-PAGE in two separate experiments.

(A)

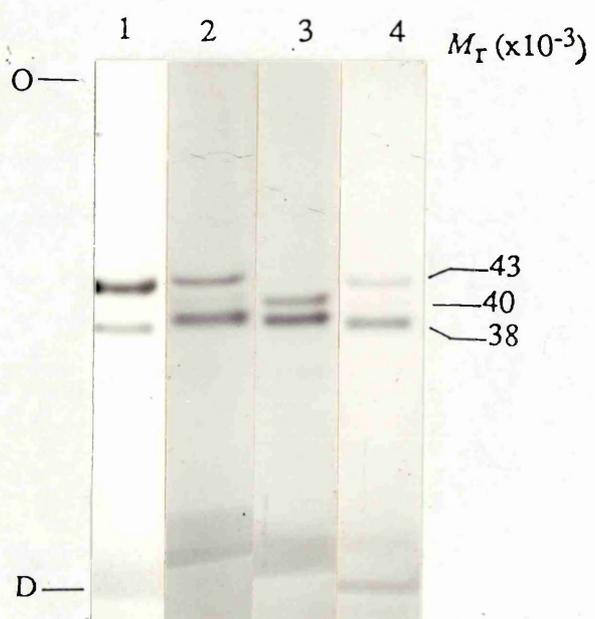
Control, lane 1; PMSF, lane 2; Bis-p-nitrophenyl phosphate, lane 3;
Eserine, lane 4

(B)

Control, lane 1; PMSF, lane 2; Bis-p-nitrophenyl phosphate, lane 3;
Eserine, lane 4



(A)



(B)

3.9 Inhibition of esterase and lipase activities in whole bacteria

The effect of PMSF, NBS, THL and 2-hydroxy-5-nitrobenzyl bromide on esterase and lipase activity in whole bacteria was determined. The esterase and lipase activities were determined in supernate and membrane fractions. Different concentrations of each reagent were tried to obtain extensive inhibition of the activities. The esterase activity was inhibited by 85% in both the supernate and the membrane fractions when bacteria were treated with PMSF (1 or 2 mM) for 1 h while no detectable effect was observed on the lipase activity in both the supernate and the membrane fractions. The inhibition of esterase and lipase activities with NBS (5 mM, bacteria treated for 1 h) was approximately 40% and 35% respectively in both supernate and membrane fractions. Increasing the NBS concentration to 10 mM increased the inhibition of esterase to 70% and of lipase to 55%. The esterase and the lipase activities were inhibited by 70% and 40% respectively in both supernate and membrane fractions with THL (0.8 or 1.6 mM, bacteria treated for 2 h). After reaction with 2-hydroxy-5-nitrobenzyl bromide (20 mM, bacteria treated for 1 h), the esterase activity was inhibited by about 80% in supernate and 20% inhibited in membranes, while the lipase activity was inhibited in supernate by 80% and in membranes by 50%. At concentrations less than 20 mM, 2-hydroxy-5-nitrobenzyl bromide only inhibited esterase activity in the supernatant.

3.10 The effect of inhibiting the esterase activity on endogenous wax ester degradation.

The rate of degradation of wax esters was determined in whole bacteria after inhibition of esterase activity by PMSF. There was no significant reduction

in the rate of wax ester degradation of the bacteria (see Figure 3.33), the esterase activity in the supernate and membranes was inhibited by 80%. The lipase activity remained at normal levels in both the supernate and the membrane fractions.

3.11 The effect of inhibiting the esterase and lipase activities on endogenous wax ester degradation.

There was no inhibitor which would inhibit only the lipase activity. The inhibitors NBS, THL and 2-hydroxy-5-nitrobenzyl bromide inhibited both esterase and lipase activities, while the lipase activity was not inhibited by more than 50-55%. Therefore wax ester degradation was determined in bacteria with esterase and lipase activity reduced by treatment with NBS (10 mM), THL (1.6 mM) and 2-hydroxy-5-nitrobenzyl bromide (20 mM). The wax ester content in the control and in the bacteria treated with these inhibitors were determined (see Figure 3.34) and the esterase and lipase activities were also assayed during this experiment. It can be seen that there was no significant effect of inhibition of esterase and lipase by THL on the rate of wax ester degradation, although, the esterase and lipase activities were inhibited by 50% and 30% respectively. When NBS and 2-hydroxy-5-nitrobenzyl bromide were used to inhibit the esterase and lipase activities, the rate of wax ester degradation was slower than in the control. The inhibition of esterase and lipase activities with NBS were 60% and 40% respectively. The esterase activity was inhibited with 2-hydroxy-5-nitrobenzyl bromide by 80% throughout the experiment but the lipase activity was only inhibited by 50% initially and after 3 h this inhibition decreased as the experiment progressed.

Figure 3.33 The effect of inhibiting the esterase activity on the degradation of endogenous wax esters in whole bacteria.

A. calcoaceticus NCIB 8250 was grown in nitrogen limited conditions and harvested as described in Methods 2.2.3.2 and 2.2.7 respectively. Samples of whole bacteria (2.5 g wet weight) were resuspended in 5 ml of 20 mM-potassium phosphate buffer pH 7.5 and treated with PMSF (1 mM) as described in Methods 2.14. The bacteria were washed with 20 mM-potassium phosphate buffer, pH 7.5 by centrifugation and resuspended in 5 ml of the same buffer. A control was prepared in the same way but without PMSF. 1.25 ml of the bacterial suspension was inoculated into 100 ml samples of starvation medium. The bacteria were starved of a carbon and energy source for various times and 25 ml of the bacterial culture removed for the determination of wax esters which were extracted as described in Methods 2.6 and analysed by g.l.c. as described in Methods 2.9. The dry weight of bacteria was determined from the absorbance of the culture using the calibration curve of dry weight against A_{500} . The remaining bacterial culture (75 ml) was used to determine esterase and lipase activities. The 100% value was 28 mg wax (g dry weight of bacteria)⁻¹.

Control •

PMSF treated □

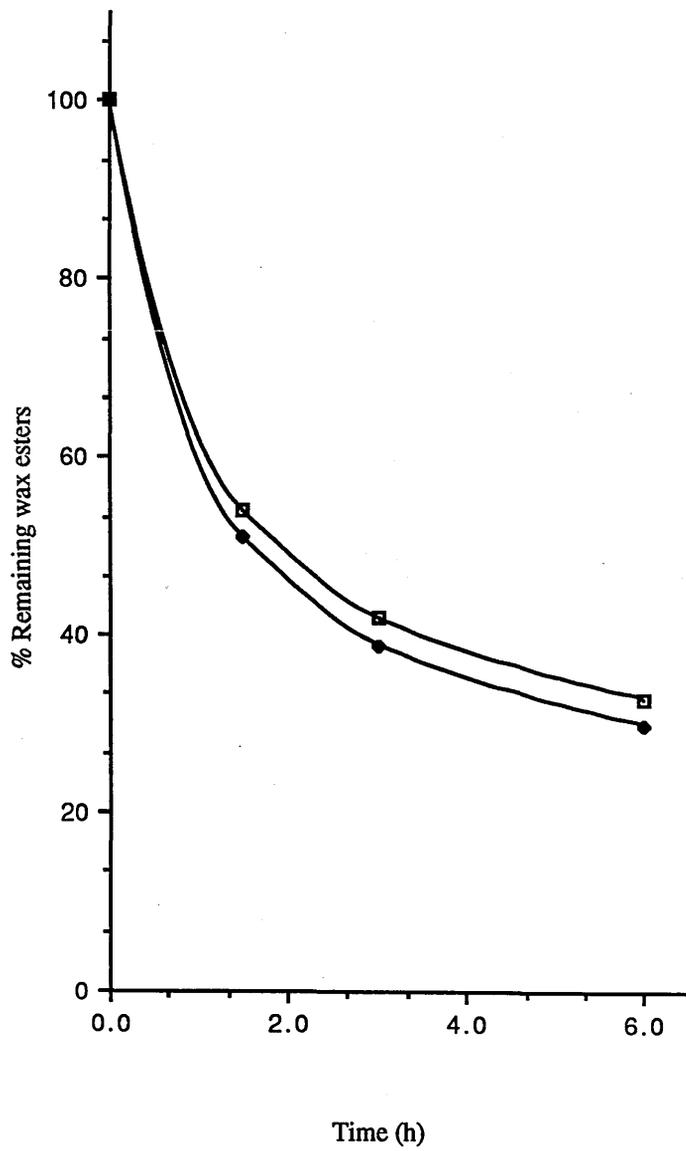


Figure 3.34 The effect of inhibiting the esterase and lipase activities on the degradation of endogenous wax esters in whole bacteria.

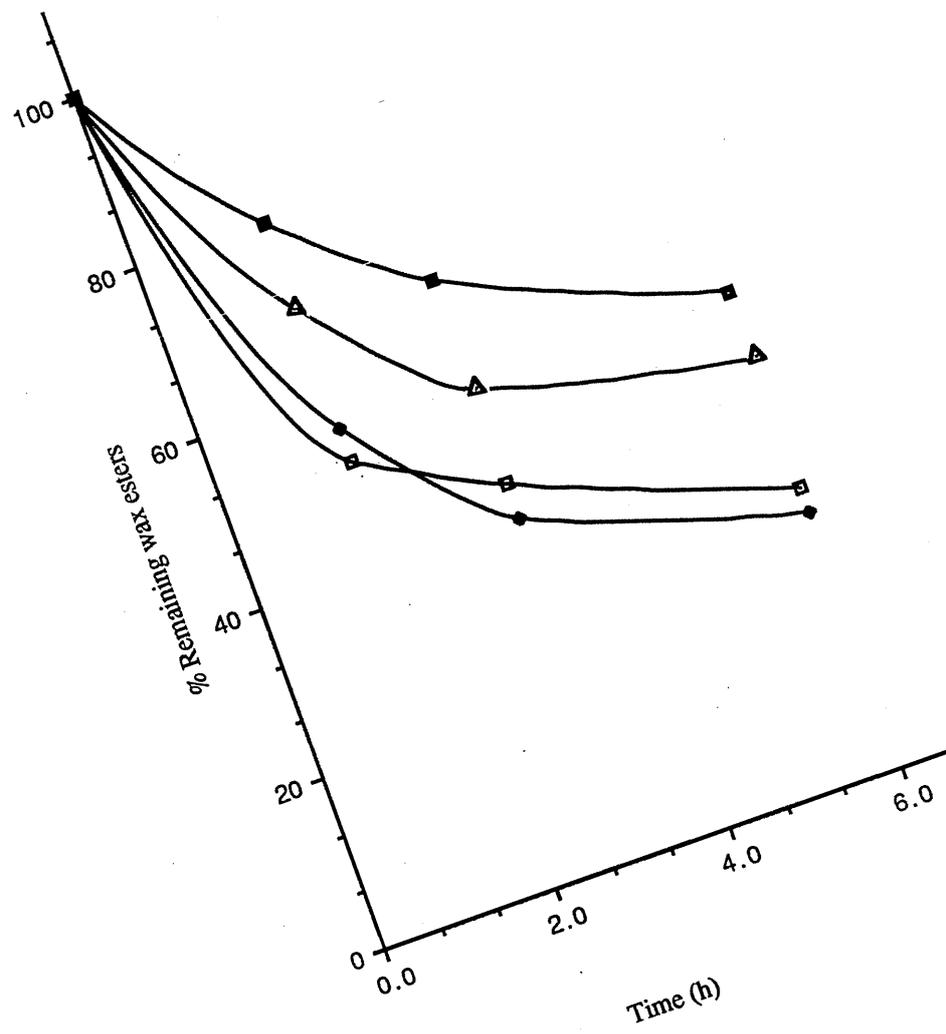
A. calcoaceticus NCIB 8250 was grown in nitrogen limited conditions and harvested as described in Methods 2.2.3.2 and 2.2.7 respectively. Samples of whole bacteria (2.5 g wet weight) were resuspended in 5 ml of 20 mM-potassium phosphate buffer pH 7.5 and treated with NBS (10 mM), 2-hydroxy-5-nitrobenzyl bromide (20 mM) or THL (1.6 mM) as described in Methods 2.20. The bacteria were washed with 20 mM-potassium phosphate buffer pH 7.5 by centrifugation and resuspended in 5 ml of same buffer. A control was prepared in the same way but without inhibitors. 1.25 ml of the bacterial suspension was inoculated into 100 ml samples of starvation medium and the bacteria were starved of a carbon and energy source for various times after which 25 ml of the bacterial culture was removed for the determination of wax esters which were extracted as described in Methods 2.6 and analysed by g.l.c. as described in Methods 2.9. The dry weight of bacteria was determined from the absorbance of the culture using the calibration curve of dry weight against A_{500} . The remaining bacterial culture (75 ml) was used to determine esterase and lipase activities. The 100% value was 28 mg wax (g dry wt bacteria)⁻¹.

Control ●

NBS treated ■

2-hydroxy-5-nitrobenzyl bromide treated ▲

THL treated □



3.12 The effect of PMSF, NBS, sodium azide, 2-hydroxy-5-nitrobenzyl bromide and THL on oxygen uptake of bacteria.

Oxygen uptake with succinate as substrate was determined in the bacteria treated with PMSF (1 mM), NBS (10 mM), sodium azide (20 mM), 2-hydroxy-5-nitrobenzyl bromide (20 mM) and THL (1.6 mM) (see Table 3.13). It is observed that oxygen uptake was totally inhibited in NBS treated bacteria, while there was no effect on oxygen uptake observed in the bacteria treated with PMSF. The oxygen consumption was decreased by about 85% in the bacteria treated with 2-hydroxy-5-nitrobenzyl bromide, 80% in the bacteria treated with sodium azide and 35% in the bacteria treated with THL.

3.13 The effect of anaerobic conditions on endogenous wax ester degradation in whole bacteria and cell free extracts.

The rate of endogenous wax ester degradation was determined in whole bacteria and cell free extracts, incubated without a carbon and energy source under anaerobic conditions. The degradation of wax esters in whole bacteria and cell free extracts starved aerobically and anaerobically is shown in Figures 3.35 and 3.36 respectively. It can be seen that there was no significant decrease in the wax ester content in the whole bacteria or cell free extracts which were starved anaerobically.

3.14 The effect of sodium azide on endogenous wax ester degradation.

The rate of endogenous wax ester degradation was determined in the bacteria treated with sodium azide (see Figure 3.37). It can be seen that there was no significant decrease in the wax ester content of the bacteria treated with sodium azide as compared to untreated bacteria.

3.15 Comparison of free fatty acid content in bacteria starved in aerobic and anaerobic conditions.

The free fatty acids were estimated in bacteria starved in aerobic and anaerobic condition. The major fatty acids present were palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1). It can be seen from Figure 3.38 that the level of free fatty acids decreased in aerobically starved bacteria, while the level of total fatty acids did not decrease significantly in anaerobically starved bacteria.

Table 3.13 Effect of PMSF, NBS, sodium azide, 2-hydroxy-5-nitrobenzyl bromide and THL on oxygen uptake of *A. calcoaceticus* NCIB 8250.

A. calcoaceticus NCIB 8250 was grown in nitrogen limited condition and harvested as described in Methods 2.2.3.2 and 2.2.7 respectively. The bacteria (0.2 g wet weight) were resuspended in 1 ml of 50 mM-potassium phosphate buffer pH 7.5 and treated with PMSF (1 mM), NBS (10 mM), 2-hydroxy-5-nitrobenzyl bromide (20 mM), sodium azide (20 mM) and THL (1.6 mM) for 2 h. The inhibitor was then removed by resuspending and centrifuging the bacteria in 50 mM-potassium phosphate buffer, pH 7.5. 20 μ l of bacterial suspension was used to measure oxygen uptake with succinate as described in Methods 2.18.

Rate of oxygen uptake

$\text{nmol min}^{-1} (\text{mg protein})^{-1}$

control	56.5
Bacteria treated with	
PMSF	55.4
NBS	0
sodium azide	9.3
2-hydroxy-5-nitrobenzyl bromide	11.5
THL	37.5

Figure 3.35 The effect of anaerobic conditions on the degradation of endogenous wax esters in whole bacteria.

A. calcoaceticus NCIB 8250 was grown in nitrogen limited conditions and harvested as described in Methods 2.2.3.2 and 2.2.7 respectively. Whole bacteria (2.5 g wet weight) were resuspended in 5 ml of 20 mM-potassium phosphate buffer, pH 7.0 and the bacterial suspension was inoculated into the starvation medium and incubated without a carbon and energy source under anaerobic conditions for various times as described in as described in Methods 2.16.2 and 25 ml of the bacterial culture were removed for the determination of wax esters which were extracted as described in Methods 2.6 and analysed by g.l.c. as described in Methods 2.9. A control was also prepared as described in Methods 2.16.1. The dry weight of bacteria was determined from the absorbance of the culture using the calibration curve of dry weight against A_{500} . The 100% value was 20 mg wax (g dry wt bacteria)⁻¹.

Control ●

Anaerobic condition. ■

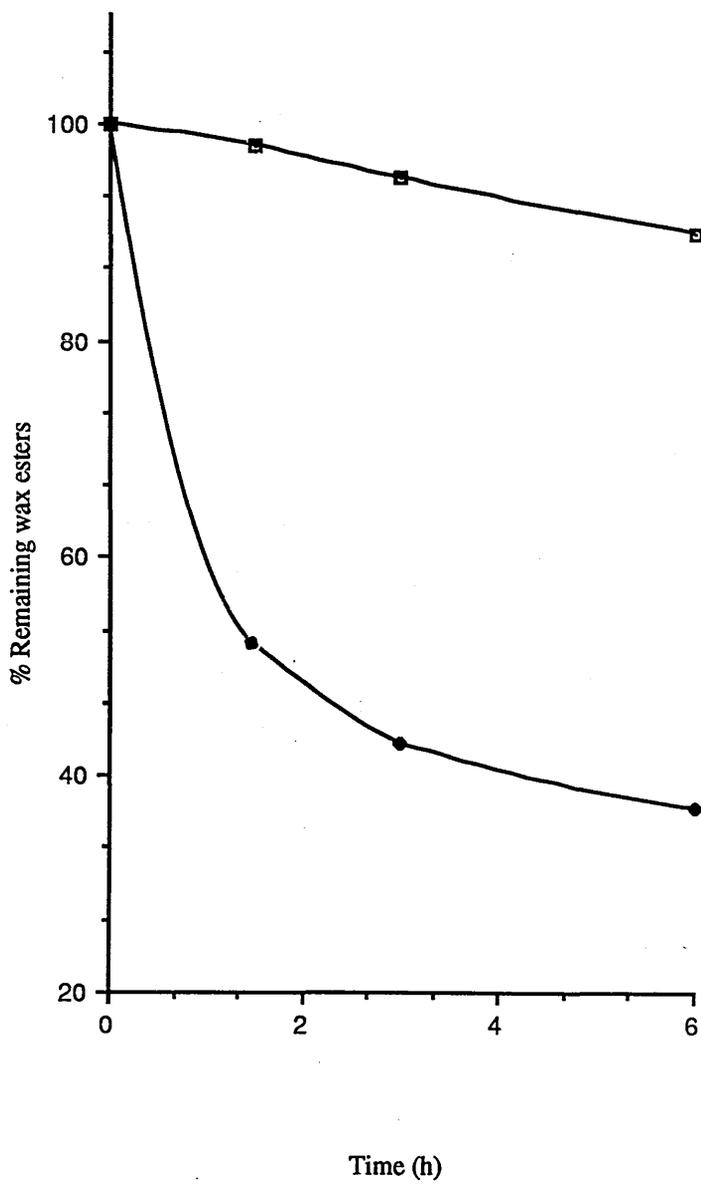


Figure 3.36 The effect of anaerobic conditions on the degradation of endogenous wax esters in cell free extracts.

Cell free extracts were prepared as described in Methods 2.2.8.2. The cell free extracts (25 ml) were starved of carbon and energy source anaerobically as described in Methods 2.16.4. A control was also prepared which was treated aerobically as described in Methods 2.16.3. Then samples (2.5 ml) were used for wax ester analysis. Wax esters were extracted as described in Methods 2.6 and analysed by g.l.c. as described in Methods 2.9.

Cell free extracts starved aerobically □

Cell free extracts starved anaerobically Δ

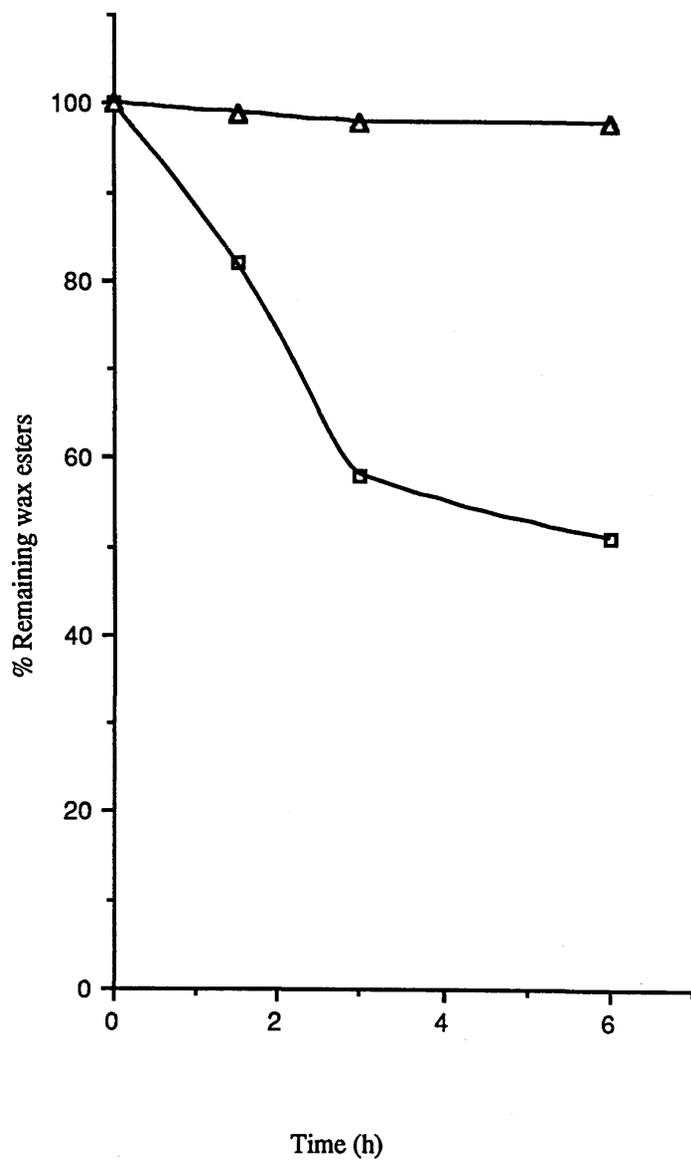


Figure 3.37 The effect of sodium azide on the degradation of endogenous wax esters in whole bacteria.

A. calcoaceticus NCIB 8250 was grown in nitrogen limited conditions and harvested as described in Methods 2.2.3.2 and 2.2.7 respectively. The bacteria (2.5 g wet weight) were resuspended in 5 ml of 20 mM-potassium phosphate buffer, pH 7.0 and 1.25 ml of the bacterial suspension was inoculated into 100 ml samples of starvation medium containing 20 mM sodium azide (see Methods 2.16.5). A control was prepared without sodium azide. The bacteria were starved of carbon and energy source for various times and 25 ml of the bacterial culture were removed for the determination of wax esters which were extracted as described in Methods 2.6 and analysed by g.l.c. as described in Methods 2.9. The dry weight of the bacteria was determined from the absorbance of the culture using the calibration curve of dry weight against A_{500} . The 100% value was $18.5 \text{ mg wax (g dry wt bacteria)}^{-1}$.

Control □

Sodium azide treated ●

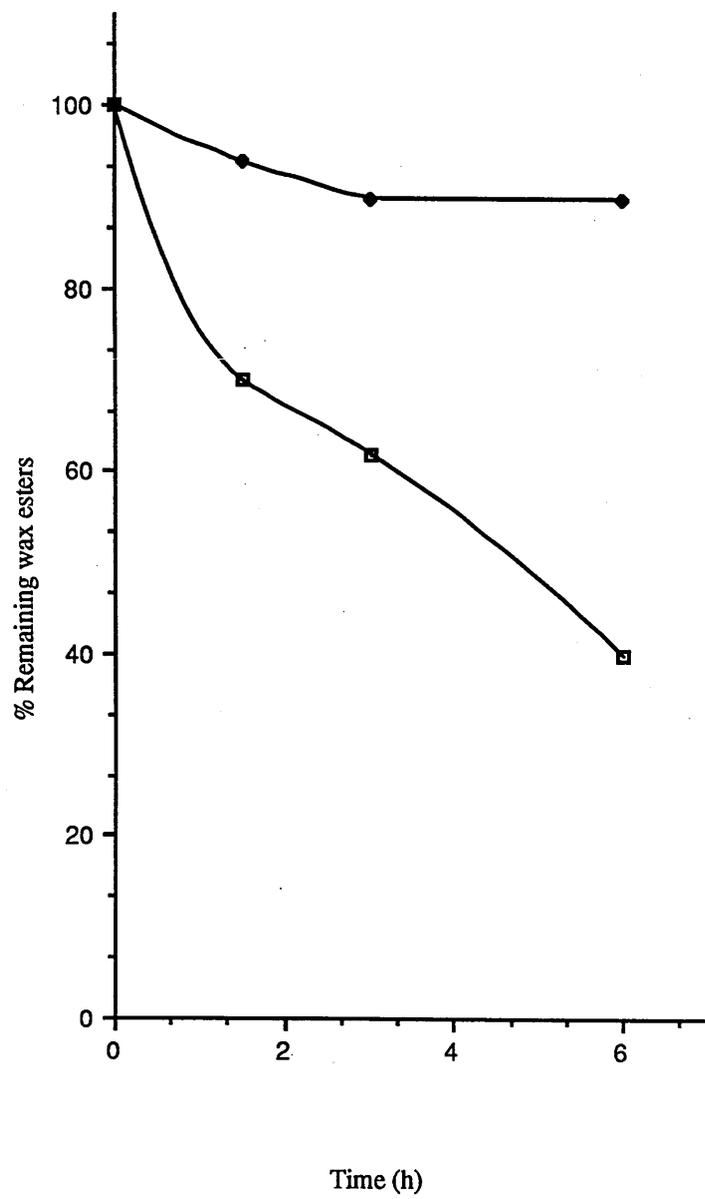
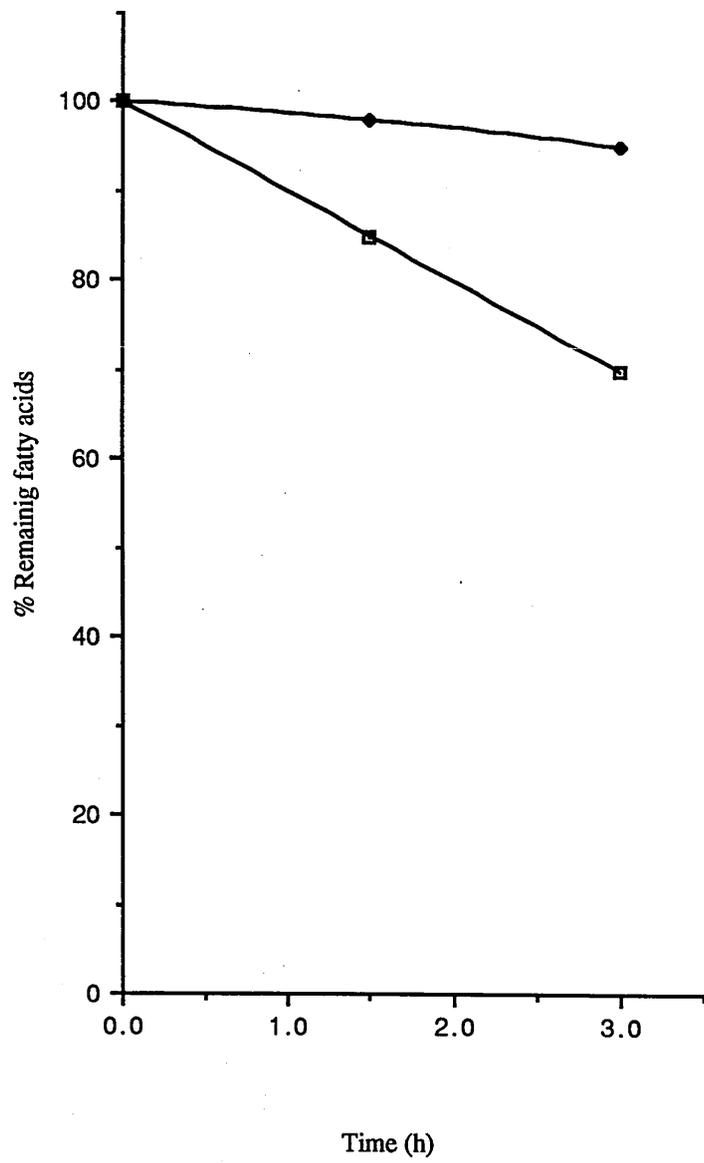


Figure 3.38 Comparison of free fatty acid content of bacteria starved in aerobic and anaerobic conditions.

The lipids were extracted from *A. calcoaceticus* NCIB 8250 degrading wax esters in aerobic and anaerobic conditions as described in the legend to Figure 3.35. Free fatty acids were separated by t.l.c and the methyl esters of the fatty acids were prepared as described in methods 2.7.1 and 2.7.2. The fatty acid methyl esters were analysed using g.l.c. as described in Methods 2.10. The authentic standard fatty acid methyl esters of palmitic acid, palmitoleic acid, stearic acid, oleic acid, and linolenic acid were used to find the retention times by g.l.c. The 100% value was 3.5 mg fatty acids (g dry weight of bacteria)⁻¹.

Aerobic condition □

Anaerobic condition ●



3.16 The effect of wax ester content on viability of *A. calcoaceticus* NCIB 8250.

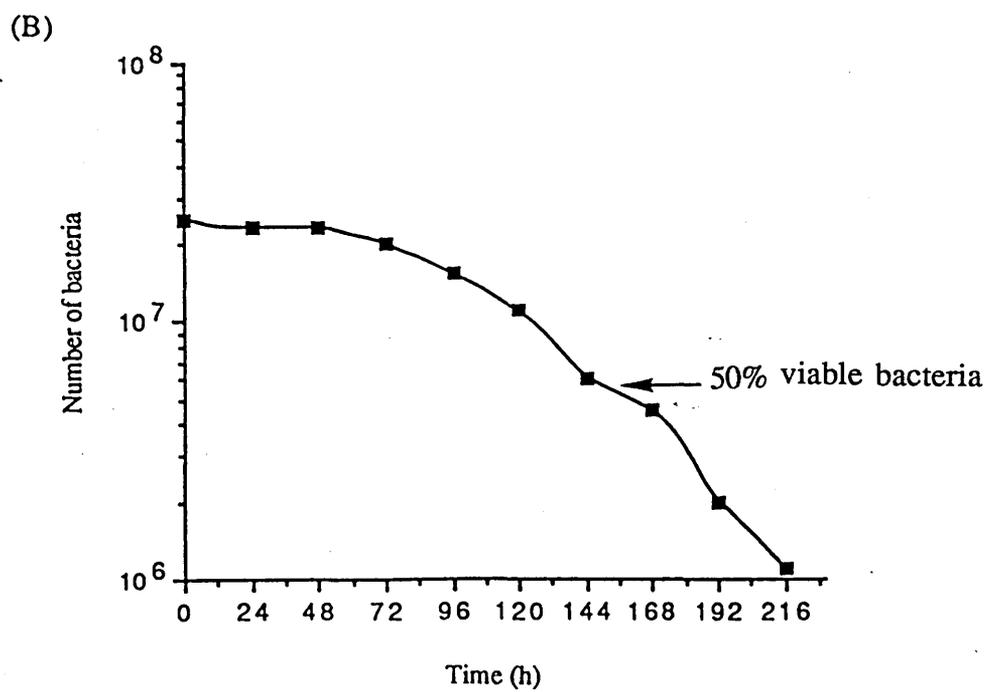
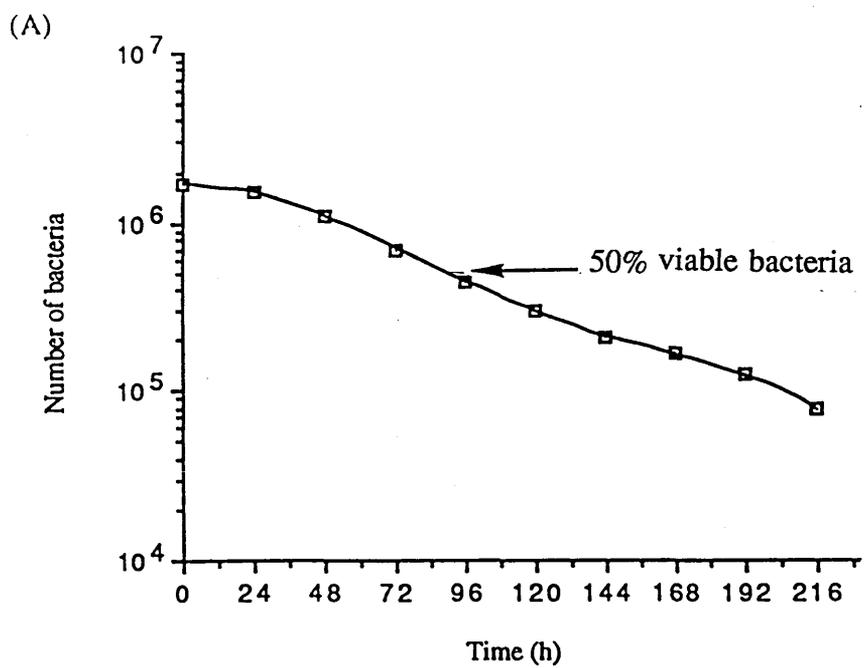
The bacteria grown in nitrogen limited conditions which have a higher content of wax esters and bacteria grown in carbon limited conditions which have a lower content of wax esters were starved of carbon and energy source and number of viable bacteria was determined at various time intervals. It was found that ^{of}the bacteria with a lower content of wax esters, 50% were viable after 84 h (see Figure 3.39 A), while those ^{of}containing an initially higher wax content, 50% were still viable after 154 h (see Figure 3.39 B).

Figure 3.39 The effect of low and high content of wax esters on the viability of *A. calcoaceticus* NCIB 8250.

The bacteria were grown in carbon and nitrogen limited conditions and harvested as described in Methods 2.2.3.2 and 2.2.7 respectively. The bacteria (0.5 g wet weight) were resuspended in 2 ml of 20 mM-potassium phosphate buffer, pH 7.0 and 1 ml of the bacterial suspension was inoculated into 100 ml samples of starvation medium without ammonium sulphate in 250 ml flasks ($A_{500} = 0.6$). The flasks were shaken for 240 h at 30 °C. The samples for bacterial viable counts were taken at the start and then after every 24 h. The samples (0.1 ml) were diluted logarithmically (1:100, 1:1000, 1:10000) into 9.9 ml of distilled water and 0.1 ml of the diluted sample was spreaded over a nutrient agar plate. The bacteria were grown at 30 °C for 36-48 h. The number of colonies were counted and multiplied by the dilution to determine the number of viable bacteria per ml of the original sample. The results are the average of two separate experiments.

Carbon limited bacteria (A)

Nitrogen limited bacteria (B)



Chapter 4

Discussion

4.1 Role of wax esters as a source of energy in

A. *calcoaceticus* NCIB 8250

Plants and animals have evolved a number of systems by which they can survive adverse conditions. In case of carbon and energy starvation, energy reserves play an important role in ensuring their survival e.g. plants accumulate starch and triacylglycerols and animals store compounds like glycogen and triacylglycerols (Stryer, 1988). However, energy reserve compounds are not limited to multicellular organisms, microorganisms also have such systems. Bacteria can store such compounds when there is more carbon and energy available than is required for new cell synthesis and they can utilize these stores during starvation. In microorganisms, glycogen, poly- β -hydroxybutyrate and polyphosphates are the common energy reserve compounds (Dawes and Senior, 1973). For example, glycogen is stored as an energy reserve by *Enterobacter aerogenes*, *Bacillus megaterium*, *Escherichia coli* and *Mycobacterium phlei*; poly- β -hydroxybutyrate is stored by *Alcaligenes eutrophus*, *Azotobacter vinelandii*, *Bacillus megaterium* and *Rhodospirillum rubrum*; polyphosphate is stored by *Enterobacter aerogenes*, *Alcaligenes eutrophus*, *Azotobacter vinelandii*, *E. coli* and *Mycobacterium phlei* (Gottschalk, 1979). Wilkinson (1959) has proposed a number of criteria which can be used to decide whether or not a compound is an energy reserve:

i) the compound should accumulate in conditions when the supply of energy from an external source is more than is required by the cell for growth and maintenance.

ii) the compound should be utilized for either growth and division or for the maintenance of viability and other processes when the supply of energy from an external source is no longer available.

iii) the compound should be degraded to yield utilizable energy for the

processes which are involved in the maintenance of organism's functions in the competition for survival with other organisms which do not possess such compounds.

A critical function of the energy reserve compound is to provide energy e.g. ATP for cell functions. Atkinson (1968) has expressed the energy status of a cell by introducing the concept of adenylate energy charge:

$$\text{Energy charge} = \frac{[\text{ATP}] + 0.5 [\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

The energy charge can have a value ranging from 0 (all AMP) to 1 (all ATP). It was proposed that ATP-generating pathways are inhibited by a high energy charge, while ATP-utilizing pathways are stimulated by the high energy charge. Growing bacteria have energy charges ranging from 0.8 to 0.9 but when the growth has stopped due to a lack of carbon and energy source the energy charge decreases toward 0.5. The viability of *E.coli* is not usually threatened significantly until the energy charge has decreased to less than half of the normal value (Gottschalk, 1979). It was proposed by Atkinson (1968) that the activity of the regulatory enzymes is most responsive to energy charge when it has values between 0.6 and 1.0 and at 0.85 the rate of ATP regeneration is equal to the rate of ATP utilization. Metabolism can be regulated by changes in energy charge and by changes in the concentration of other key metabolites such as NAD, NADPH etc. One of the results of the degradation of energy reserve compounds should be the maintenance of the energy charge at a high value to maintain viability. In an obligate aerobe such as *A. calcoaceticus*, oxygen consumption is a good measure of the generation of energy within the bacterium.

During starvation, the production of new cells ceases while the vital metabolic processes receive energy from the degradation of stored compounds. The endogenous metabolic rate of a microorganism is defined as the total metabolic reactions that occur when it is starved of an external source of carbon and energy. The energy supply for these processes can be maintained by the degradation of RNA, proteins or specific energy reserve compounds if stored by the organism (Dawes, 1986). The endogenous metabolic rate has been reported to be lower in those organisms which can survive longer starvation periods than those which suffer short starvation periods e.g. *E. coli* has to face short intervals of starvation in the gut and has a higher endogenous metabolic rate than the soil bacterium *Arthrobacter crystallopoietes* which can survive long starvation periods (Dawes, 1986). The maintenance energy of a cell, is defined as the energy required for essential chemical and mechanical processes, e.g. the turnover of macromolecules, osmotic regulation, maintenance of intracellular pH and motility, that is everything except those processes required for the production of new cells (Dawes, 1986). The maintenance energy of bacteria in terms of oxygen consumption can be estimated by measuring the relationship between oxygen consumption and specific growth rate during carbon or nitrogen limitation in continuous culture (Hardy and Dawes, 1985).

In *A. calcoaceticus* wax esters accumulate in nitrogen limited conditions when there is sufficient supply of carbon and energy source (Fixter *et al.*, 1986). The accumulated endogenous wax esters are metabolised and used as a carbon and energy source during starvation and have been proposed as an energy reserve compound in *A. calcoaceticus* NCIB 8250 by Fixter *et al.*, (1986). The wax esters have the advantage of being more reduced than glycogen and triacylglycerols and thus produce more ATP than glycogen and triacylglycerols when completely oxidised (see introduction). As a preliminary

part of this study the rate of wax ester degradation in whole bacteria was measured. Wax ester composition during starvation was also determined in terms of the chain length and degree of unsaturation and extends the data given previously (Fixter *et.al.*, 1986). The waxes which were identified in *A. calcoaceticus* NCIB 8250 were C₃₀, C₃₂, C₃₄ and C₃₆, saturated, monounsaturated and diunsaturated similar to those reported by Fixter *et.al.*, (1986). The total amount of wax ester accumulated was about 30 to 35 mg (g dry wt bacteria)⁻¹ (see Table 3.3), which is lower than the amount reported previously by Fixter *et.al.*, (1986). This lower amount of wax esters may be due to more time spent washing the bacteria. The fast initial rate of wax ester degradation means that the bacteria even at low temperatures may have degraded some waxes prior to the collection of a sample for wax ester estimation.

The initial and final rates of degradation of total wax esters observed in whole bacteria were 11.5 mg (g dry wt bacteria)⁻¹ h⁻¹ [22.5 μmol (g dry wt bacteria)⁻¹ h⁻¹] and 0.83 mg (g dry wt bacteria)⁻¹ h⁻¹ [1.63 μmol (g dry wt bacteria)⁻¹ h⁻¹] respectively (see Table 3.1), these rates were in close agreement with the rates (initial and final rates, 10.0 and 1.0 mg (g dry wt bacteria)⁻¹ h⁻¹ respectively) which can be calculated from a similar experiment on the degradation of wax esters reported by Fixter *et.al.*, (1986). The initial rate of degradation of individual wax esters i.e. C_{32:0}, C_{34:0} and C_{36:0} were 6.16, 5.11 and 1.23 μmol (g dry wt bacteria)⁻¹ h⁻¹ respectively (see Table 3.2), which show a decrease in the rate of degradation with an increase in the chain length of the wax ester. However the C_{32:0} wax is present in higher amounts than the other waxes and therefore its higher rate of degradation may just be a reflection of its higher concentration in the bacterium. The amount of unsaturated wax esters was lower than the saturated wax esters and the majority of these disappeared within 4 h.

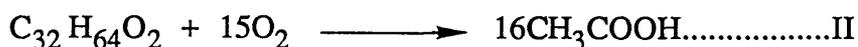
Does the degradation of wax esters provide all or a significant part of the carbon containing substrate required to maintain endogenous respiration or the oxygen consumption required for maintenance energy ? Results showed that C_{32:0} is the major wax degraded and calculations are therefore based on the oxidation of this wax ester.

The stoichiometry of the complete oxidation of C_{32:0} wax is:



It has been shown that in *A. calcoaceticus* NCIB 8250 that about 40% of the wax esters are totally oxidised and that the rest (60%) are converted to water soluble materials (Fixter *et.al.*, 1986). If it is assumed that water soluble materials from wax ester oxidation were mostly acetate residues produced by the oxidation of fatty acids and fatty alcohols, the oxygen consumption during incomplete wax ester oxidation could also be calculated.

The stoichiometry of the oxidation of wax esters to acetate is:



Considering these factors, the consumption of oxygen for the oxidation of wax esters was 27.8 μmol (μmol wax)⁻¹ (40% of the wax esters being oxidised to CO₂ and 60% of the wax esters being converted to acetate). Thus, the rate of oxygen consumption during the initial rapid phase of wax degradation would be 0.63 mmol oxygen (g dry wt bacteria)⁻¹ h⁻¹, and during the slow phase of wax ester degradation the oxygen consumption would be 0.05 mmol oxygen (g dry wt bacteria)⁻¹ h⁻¹ calculated on the basis of oxygen consumption by the wax ester oxidation obtained from equations I and II. The endogenous rate of respiration

of wax ester containing *A. calcoaceticus* NCIB 8250 was constant during the first 4 h of starvation and had a value of about 0.13 mmol oxygen (g dry wt bacteria)⁻¹ h⁻¹. Thus, the initial rate of wax degradation is about six times higher than that required to maintain endogenous respiration. At later stages the rate of wax degradation is 2-3 times lower than the measured rate of endogenous respiration. This could mean that most of the wax ester's carbon is being used for synthesis of other carbon compounds during the initial rapid phase of wax ester degradation (Nagi, 1981). Maintenance energy for *A. calcoaceticus* NCIB 8250 has been estimated to be between 1.5–2.1 mmol oxygen (g dry wt bacteria)⁻¹ h⁻¹ on the basis of continuous culture experiments (Hardy and Dawes, 1985). Thus the initial rate of wax ester degradation would supply approximately 40% of the maintenance energy oxygen consumption. The lower rate of wax degradation could not possibly provide the required maintenance energy.

The large difference found between the maintenance energy calculated from continuous culture experiments and the endogenous rate of respiration is a reflection of many competing factors. In the simplest possible terms, maintenance energy supplies the energy required to ensure that the bacterium could immediately on a change to favourable circumstances start to grow. Endogenous respiration on the other hand is a reflection of the balance between ATP producing and consuming reactions in a bacteri^{um} starved of an exogenous substrate. In these circumstances the bacteri^{um} might well have switched off some of its ATP/energy consuming reactions and the fact that during such starvation there is a slow reduction in the viability of bacterial cultures indicates that endogenous respiration might well supply less energy than that required for maintenance of viability which is in the simplest terms maintenance energy. This would indicate that even the relatively small amount of wax degraded in these

experiments could play an important role in the short term, in providing the substrate required for the production of maintenance energy.

As it was observed that with a high wax content only 50% of the bacteria had lost viability by 154 h while with a low wax content 50% of the bacteria were not viable at 84 h (see Results 3.16), there is evidence for a role of waxes in maintaining viability. As most of the wax ester had disappeared within 6 h of starvation, the role of waxes can not be simply to provide a respiratory substrate during long periods of carbon and energy source starvation, but perhaps they provide energy substrates for short period of metabolic adjustment required to survive during starvation. The different shapes of the survival curves also indicate that the mechanism by which waxes increase the survival of bacteria may be complex.

4.2 β -Oxidation enzymes in *A. calcoaceticus* NCIB 8250.

Succinate is a good carbon source for the study of inducible enzymes in NCIB 8250 as it gives catabolite repression (Hills and Fewson, 1983). The activities of β -oxidation enzymes in *E. coli* K12 grown on oleic acid and *A. calcoaceticus* NCIB 8250 grown on oleic acid and succinate were determined (see Table 3.4). It was found that the activities of all β -oxidation enzymes except β -hydroxyacyl CoA dehydrogenase in *A. calcoaceticus* NCIB 8250 even when it was grown on succinate were higher than that of *E. coli* K 12. But the activities of these enzymes were lower in NCIB 8250 grown on succinate than that of NCIB 8250 grown on oleate. This suggests that the enzymes of fatty acid oxidation system are meso-constitutive in *A. calcoaceticus* NCIB 8250 in contrast to *E. coli* K12 which has an inducible system, the activity of fatty acid oxidation enzymes increasing 200-fold in *E. coli* K12 grown on long chain fatty acids instead of on glucose (Overath *et al.*, 1967) which only appears

when the organism is grown on fatty acids. It is interesting to note that one of the key enzymes, fatty acyl CoA synthetase, has the lowest activity. This enzyme also plays a role in the incorporation of exogenous fatty acids into wax esters (see Introduction sections 1.3.3.2 and 1.3.4). This low activity may indicate that determination of rates of synthesis of wax esters using the incorporation of external fatty acids (Nagi, 1981) may be simply a reflection of changes in the activity of this enzyme.

4.3 Esterases and lipases in *A. calcoaceticus*.

Degradation of waxes is likely to be dependent on an esterase or lipase activity in bacteria and therefore a study of such enzymes was undertaken. Wax esters are found in a variety of marine animals (Bauermeister and Sargent, 1979), plants (Kolattukudy *et al.*, 1976) and in some microorganisms (Weete, 1976; Albro, 1976). The endogenous wax esters in marine animals are often a carbon and energy reserve (Lee *et al.*, 1974). Studies on marine animals e.g. *Euchaeta japonica* (Lee *et al.*, 1974), *Gaussia princeps* (Lee and Barnes, 1975) *Euchaeta norvegica* and *Calanus* species (Sargent and Falk-Petersen, 1988) have shown that the accumulated wax esters were utilized during starvation, but the enzyme or enzymes involved in hydrolysis of endogenous wax esters were not detected. Certain marine zooplankton e.g. calanoid copepods contain 70% of their dry weight as wax esters at certain stages of their life cycle (Lee, 1974) and such zooplankton are common dietary material for many marine animals (Bauermeister and Sargent, 1979). The lipids stored in zooplankton are utilized as an energy source by marine fishes and the digestive enzymes responsible for wax ester hydrolysis have been detected, for instance in *Cyprinus carpio* (Kayama *et al.*, 1979), *Engraulis mordax* (Patton *et al.*, 1975) and *Salmo gairdneri* (Tocher and Sargent, 1984).

The endogenous wax esters stored in plants also serve as a carbon and energy source e.g. seeds of jojoba contain 50 to 60% of their fresh weight as wax esters, which are present in the cotyledons (Yermanos and Duncan, 1976). It has been reported that during germination the accumulated wax esters were metabolised into sugars and used for the growth of seedlings (Huang *et al.*, 1978). A wax ester hydrolase was identified and shown to be associated with the membrane of the wax bodies, the organelle storing wax esters (Huang *et al.*,

1978). Other plants which do not store waxes as energy reserves still contain complex wax esters in their cuticles i.e. cutin and suberin. Some plant pathogens have been reported to grow on cutin as the sole source of carbon by producing extracellular enzymes capable of degrading these waxes (Hankin and Kolattukudy, 1971; Shishiyama *et.al.*, 1970). Plant waxes present in the soil were degraded by a *Pseudomonas* sp and were utilized as the sole source of carbon for growth (Hankin and Kolattukudy, 1969). Two isoenzymes of cutinase and a non-specific esterase (4-nitrophenyl palmitate hydrolase) have been purified from the culture media of *Fusarium solani f. pisi*, a pea plant rotting fungus, grown on cutin (Purdy and Kolattukudy, 1975). The activities of cutinase and the nonspecific esterase could be assayed with 4-nitrophenyl esters of fatty acids from C₂ to C₁₈. Cutinase showed activity with short chain esters, its activity being highest against 4-nitrophenyl acetate, while the nonspecific esterase was active against all esters from C₂ to C₁₈ but its highest activity was with 4-nitrophenyl butyrate. The non-specific esterase showed a greater hydrolytic activity than cutinase against 9-hexadecanoyloxyheptadecane (or 9-heptadecenyl hexadecanoate or dioctyl methyl hexadecanoate), cholesteryl hexadecanoate, hexadecyl hexadecanoate, cyclohexyl hexadecanoate and methyl hexadecanoate. All three enzymes were reported to be serine hydrolases because they were inhibited by di-isopropyl fluorophosphate (Purdy and Kolattukudy, 1975).

Thus, in the utilisation of waxes by *A. calcoaceticus*, an esterase or lipase activity would be expected to play a role. However, very little is known about the degradation of endogenous wax esters by microorganisms. As endogenous wax esters are oxidised by *A. calcoaceticus* NCIB 8250 (Fixter *et.al.*, 1986), it was important to find out whether there are any hydrolytic enzymes with the capability of hydrolysing wax esters in this organism and

whether they play a role in the regulation of wax ester metabolism. The supernates of *A. calcoaceticus* NCIB 8250 were able to hydrolyse nitrophenyl esters of fatty acids of chain length from C₂ to C₁₈ and as well as some aliphatic esters (see Table 3.5). It can be concluded from these results that the hydrolytic activities in *A. calcoaceticus* NCIB 8250 may be due to an esterase or esterases with broad specificity. However, evidence was obtained for two distinct types of esterase activities in *A. calcoaceticus* NCIB 8250 and in some other strains of *Acinetobacter*, one enzyme most being active with short chain nitrophenyl ester (i.e. 4-nitrophenyl acetate) and aliphatic esters, whereas the other esterase activity most being active against long chain nitrophenyl esters (i.e. 4-nitrophenyl hexadecanoate). The evidence for these being at least two separate enzyme activities is based on their sensitivity to different types of inhibitors. The short chain esterase lost most of its activity when treated with serine reactive reagents i.e. PMSF, while the long chain esterase was insensitive to these type of reagents (see Figure 3.3). The fact that two peaks for esterase activity and one for lipase activity were obtained by gel permeation chromatography of the supernate is further evidence for two separate enzyme activities (see Figures 3.13 and 3.23). Furthermore, another indication of the presence of two activities on separate enzyme molecules is that Triton X-100 inhibits the activity with short chain esters whereas Triton X-100 increases activity with long chain esters (see Figures 3.8 and 3.18).

4.4 The esterase and lipase activities in *A. calcoaceticus* NCIB 8250.

Some esterolytic enzymes are dependent on culture conditions (Shabtai and Gutnick, 1985; Breuil and Kushner, 1975a). In *A. calcoaceticus* O16 grown on a complex medium with a high concentration of yeast extract, the

extracellular esterase activity was higher than when this strain was grown on a complex medium containing a lower yeast extract concentration or on a medium based on amino acids. The esterase activity was cell bound in early stages of growth but significant amounts of esterase activity were released into the medium in the late growth phase (Breuil and Kushner, 1975a). Similarly, the esterase activity of *A. calcoaceticus* RAG-1 grown on ethanol-mineral salts medium, was initially cell bound but later was released into the culture medium along with an emulsan (Shabtai and Gutnick, 1985). Unlike these strains of *Acinetobacter*, the esterase activity of *A. calcoaceticus* NCIB 8250 was not released into the culture medium at any stage.

The total esterase activity assayed in whole bacteria was not found to be significantly different in any of the culture conditions employed in this study (see Table 3.7). However the results show that the distribution of esterase activities between the supernate and membranes of *A. calcoaceticus* NCIB 8250 was dependant on culture conditions. The total supernatant esterase activity was higher in exponentially growing and nitrogen limited cultures than that of carbon limited cultures, whereas the total membrane esterase activity was higher in carbon limited cultures than exponentially growing and nitrogen limited cultures. It has been shown that *A. calcoaceticus* NCIB 8250 grown in carbon limited cultures had a low rate of wax ester synthesis but a high rate of degradation due to the lack of a carbon and energy source (Nagi, 1981). The high levels of esterase activities in membranes of carbon limited bacteria may be due to the lack of a carbon and energy source causing transfer of enzyme from supernate to membranes. This kind of phenomenon is seen with other lipid metabolising enzymes e.g. the phosphatidate phosphohydrolase in mammalian liver (Brindley, 1984). The studies on the distribution of the esterase activity within the membrane system showed some activity associated with both the inner and outer

membranes. Although the distribution of esterase activity depends on the culture conditions of the bacteria, the esterase was predominantly associated with the inner cytoplasmic membrane. Thus, the location of the esterase in the bacterium is such that it could degrade endogenous wax esters.

A very high molecular mass for the esterase from *A. calcoaceticus* RAG-1 was reported (Shabtai and Gutnick, 1985) and may be due to the binding of esterase with secreted emulsan, which is a lipid-saccharide complex. Recently the esterase gene from *A. calcoaceticus* RAG-1 has been cloned into *E. coli* (Reddy *et al.*, 1989). The translated product from the plasmid showed that the esterase was a peptide of M_r 32,500, which is presumed to be the subunit of the esterase activity complexed with emulsan (Shabtai and Gutnick, 1985). When the molecular mass of the esterase activity from the same strain was determined on SDS-PAGE gel, it showed two components of molecular mass of about 40,000 and 43,000 (see Figure 3.31). Perhaps even on SDS-PAGE gel there may be other material bound to the esterase "subunits". This could be traces of the emulsan identified by Shabtai and Gutnick (1985) in the native form. Similarly the molecular mass by gel permeation chromatography of the supernate of *A. calcoaceticus* NCIB 8250 showed esterase activity in a high and a low molecular mass forms (see Results 3.5.1.10). It may be possible that the high molecular mass of esterase activity in *A. calcoaceticus* NCIB 8250 is due to association with lipophilic saccharides or lipopolysaccharide or undefined lipids in a high molecular mass complex as is the case with strain RAG-1.

The lipase activity is, like the esterase activity, distributed between membrane and soluble forms in *A. calcoaceticus* NCIB 8250 (see Table 3.8). There was no significant difference observed in the total lipase activity in any of the culture conditions, however, the total supernate lipase activity was higher in

nitrogen limited bacteria than in exponentially growing and carbon limited bacteria. The total membrane bound lipase activity was also higher in the membranes of exponentially growing and carbon limited bacteria than nitrogen limited bacteria. The higher level of total lipase activity in the membranes of exponentially growing and carbon limited bacteria than in the nitrogen limited bacteria may be a reflection of a higher rate of degradation of wax esters in these conditions as reported by Nagi, (1981). The distribution of lipase activity within the membrane system was similar to that of the esterase activity in nitrogen limited bacteria i.e. higher in the inner membranes than outer membranes, but equal in both inner and outer membranes in carbon limited bacteria. The lipase activity present in the outer membrane is unlikely to be involved in wax ester degradation as the energy reserve waxes are thought to be located in intracellular inclusions (Fixter, 1976).

Very high molecular mass has also been reported for lipases from *A. calcoaceticus* O16 (Breuil and Kushner, 1975b), *Pseudomonas aeruginosa* (Stuer *et al.*, 1986) and *Staphylococcus aureus* (Jurgens and Huser, 1981). The activities of esterase, lipase and a wax ester hydrolase of *Cyprinus carpio* hepatopancreas and rat liver were eluted near the void volume on a Sephadex G-200 column (exclusion limit of Sephadex G-200 $M_r = 800,000$) (Kayama *et al.*, 1979). Similarly, in *A. calcoaceticus* NCIB 8250 the lipase activity was eluted as a protein of high molecular mass by gel permeation chromatography (see Results 3.5.2.10). It is possible that the very high molecular mass of the lipase activity is due, like that of the esterase activity to its association with lipophilic saccharides or lipopolysaccharide or undefined lipids in a high molecular mass complex.

4.4.1 Esterase and lipase activities in other strains of *A. calcoaceticus*.

Esterase activities were also determined in strains of *Acinetobacter* other than NCIB 8250 (see Tables 3.6, 3.11a and b). Although esterase activity was found in all strains tested i.e. ATCC 23055 the neotype strain, EBF 65/65, and HO1-N, it was lower than in strain NCIB 8250. The esterase activity from other strains was also sensitive to serine reactive reagents, showing that esterases of *Acinetobacter* sp may have some similarities. These strains accumulate wax esters and these activities may be associated with the degradation of wax esters.

Geigert *et al.*, (1984) have isolated a series of mutants of *A. calcoaceticus* HO1-N, e.g. CP 179-22 which when grown on n-hexadecane can synthesize thirty times more wax than the parent strain. The basis of the selection of the mutants was that they grew on sodium acetate rather on cetyl palmitate as the carbon source. It was proposed that the reason for such mutant phenotypes was that they have low levels of activities of degradative enzymes for wax esters as they do not grow on wax esters. To further explore the role of esterase or lipase activities in wax ester degradation in *Acinetobacter* strains it was interesting to determine whether there were significant changes in the esterase and lipase activities of the parent and mutant strains. There was no great difference observed in the esterase activities of the mutants and the parent strain when they were grown on complex medium (see Table 3.11a). The results also showed that esterase activities in both the mutant and the parent strain were lower than those of NCIB 8250 grown on complex medium (see Table 3.11a). The lipase activities were about same in all mutants and the parent strain when the strains were grown on complex medium (see Table 3.11a). The lipase activities of these strains were less than that of strain NCIB 8250 grown on on complex

medium (see Table 3.11a). More experiments were carried out on mutant CP179-22 grown in acetate-minimal salts medium to see if the culture conditions effected the esterase activity because when *A. calcoaceticus* NCIB 8250 and RAG-1 were grown on complex medium they showed about 50% of the esterase activity present when they were grown on succinate-minimal salts medium (see Table 3.6). However, CP179-22 did not show an increase in activity when grown on acetate-salts medium (see Table 3.11b). These results show that there are factors other than esterase or lipase activities, which change the content or ability to accumulate wax esters in these mutants.

4.4.2 The effect of inhibitors on esterase and lipase activities.

An active site serine is important in many esterases and the mechanism of these enzymes is similar to serine proteases where aspartate and histidine together with the active site serine form a catalytic triad. Brenner (1988) proposed that the serine protease and serine esterase enzyme active sites share the consensus sequence "G X S X G". A triad of aspartate-histidine-serine is also found in the lipase from *Mucor miehei* and the separation of these amino acids are 59 residues from serine to aspartate and 54 residues from aspartate to histidine (Brady *et al.*, 1990). In human pancreatic lipase (Winkler, 1990) the separation of these amino acids are serine-aspartate 24 residues and aspartate-histidine 87 residues. A similar consensus sequence for the active site serine can be seen in the esterase of *A. calcoaceticus* RAG-1, glycine (129), aspartate (130), serine (131), cysteine (132) and glycine (133) (Reddy *et al.*, 1989). The serine present in the consensus sequence does not have aspartate and histidine residues at either positions 24 or 87 residues respectively from serine as found in human pancreatic lipase (Brady *et al.*, 1990), but an aspartate is present 25

residues from serine and a histidine is found 92 residues from this aspartate. If the sequence of *A. calcoaceticus* RAG-1 is compared with that of the lipase from *Mucor miehei* (Winkler *et al.*, 1990), there is no aspartate 59 residues from serine but there is an aspartate 66 residues from serine and there is a histidine 51 residues from aspartate. Thus, there is the possibility that the esterase of RAG-1 uses the same type of mechanism as other serine esterases. Based on these reasons it is not surprising that the esterase activity of NCIB 8250 is sensitive to serine reactive agents and it would be expected that aspartate and histidine residues may also play a role in the esterase of NCIB 8250.

The esterase and lipase activities of *A. calcoaceticus* NCIB 8250 have been differentiated on the basis of the presence of a reactive serine residue at the active site of the esterase (see Results 3.5.1.6 and 3.5.2.6). The inhibition of esterase activity with bis-*p*-nitrophenyl phosphate and eserine (at high concentrations) further confirms the presence of a serine at the active site. The sulphhydryl group of cysteine ionizes to produce a thiolate ion which is highly reactive with alkyl halides, such as iodoacetate and iodoacetamide (Creighton, 1983). Thiols also form complexes with metal ions and the most stable complexes being with divalent mercury (Creighton, 1983). A partial inhibition of esterase and lipase activities with alkyl halides and a complete inhibition of the esterase with mercuric chloride was found in *A. calcoaceticus* NCIB 8250 (see Results 3.5.1.7 and 3.5.2.7). The indole ring of tryptophan can be irreversibly oxidised by *N*-bromosuccinamide, however, NBS is not very specific because it can also oxidise methionine and cysteine residues. A more specific reagent is 2-hydroxy-5-nitrobenzyl bromide (Koshland's reagent) which reacts only with tryptophan residues in neutral conditions (Horton *et al.*, 1965). Both esterase and lipase activities were partially inhibited by NBS and 2-hydroxy-5-nitrobenzyl bromide (see Results 3.5.1.8 and 3.5.2.8). The inhibition of esterase and lipase activities with sulphhydryl reagents and tryptophan reactive

reagents indicates that cysteine and tryptophan residues are present near the active site or that their modification affects catalytic activity indirectly. Thus addition of a bulky group or metal ion at the cysteine (132) in the serine consensus sequence could be expected to effect the activity. Such cysteine or tryptophan residues could be expected in the esterase of *A. calcoaceticus* NCIB 8250. Tetrahydrolipstatin, a derivative of lipstatin, is a specific covalent inhibitor of lipase and inhibits pancreatic lipase, human gastric lipase, carboxyl ester lipase (cholesterol esterase) and the bile salt stimulated lipase of milk (Borgstrom, 1988). The esterase and lipase activities of *A. calcoaceticus* NCIB 8250 were inhibited with THL by about 80% and 40% respectively (see Results 3.5.1.9 and 3.5.2.9) but at very much higher concentrations of THL than those used by Hadvary *et.al.*, (1988). The lipase of *Staphylococcus aureus* has been reported to be insensitive to THL at concentrations of THL which were lower than used for the lipase of *A. calcoaceticus* NCIB 8250 (Borgstrom, 1988).

4.4.3 Multiple forms of esterase activities in *A. calcoaceticus.*

Esterase activities from various strains of *Salmonella*, *Proteus* and *Providencia* have been resolved into distinct bands by electrophoresis on native gels and then located by using 1-naphthyl acetate and 2-naphthyl acetate as substrates (Goulet, 1975, 1977). The relative molecular mass of these esterases in native form are in the range of M_r 15,000 to 90,000. The esterase activity of *A. calcoaceticus* NCIB 8250 did not separate into distinct bands on non-denaturing gels (see Figure 3.26) and this is probably a reflection of the fact that the esterase is present as a high molecular mass complex, as was shown by its behaviour on gel permeation chromatography (see Results 3.5.1.10).

It is known that native polyacrylamide gel electrophoresis is not the only technique to resolve enzyme activity and individual enzymes have been detected after electrophoresis in denaturing conditions with sodium dodecyl sulphate. The limitations upon detecting enzymes after SDS-PAGE are related to the renaturation of the protein after electrophoresis. In particular, the reformation of correct inter- and intra-polypeptide chain disulphide bonds is critical and this limits the utility of this technique. The activity of a major endonuclease, a hydrophobic membrane protein and a major exonuclease from *Diplococcus pneumoniae* has been detected after electrophoresis of crude bacterial extracts on SDS-PAGE gels. Furthermore, multiple bands of DNases and RNases were revealed by electrophoresis of the crude extracts from *E.coli*, *Haemophilus influenzae*, *Bacillus subtilis*, and HeLa cells on SDS-PAGE gels (Rosenthal and Lacks, 1977). After SDS-PAGE and removal of SDS, it was possible to detect activity in samples of amylases, proteases, glucose-6-phosphate dehydrogenase, bovine heart lactate dehydrogenase and equine liver alcohol dehydrogenase, although in the case of the latter two enzymes only a small amount of activity could be detected (Lacks and Springhorn, 1980). Among the proteases only thermolysin, which does not contain any disulphide bonds, could be detected after SDS-PAGE denaturation, whereas, chymotrypsin, pronase and trypsin showed activity only when their disulphide bonds were not reduced with β -mercaptoethanol before SDS-PAGE. Anfinsen and White (1961) have proposed from their work on RNase that a polypeptide chain can carry within itself the ability to reform as an active enzyme even when disulphide bridges in the native protein have been broken. This may be the reason for the renaturation of denatured disulphide-bonded enzymes (Lacks and Springhorn, 1980). It was proposed by Lacks and Springhorn (1980) that proteases, particularly trypsin (a disulphide-bonded enzyme) when denatured in the presence of β -mercaptoethanol may not have ability to reform like RNases when completely

unfolded. In the case of oligomeric dehydrogenases whose activity appears to be only partially restored, it has been proposed that the reassociation of their identical subunits into an active oligomer is retarded by the presence of the gel matrix (Lacks and Springhorn, 1980). The search for a better resolution of the esterase activity of *A. calcoaceticus* NCIB 8250 by electrophoresis led to the use of SDS-PAGE, which appeared to be the effective way to resolve esterase activity on gel. Esterase activities in distinct bands could be obtained after SDS-PAGE provided that the SDS was removed by washing (see Figure 3.27). The results obtained from test tube assays of esterase activity with 0.05% (w/v) SDS showed inhibition by about 25% (see Figure 3.8). Therefore, 2% (w/v) SDS in the protein sample should inhibit the esterase activity and this inhibitory effect of high concentration of SDS is the explanation of the absence of activity when the gel was washed for less than 2 h. Thus, it can be concluded from the behaviour of the esterase activities on SDS-PAGE gel that:

I) the enzymes may be present in an aggregated form with other protein molecules *in vivo*.

or

II) the enzymes are associated with a lipophilic saccharide or undefined lipids in a complex, which are removed using SDS.

or

III) the enzymes may be either very large oligomers having multiple identical subunits or if the oligomers are composed of different subunits, each subunit has activity when separated.

Most probably the native form of the esterase activity in *A. calcoaceticus* NCIB 8250 is a complex of the esterase activities separated by SDS-PAGE and other components such as lipids but it is possible that the native esterase is a hetero-oligomer composed of the different forms of esterase separated by SDS-PAGE gel.

Different strains of *Acinetobacter* also showed multiple forms of esterase activity on SDS-PAGE (see Figure 3.31). Esterase activity from *A. calcoaceticus* RAG-1 was separated into two bands of M_r about 40,000 and 43,000. The molecular mass of esterase activity on SDS-PAGE is different to the molecular mass (32,500) of the peptide reported after sequencing of gene (Reddy *et al.*, 1989). This raises the possibility that SDS treatment does not remove all the lipid present in the native complex. Phospholipid e.g. dipalmitoyl phosphatidyl ethanolamine has M_r of about 700 and lipopolysaccharide with one O chain substitution M_r about 2,700 (Hammond *et al.*, 1984). Thus the presence of about 4 moles of phospholipid or about 1 mole of lipopolysaccharide per mole of esterase component would increase M_r by about 3,000 and this is a possible explanation of the differences in molecular mass. Absence of components of M_r 38,000 and 43,000 in ATCC 23055 and presence of two extra components of M_r 20,000 and 50,000 in HO1-N indicates that there may be a large diversity in the esterases of *Acinetobacter* strains.

4.4.4 Substrate specificity and inhibitor sensitivity of esterase activities separated by SDS-PAGE.

When the esterase activities from *E. coli*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* were resolved into bands of different molecular mass by electrophoresis on an acrylamide-agarose gel, the bands showed different specificity with various synthetic substrates e.g. 1-naphthyl acetate and 2-naphthyl acetate and also differential sensitivity to inhibitors (Goulet, 1973; Goulet and Picard, 1984). In *A. calcoaceticus* NCIB 8250, esterases separated on SDS-PAGE also showed different activities with 1-naphthyl acetate, 2-naphthyl acetate (see Figures 3.27) and ethyl butyrate esterase activity was most obvious in the component of M_r 43,000 (see Figures 3.30). Different activities

with these substrates could also be due to varying amounts of lipid bound to protein. However it is not easy to see how the presence of differing amounts of lipid bound to protein should change the esterase activities in the way observed

Attempts to see whether or not the esterase activities separated by SDS PAGE have different sensitivities to inhibitors which could indicate that each component constituted a different enzyme activity gave in some cases confusing results. With PMSF which is an effective inhibitor of the total esterase activity in supernates when assayed with 4-nitrophenyl acetate, the major effect on activities in the gels was to reduce the staining of the major esterase components with both 1-naphthyl acetate and 2-naphthyl acetate as substrate (see Figure 3.32 A and B). Different effects were obtained with the two other serine reactive inhibitors used, bis-*p*-nitrophenyl phosphate and eserine (see Figure 3.32 A and B). With bis-*p*-nitrophenyl phosphate only the major band showed a reduction in activity with both the substrates, but other bands may be more intensely stained. Eserine also reduced the activity of the major esterase activity component with both the substrates, but with 1-naphthyl acetate it also reduced the activity of other two components. These results should be interpreted with caution because of the apparent stimulation of the activity of certain bands by one inhibitor. Overall this indicates that the M_r 43,000 component is more sensitive to these inhibitors than the lower molecular mass components and with 2-naphthyl acetate as substrate the M_r 38,000 is relatively resistant to these inhibitors. The M_r 40,000 component showed the most bizarre response in that its activity was apparently increased by bis-*p*-nitrophenyl phosphate. Overall these results show that there are differences in the sensitivity to the inhibitors of the esterase components separated by SDS-PAGE. This together with the differences in substrate specificity of the electrophoretic forms of the esterase indicates that these electrophoretic forms are probably unrelated esterase activities rather than a family of esterase activities derived by proteolytic cleavage of the high molecular

mass form or the same esterase species with different amounts of lipid associated to give a series of components on SDS-PAGE.

The inability to demonstrate lipase activity with naphthyl hexadecanoate either on non-denaturing or denaturing gels at concentrations of acrylamide from 3.5 to 12.5% may be due to a lower activity of lipase than esterase. Although the lipase activity was less sensitive to SDS than that of the esterase, it has about ten times lower activity than that of the esterase in supernates and this could be the reason why it could not be detected on the gel. Another possible reason for not obtaining lipase activity on such a gel may be because of the large particle size of the substrate emulsion (substrate was emulsified using either Triton X-100 or deoxycholic acid) which would prevent the substrate entering the gel.

4.4.5 The effect of culture conditions on the esterase activities separable by SDS-PAGE.

SDS-PAGE electrophoresis of supernates of *A. calcoaceticus* NCIB 8250 grown in different culture conditions showed differences in the activity of the separated esterase activities stained with 1-naphthyl acetate or 2-naphthyl acetate (see Figures 3.29). The membrane fraction also showed a similar esterase banding pattern to that of supernates under the same culture conditions. A major difference was observed between the bands of esterase activities from carbon limited bacteria and those from exponentially growing and nitrogen limited bacteria (see Table 3.12). The activity of the highest molecular mass esterase band was decreased in carbon limited bacteria compared to this band in exponentially growing bacteria and nitrogen limited bacteria. On the other hand the activity of the lowest molecular mass band was increased in this condition compared to the other two conditions. High rates of wax esters degradation in carbon limited bacteria may be a reflection of a change of high molecular mass

subunit to low subunit molecular mass esterase activity. Experiments were carried out to determine whether there are any effects on the intensities of the separated bands if the bacteria grown in nitrogen limited conditions were transferred to carbon limited conditions or vice versa. No significant difference in the intensities of bands was shown on changing the cultures from one condition to the other condition. This means the separated bands of esterase activity were not likely to be the products of proteolytic cleavage and possibly are independently synthesised enzyme molecules.

4.5 The role of esterase and lipase activity in wax ester degradation.

In order to look at the role of esterase and lipase activities in wax ester catabolism, the work was split into two stages. In the first stage, an attempt was made to find individual inhibitors for both the esterase and the lipase which gave extensive inhibition in whole bacteria, so that their effect on the wax degradation could be studied easily. The results showed that in whole bacteria the inhibitors tested like PMSF inhibited the esterase activity almost completely while none of the inhibitors tested was effective against only the lipase activity because compounds which inhibited the lipase activity also inhibited the esterase activity to about the same extent. The efforts to find inhibitors which would completely inhibit only the lipase activity remained unfruitful. Therefore, those inhibitors which gave severe inhibition of both activities or just the esterase were selected for further study. In the second stage, the role of esterase and lipase activities in wax ester degradation was investigated by determining the effects of inhibitors of esterase and lipase activity on wax ester degradation. The results obtained from these studies in whole bacteria showed that there were only two ways to study

the effect of inhibiting the esterase and lipase activities on wax ester degradation. Firstly, the effect of inhibition of only esterase activity by using PMSF, and secondly the effect of inhibition of both esterase and lipase activities by using NBS, 2-hydroxy-5-nitrobenzyl bromide and THL, on wax degradation could be investigated. Although the esterase and lipase activity determinations show that esterase activity was inhibited by 80% with PMSF while the lipase activity remained essentially uninhibited during the course of experiment, wax ester degradation was not reduced (see Figures 3.33). These results show that the esterase activities are not involved in wax ester degradation. On the other hand partial inhibition of esterase and lipase activities reduced the wax ester degradation to some extent (see Figures 3.34). Due to the reduction in wax ester degradation with NBS and 2-hydroxy-5-nitrobenzyl bromide, it would appear that the lipase type activity is more important in wax degradation because selective inhibition of the esterase by PMSF does not reduce wax degradation .

4.5.1 Complications in using inhibitors to study the role of esterase and lipase activities in wax ester degradation.

There are, however, considerable difficulties in interpreting the results obtained using these inhibitors. All the inhibitors but especially NBS are reactive compounds and could have other effects on the bacterium's biochemistry. Therefore the effect of these inhibitors on respiration of the bacteria was determined by measuring the rate of oxygen consumption in the presence of a carbon source. The results demonstrated that NBS totally inhibits the consumption of oxygen, while 2-hydroxy-5-nitrobenzyl bromide inhibits the consumption of oxygen by the bacteria by more than 50% (see Table 3.13). There was no significant degradation of wax esters in anaerobic conditions (see

Figure 3.35) which shows that the oxidative metabolism must be maintained in order for wax esters to be degraded. Similarly, the inhibition of wax ester degradation due to inhibition of respiration by sodium azide confirms that inhibition of respiration prevents the degradation of wax esters (see Figure 3.37). The results showed that there is a requirement for an operating respiratory chain during wax ester degradation and the reduction in the degradation of wax esters caused by inhibitors like NBS and 2-hydroxy-5-nitrobenzyl bromide may not be due to esterase or lipase inhibition but is more likely to be due to these inhibitor's effect on respiration. There was however another possible way to solve this problem, as the experiments with cell free extracts showed that it is possible to follow up the wax ester degradation in cell free extracts during carbon and energy starvation (see Figure 3.1). It was thought that the products of the action of esterolytic enzymes i.e. fatty acids and fatty alcohols could be inhibiting the hydrolytic enzymes in anaerobic conditions in the closed environment of the bacterium and this effect could be diluted out by using cell free extracts. However the degradation of wax esters in cell free extracts is also inhibited by anaerobiosis making this approach invalid (see Figure 3.36). The results showed that fatty acids were removed at a very much slower rate in anaerobic conditions than in aerobic conditions (see Figure 3.38). This indicates that the products of wax ester hydrolysis are not being consumed. Fatty alcohols have been reported to inhibit the mammalian triacylglycerol lipase (Mattson *et al.*, 1970). A similar effect of fatty alcohols on the fish lipase was proposed (Bauermeister and Sargent, 1979). Thus it may be possible that the fatty acids and fatty alcohols are inhibiting the lipase activity under anaerobic conditions in *A. calcoaceticus* NCIB 8250.

4.6 General conclusions and future work.

Endogenous respiration during wax degradation and viability determination of the bacteria containing a low or high content of wax ester after carbon and energy starvation indicate that the wax esters could have some role in maintaining the viability and that wax ester fulfill a basic criteria for energy reserves in *A. calcoaceticus* NCIB 8250. The activities of β -oxidation enzymes in *A. calcoaceticus* NCIB 8250 are found at sufficiently high levels even in the uninduced state to ensure that the products of wax ester hydrolysis can be oxidised via β -oxidation system to produce energy.

Esterase and lipase activities were found in *A. calcoaceticus* NCIB 8250 which can be differentiated using PMSF. Multiple forms of the esterase activities can be detected on SDS-PAGE gels. In different growth conditions there are changes in the relative activities of the esterase components separated by SDS-PAGE. Although the specific activity of the esterase and lipase do not change in different culture conditions, the distribution of esterase and lipase activities found in *A. calcoaceticus* NCIB 8250 were dependent on culture conditions i.e. exponentially growing, carbon or nitrogen limited conditions. Furthermore a shift of esterase or lipase activity from supernate to membranes was found under these conditions. The turnover of wax esters during such conditions has already been reported to be different (Nagi, 1981). The accumulation of a high content of wax esters in the mutants derived from *A. calcoaceticus* HO1-N was suggested to be due to low levels of wax hydrolysing enzymes (Geigert *et al.*, 1984), but no such differences were observed in the activities of the esterase and lipase in these mutants compared to the parent strain indicating that the extra accumulation of waxes in the mutants compared to the parent strain is not due to loss of esterase or lipase activities.

Although there is indirect evidence for the involvement in wax ester degradation of such esterolytic activities in *A. calcoaceticus* NCIB 8250, particularly the lipase, a direct demonstration of the role of the esterase and lipase activity in wax ester degradation is still lacking.

The role of the esterase and lipase activities in wax degradation could be further explored by:

(a) Purification and characterisation of the esterase and lipase so that their substrate specificity and kinetics can be investigated in detail.

(b) Investigation of the levels of metabolites particularly fatty acids and fatty alcohols during the transition between aerobic and anaerobic conditions as this could give an indication of rate controlling steps.

(c) The role of fatty alcohol dehydrogenase and fatty acid oxidation in removing metabolites of wax ester oxidising enzymes should be further explored as there is some evidence that these enzymes play an important role in the regulation of wax ester metabolism.

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