THE UNIVERSITY OF GLASGOW

Effects of culture conditions on the lipid composition of *Acinetobacter* sp. NCIB 8250

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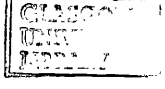
by

Martin J. M^{c}Avoy

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346



To my Mothter, may she live in peace
and my Father, may he rest in peace

Summary

1 This thesis describes the effects of a wide variety of growth conditions on the wax ester content and composition of *Acinetobacter* sp. NCIB 8250. It also describes the effects of these different growth conditions on the fatty acid composition of both the wax esters and the phospholipids found in this bacterium.

2 When *Acinetobacter* sp. NCIB 8250 was grown in continuous culture at 30 °C under ammonium limitation the wax ester content was significantly greater at low specific growth rates [approximately 40 - 45 mg (g dry weight)$^{-1}$] than at specific growth rates approaching the maximum specific growth rate [1 - 2 mg (g dry weight)$^{-1}$]. This trend in the wax ester content was true for all ammonium-limited cultures at all growth temperatures investigated.

3 Wax esters were not accumulated to any great extent when *Acinetobacter* sp. NCIB 8250 was grown in succinate-limited continuous culture.

4 Using continuous culture it was shown that both the specific growth rate and the growth temperature affected the wax ester composition. When the specific growth rate was increased, there was a decrease in the overall chain length of the wax esters but an increase in their degree of unsaturation. As the growth temperature was increased there was no discernible change in the overall chain length but the wax esters became more saturated at the elevated growth temperatures.

5 In continuous culture only the growth temperature had a significant effect on the fatty acid composition of the wax esters, the affect being an increase in the degree of unsaturation as the growth temperature was lowered. However, the increase in the degree of unsaturation in the fatty acid composition was insufficient to account for the increased degree of unsaturation in the wax ester composition, indicating that there was also a change in the degree of unsaturation in the fatty alcohol composition.

6 When *Acinetobacter* sp. NCIB 8250 was grown at 30 °C in continuous culture the fatty acid composition of the phospholipids was shown to be dependent on the growth temperature, becoming more unsaturated as the growth temperature decreased. However, the growth rate and nutrient limitation did not have a significant affect on the fatty acid composition of the phospholipids.

7 When *Acinetobacter* sp. NCIB 8250 was grown at 30 °C in batch culture using a medium with a low concentration of $(NH_4)_2SO_4$ the wax ester content during stationary phase was greater [10 - 15 mg (g dry weight)$^{-1}$] than during the exponential phase [0·5 - 1 mg (g dry weight)$^{-1}$]. Also, in batch culture exponential and stationary phase bacteria, grown on the medium with a low concentration of succinic acid there was only 0·2 - 0·5 mg wax esters (g dry weight)$^{-1}$ accumulated.

8 The wax ester composition of *Acinetobacter* sp. NCIB 8250 grown in batch culture was dependent on the growth temperature and on the phase from which the culture was harvested. At low growth temperatures the wax esters were significantly more unsaturated than when the bacterium was grown nearer its optimum growth temperature. Also, when cultures were harvested from stationary phase the wax ester composition became more saturated and consisted predominantly of shorter chain length wax esters when compared with the wax ester compositions of bacteria harvested during the exponential phase.

9 The fatty acid composition of the wax esters of *Acinetobacter* sp. NCIB 8250 harvested from stationary phase was predominantly $C_{16:0}$ whereas the fatty acids of the wax esters in the bacteria harvested from exponential phase were more unsaturated and the predominant fatty acid was $C_{18:0}$.

10 Unambiguous evidence is shown for the presence of diglycerides in the lipids extracted from *Acinetobacter* sp. NCIB 8250. However, it is not clear whether these compounds originated from the phospholipids or whether they are present in the bacterium as diglycerides.

Acknowledgements

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I am forever indebted to Alan Scott, James Jardine and Patrick Ferry for sharing their superb technical expertise with me and to Dr. El-Mansi M.T. El-Mansi, for sharing!

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I produced this thesis on my Apple Macintosh™ using Word™ and CricketGraph™

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Abbreviations

ACP	acyl carrier protein
AMP	adenosine monophosphate
ATCC	American Type Culture collection
ATP	adenosine triphosphate
CDP	cytidine diphosphate
cli	chain length index
CMP	cytidine monophosphate
CoA	co-enzyme A
CTP	cytidine triphosphate
D	dilution rate
DNA	deoxyribonucleic acid
glc	gas liquid chromatography
FAME	fatty acid methyl esters
M_r	molecular weight
μ	specific growth rate
μ_{max}	maximum specific growth rate
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
NCIB	National Collection of Industrial Bacteria
nd	not determined
ND	not detected
OD_{500}	optical density measured at 500 nm
PAGE	polyacrylamide gel electrophoresis
rRNA	ribosomal ribonucleic acid
tlc	thin layer chromatography
ui	unsaturation index

Chapter One

Introduction

1.1 The genus *Acinetobacter*

Brisou & Prévot (1954) originally proposed the name *Acinetobacter* for a heterogeneous collection of non-motile, Gram-negative saprophytes which could be distinguished from other similar bacteria by their lack of pigmentation (Ingram & Shewan, 1960). The Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria subsequently proposed that the genus *Acinetobacter* should include only the oxidase-negative strains (Lessel, 1971), a decision that has been supported by use of transformation tests (Juni, 1972). As current members of the genus *Acinetobacter* have previously been classified under a variety of different names e.g. *Herellea vaginicola* and *Mima polymorpha* (Debord, 1939), *Moraxella glucidolytica* and *Moraxella lwoffii* (Piéchaud *et al.*, 1956; Brisou, 1957) and *Micrococcus calcoaceticus* (Juni, 1978) much of the early literature concerning this group of micro-organisms is difficult to interpret due to confusion over nomenclature and the lack of a widely accepted classification system.

However, the current accepted definition of the genus *Acinetobacter*, which is classified in the family Neisseriaceae, allows unambiguous identification of strains down to the genus level (Grimont & Bouvet, 1991). Thus, acinetobacters are defined as being: strictly aerobic, non-motile, oxidase negative coccobacilli; Gram-negative but sometimes difficult to destain; grow well on complex media between 20 °C and 30 °C without growth factor requirements; nitrates are rarely reduced; extracted DNA is able to transform BD413 *trp*E27 and the G+C content of the DNA is between 39 and 47 mol % (Grimont & Bouvet, 1991). Recently, a subdivision of the genus *Acinetobacter* was proposed (Bouvet & Grimont, 1986; Grimont & Bouvet, 1991; Tjernberg & Ursing, 1989). This delineation divides the genus into 17 genospecies based on a combination of phenotypic properties (e.g. carbon source utilization tests) and identification of genotypic species by means of genetic transformation, DNA hybridization and rRNA sequence comparison; however, only seven of the 17 identified genospecies have been named (Grimont & Bouvet, 1991). One of the un-named genospecies is group 11, the reference strain for which is the strain NCIB 8250 (Grimont & Bouvet, 1991). This strain has for many years been described as *A. calcoaceticus* but that name has been reserved for those strains identified as belonging to genospecies group 1. Therefore, throughout this thesis the strain NCIB 8250 will be referred to as *Acinetobacter* sp. NCIB 8250.

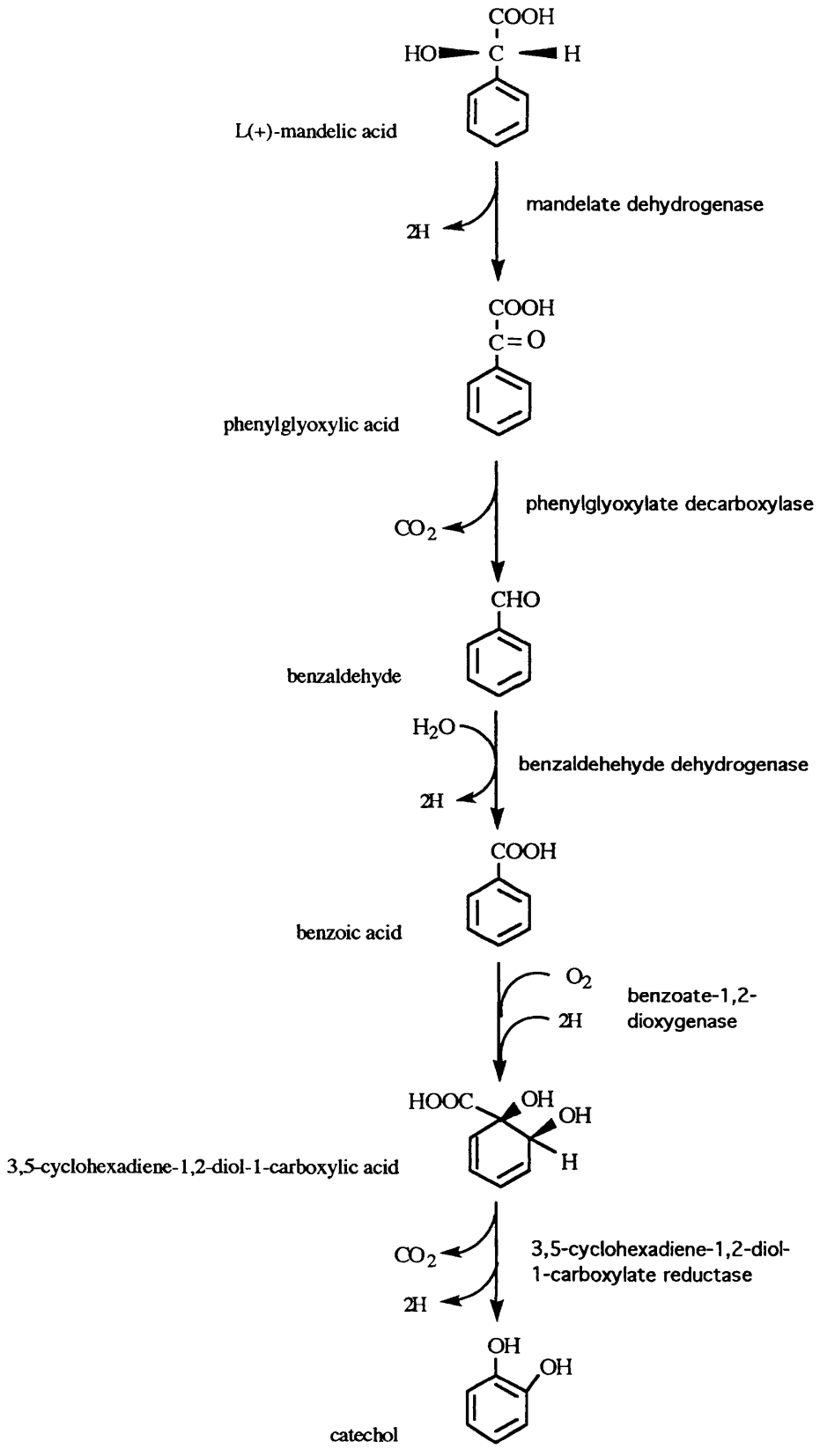
Acinetobacter species are widely distributed in nature and can be easily isolated from soil and water (Baumann *et al.*, 1968; Henriksen, 1973), foodstuffs (Koburger, 1964; Gallagher, 1971; Eribo & Jay, 1985), livestock (Dickie & Regnier, 1978),

petroleum-contaminated habitats (Einsele, 1983), sewage (LaCroix & Cabelli, 1982) and from hospital environments (Glew *et al.*, 1977; French *et al.*, 1980; Ghoneim & Halaka, 1980). A major biochemical feature of the genus *Acinetobacter* is that strains are metabolically very versatile and are capable of growing in a simple mineral medium containing a single carbon and energy source. Such compounds include aliphatic alcohols, some amino acids, dicarboxylic and fatty acids, un-branched hydrocarbons, some diols, many aromatic compounds such as mandelate, benzoate and cyclohexanol. However, only a few strains have been isolated that can utilize carbohydrates (Fewson, 1967; Baumann *et al.*, 1968; Juni, 1978) this may be because these strains are unable to synthesis the prosthetic group, pyrrolo-quinoline quinone, required by glucose dehydrogenase (van Schie *et al.*, 1984). Hence, due to the diversity of compounds utilized, some of which are usually toxic to other bacteria, members of the genus *Acinetobacter* are particularly suited to the study of a variety of unusual peripheral biochemical pathways.

One such pathway is that of mandelate degradation. This pathway has been most notably studied in *Acinetobacter* sp. NCIB 8250 (Fewson, 1991). In this peripheral pathway L(+)-mandelate is converted into phenylglyoxylate by the stereospecific L(+)-mandelate dehydrogenase. Phenylglyoxylate is decarboxylated to benzaldehyde which is then reduced to benzoic acid. This intermediate is sequentially oxygenated, reduced, decarboxylated and dehydrogenated, by two separate enzymes, to give catechol (Figure 1.1) a key intermediate through which a wide variety of recalcitrant aromatic compounds feed into the central metabolic pathway (Fewson, 1991). The enzymes involved in the conversion of L(+)-mandelate to benzoic acid have been studied both as enzymes i.e. to characterize their prosthetic group requirements, pH optima, substrate specificity etc. and as a means to a better understanding of the mechanisms involved in the evolution of enzymic pathways (Chalmers & Fewson, 1989).

Members of the genus *Acinetobacter* are also of great interest as they are the only genus of bacteria known that accumulate wax esters as an energy reserve (Fixter & Fewson, 1974; Fixter & McCormack, 1976), although the accumulation of wax esters as an energy reserve has been reported in marine animals, (Sargent *et al.*, 1976) plants (Kolattukudy, 1967; Yermanos, 1975) and other single cellular organisms (Kolattukudy, 1970; Kawabata & Kaneyama, 1989). Some *Acinetobacter* species have also been shown to be capable of accumulating poly-β-hydroxybutyrate and polyphosphates (Fuhs & Chen, 1975; Lotter *et al.*, 1989) and these are also regarded as being energy reserves (Fixter & Sherwani, 1991). However, it is unclear whether accumulation of these compounds is a general feature of the genus and that all strains could accumulate them under the appropriate physiological conditions or

Figure 1.1 Dissimilation of L(+)-mandelate to catechol



whether this aspect of the micro-organisms biochemistry is limited to a few specific genospecies.

1.2 Wax esters

Wax esters are the esterified product of a fatty alcohol and a fatty acid. Two types of short hand notation will be used throughout this thesis. For molecular classes of wax esters the number of carbon atoms is given and after a colon the total number of double bonds is given. $C_{32:1}$ would represent a wax ester with 32 carbon atoms and one double bond. For individual species, a similar notation is used for the fatty alcohol and fatty acid moieties, the fatty alcohol moiety is always given first (Aasen *et al.*, 1971). Therefore, $C_{16:0}$ - $C_{18:1}$ would represent a wax ester containing a saturated fatty alcohol with 16 carbon atoms and a mono-unsaturated fatty acid with 18 carbon atoms. Further examples of this notation are shown in Figure 1.2a.

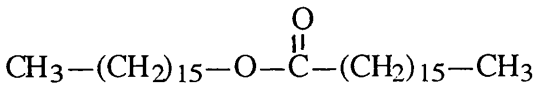
1.2.1 Structure and properties

Two classes of wax esters are found in nature, simple and complex. Simple wax esters are straight chain primary alcohols esterified to straight chain fatty acids, either or both chains may possess double bonds. Simple wax esters are usually found in marine animals and bacteria (Sargent *et al.*, 1976; Fixter *et al.*, 1986) and examples of such wax esters are shown in Figure 1.2a. In complex wax esters the alcohol and/or the acid moiety have a more complex structure typified by the presence of side chains off the main acyl backbone. An example of such wax esters is found in *Mycobacterium tuberculosis* (Asselineau, 1966) where the alcohol moities are C_{33} - C_{35} branched chain diols esterified to C_{29} - C_{32} branched chain fatty acids (Figure 1.2b). Another type of complex wax ester is one that contains a hydroxyl group as well as a carboxylic acid group and in this case an alcohol would be esterified to the carboxylic group and a second fatty acid esterified to the hydroxyl group (Figure 1.2b). Wax esters such as these are found in the lipids of animal skins (Nicolaides *et al.*, 1972; Downing, 1976) and in plants, which have two main groups of wax esters, cutin and suberin. Cutin consists of polyesters of hydroxy fatty acids and is a structural component of the plant cuticle. Suberin is its counterpart located in the underground portions of the plant although suberin differs from cutin as it contains predominantly ω-hydroxy and dicarboxylic acids (Kolattukudy *et al.*, 1976).

Wax esters are often compared with triglycerides as these lipids contain acyl chains and they are also storage compounds. However, wax esters and triglycerides have

Figure 1.2 Simple and complex wax esters

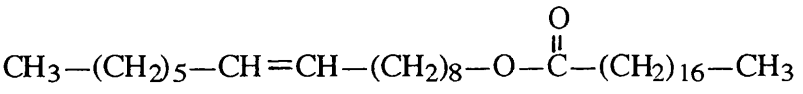
a) examples of simple wax esters



$C_{16:0}$ - $C_{16:0}$

$C_{32:0}$

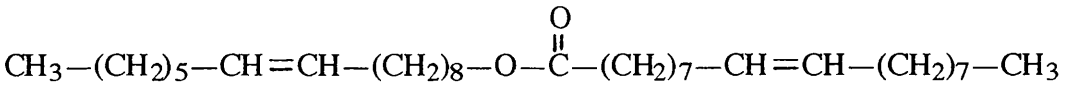
hexadecanyl hexadecanoate



$C_{16:1}$ - $C_{18:0}$

$C_{34:1}$

cis-Δ^9-hexadecenyl octadecanoate



$C_{16:1}$ - $C_{18:1}$

$C_{34:2}$

cis-Δ^9-hexadecenyl *cis*-Δ^9-octadecenoate

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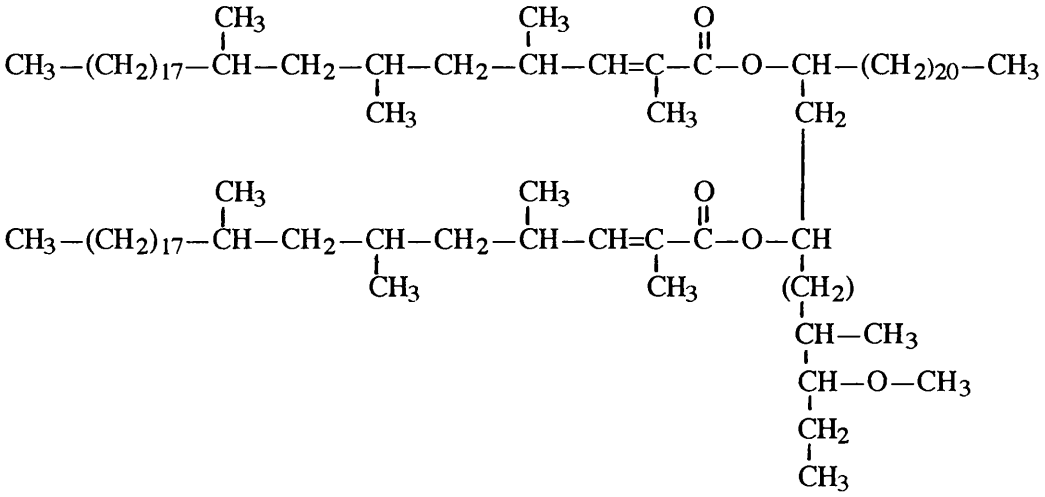
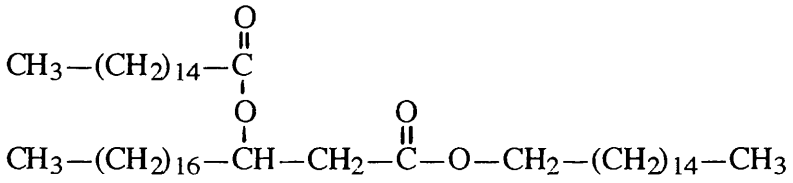
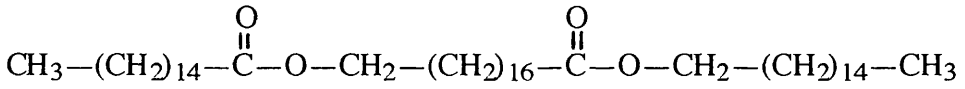
b) examples of complex wax esters



Phthiocerol dimycocerosate



A β-hydroxy acid wax diester



An ω-hydroxy acid wax diester

very different physico-chemical properties. Wax esters do not contain glycerol and they have two acyl chains per ester linkage, making wax esters much more hydrophobic then triglycerides. This difference in their hydrophobicity underpins the means by which these two types of lipids are separated using chromatographic techniques. The use of non-polar solvents, such as toluene, move wax esters with or near to the solvent front whereas triglycerides move more slowly. With the use of more polar solvents the reverse would be true, i.e. the triglycerides would elute nearer to the solvent front than the wax esters. Also, wax esters have a lower specific gravity than triglycerides and in marine animals this has the advantage over a similar volume of triglycerides as the wax esters would provide a source of bouyancy (Lewis, 1970) as well as acting as an energy reserve. Also, as wax esters are highly reduced they produce more energy, in terms of ATP, than triglycerides or glycogen. Wax esters can be calculated to produce 8·35 mol (g atom carbon)$^{-1}$ compared to 7·98 mol (g atom carbon)$^{-1}$ and 6·00 mol (g atom carbon)$^{-1}$ from triglycerides or glycogen, respectively (Sherwani, 1990). However, like triglycerides, wax esters do not form bilayers in water although at low concentrations wax esters can insert readily into phospholipid bilayers.

1.2.2 Distribution and importance

Wax esters are ubiquitous throughout nature and have been found in nearly all organisms from bacteria and yeast through to higher plants and animals, although the quantities of wax esters present are usually very low, perhaps only 0·02 - 0·2 % of the total lipid (Naccarato *et al.*, 1972; Blomquist *et al.*, 1972; Yermanos, 1975; Downing, 1976; White *et al.*, 1987). However, in some organisms wax esters are present in much greater quantities. For instance, in the bacterium *Micrococcus cryophilus* ATCC15174, wax esters represent up to 14 % of the total lipid (Russell, 1974; Lloyd & Russell, 1983). Russell and co-workers have studied the effect of growth temperature on the fatty acid composition of the phospholipids (Russell, 1971 & 1974; Foot *et al.*, 1983a; McGibbon & Russell, 1983; 1985; McGibbon *et al.*, 1985; Sandercock & Russell, 1980) and showed that throughout the growth temperature range (25 °C to minus 4 °C) 95 % of the fatty acids in the phospholipids were mono-unsaturated. They also showed that whilst decreasing the growth temperature had very little effect on the degree of unsaturation, decreasing the growth temperature from 20 °C to 1 °C increased the percentage of shorter chain fatty acids. After investigating the effect of growth temperature on the wax ester composition they proposed that the wax esters might contribute to the maintenance of membrane fluidity (Russell & Volkman, 1980) as a decrease in the growth

temperature increased the degree of unsaturation in the fatty alcohol and fatty acid moieties of the wax esters which they showed where located in both the inner and outer membranes (Lloyd & Russell, 1984). However, later studies using fluorescence polarisation and differential scanning calorimetry showed that the wax esters where not involved (McGibbon *et al.*, 1985) and that the fatty acid composition of the phospholipids was sufficient to maintain membrane fluidity throughout the growth temperature range of the bacterium. It has been proposed that in *Acinetobacter* sp. NCIB 8250 wax esters act as an energy reserve (Fixter & Fewson, 1974; Fixter *et al.*, 1986) and as *M. cryophilus* ATCC15174 is a Gram-negative coccus of uncertain taxonomic status and resembles *Acinetobacter* species (Russell, 1974), the wax esters in this bacterium may also be an energy reserve. However, as *M. cryophilus* ATCC15174 has never been grown under nitrogen limitation (N.J. Russell, personal communication) it remains unclear what role the wax esters have in this bacterium.

Mature jojoba seeds also contain significant amounts of wax esters (Yermanos, 1975) and the biosynthesis of these wax esters has been the subject of much research (Ohlrogge *et al.*, 1978; Pollard *et al.*, 1979; Wu *et al.*, 1981). Moreover, jojoba seeds cannot synthesis triglycerides and therefore jojoba is unique within its phylum (Wu *et al.*, 1981). In mature seeds the wax esters which act as an energy reserve in this plant account for approximately 50 % of the dry weight (Wu *et al.*, 1981), and act as an energy reserve. Wax esters are also abundant in *Euglena gracilis* and have been reported to serve as an energy reserve in this organism (Rosenberg & Pecker, 1964; Inui *et al.*, 1982). In fact *E. gracilis* has two types of reserve compounds; wax esters, which can constitute up to 50 % of the total lipid (Rosenberg, 1963) and paramylon, a β-1,3-glucan (Inui *et al.*, 1982; Kawabata *et al.*, 1990). Each compound accumulates under different growth conditions; paramylon under aerobic conditions and wax esters under anaerobic conditions or in the dark. These two compounds are also interconvertible. For instance, if *Euglena gracilis* is grown in a carbon rich medium under aerobic conditions paramylon is accumulated but on transfer to anaerobic conditions the paramylon is converted to wax esters, a process referred to as wax ester fermentation. This process is accompanied by a net synthesis of ATP (Inui *et al.*, 1982).

Before a compound can be considered as an energy reserve if must fulfil certain criteria (Wilkinson, 1959). These are (i) that the compound should accumulate when the growth of the organism is limited by a nutrient other than that required to synthesis the energy reserve, (ii) that the accumulated compound is degraded when the exogenous supply becomes insufficient, and (iii) that utilizable energy is obtained from the degradation of the stored compound.

Strains of *Acinetobacter* have been shown to accumulate significant amounts of wax esters (Fixter & McCormack, 1976) and in *Acinetobacter* sp. NCIB 8250 the wax esters have been shown to fulfil the requirements of an energy reserve (Fixter & Fewson, 1974, Fixter *et al.*, 1986; Fixter & Sherwani, 1991). In this particular strain, under nitrogen limitation, wax esters can accumulate to 40 - 60 mg (g dry weight)$^{-1}$ in stationary phase cultures whereas under carbon limitation the wax ester content is less than 1 mg (g dry weight)$^{-1}$. Also, when *Acinetobacter* sp. NCIB 8250 was grown to stationary phase under nitrogen limitation, harvested and then resuspended in phosphate buffer the wax esters were degraded to water soluble products and presumably produces energy (Fixter *et al.*, 1986).

Several researchers have investigated the cellular location of wax esters. Lloyd & Russell (1984) showed that in *M. cryophilus* ATCC 15174 the wax esters were located in both the inner and outer membranes whereas Thorne *et al.* (1973), who only studied the outer membrane of *Acinetobacter* sp. MJT/F5/199A found wax esters to be present in this membrane. Fixter (1976) obtained electron micrographs of *Acinetobacter* sp. NCIB 8250 and these show that under nitrogen limitation wax ester accumulation caused the appearance of inclusions (Fixter, 1976; Fixter & Sherwani, 1991) that were absent in bacteria grown under carbon limitation (Figure 1.3). Singer *et al.* (1985) have also show the presence of inclusions in *Acinetobacter* sp. HO1-N grown on hexadecanol. These authors also observed an increase in the total amount of phospholipid present and showed that this coincided with the appearance of intracellular membranes surrounding the wax ester inclusions (Singer *et al.*, 1985).

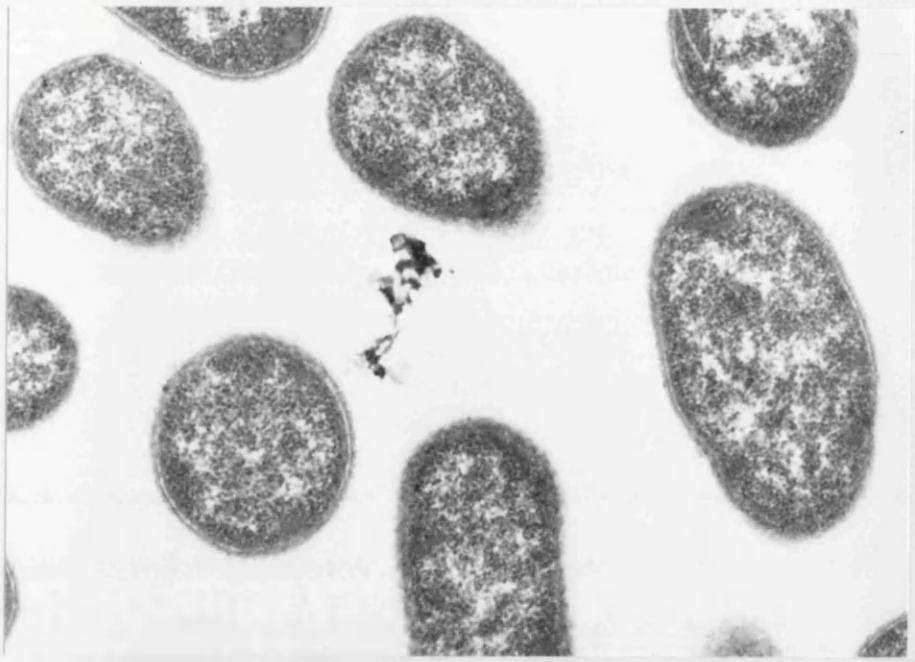
1.2.3 Biosynthesis of fatty acids, fatty alcohols and wax esters

1.2.3.1 Biosynthesis of fatty acids

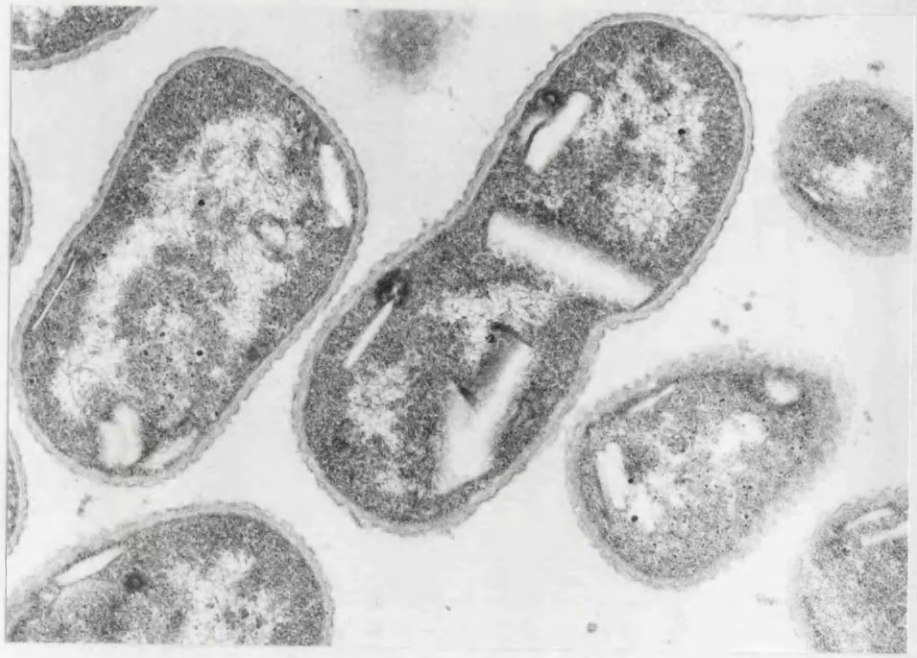
The biosynthesis of fatty acids has been studied in considerable detail in both prokaryotes and eukaryotes. These studies have revealed many similarities and differences in the biosynthesis of fatty acids between the two groups of organisms and has led to the recognition of two classes of fatty acid synthases, known as type I and type II fatty acid synthases (McCarthy & Hardie, 1984). The main structural characteristic of type I fatty acid synthases is that the enzymes exist as a multifunctional polypeptide, although in some organisms the different active sites of fatty acid synthesis are located on more than one multifunctional polypeptide (Buckner & Kolattukudy, 1976; Bloch & Vance, 1977; Sonnenborn & Kunau, 1982). Also, type I fatty acid synthases are found predominantly in animals. Type II fatty acid synthases, however, are characterised by having each reaction catalysed by

Figure 1.3 Electron micrographs of *Acinetobacter* sp. NCIB 8250

a) from carbon-limited stationary phase



b) from nitrogen-limited stationary phase



a discrete, separate enzyme and they are found mostly in micro-organisms and plants (Ohlrogge, 1982; Fulco, 1983), although there are some exceptions; for instance, *Mycobacterium smegmatis*, an advanced prokaryote, has been shown to possess a type I fatty acid synthase (Wood *et al.*, 1978).

The synthesis of fatty acids has been well characterised both biochemically and genetically (Volpe & Vagelos, 1976; de Mendoza & Cronan, 1983) in a wide range of organisms. One of the similarities of fatty acid synthase systems is that the majority use acetyl-ACP and malonyl-ACP as the building blocks for the synthesis of fatty acids. Acetyl-ACP and malonyl-ACP are themselves derived from acetyl-CoA and malonyl-CoA via two specific CoA-ACP transacylases (Figure 1.4b); malonyl-CoA being formed by the carboxylation of acetyl-CoA by the enzyme acetyl-CoA carboxylase. In *E. coli* this enzyme is a multi-protein complex and consists of biotin carboxylase, biotin carboxylase carrier protein and carboxyl transferase subunits α and β and catalyses two half reactions to give malonyl-CoA (Figure 1.4a).

The acetyl-CoA-ACP and malonyl-CoA-ACP transacylases are regarded as being part of the fatty acid synthase whereas the acetyl-CoA carboxylase is regarded as an enzyme separate from the fatty acid synthase (Rock & Cronan, 1985). Although there are different fatty acid synthase systems known, they all follow the same basic pattern of reactions. Thus, after acetyl-CoA and malonyl-CoA have been converted to their ACP derivatives the enzyme acetoacetyl-ACP synthase condenses them (Jackowski & Rock, 1987) with the concomitant loss of the CO_2 that had been attached to the malonyl unit by acetyl-CoA carboxylase (Figure 1.4b). The β-keto-acyl-ACP formed is reduced by an NADPH-dependent β-ketoacyl-ACP reductase and water is removed, producing a *trans*-Δ^2-acyl-ACP. This step is catalysed by the enzyme β-hydroxyacyl-ACP dehydrase and the double bond produced is reduced by an enoyl-ACP reductase (Figure 1.4b) (Volpe & Vagelos, 1976). The saturated C_4-ACP moiety is then condensed with another unit of malonyl-ACP by a β-ketoacyl-ACP synthase and the cycle of reduction, dehydration and reduction followed to produce an acyl chain elongated by two more carbons. This cycle of reactions continues, usually until a saturated C_{16}-ACP has been formed. The series of reactions described above are the basic route by which both facultative anaerobes, such as *E. coli* and obligate aerobes, such as *Acinetobacter* species synthesis saturated fatty acids, although these different classes of organisms have different ways of synthesizing unsaturated fatty acids (Volpe & Vagelos, 1976; de Mendoza & Cronan, 1983; Rock & Cronan, 1985).

Figure 1.4 The biosynthesis of fatty acids

a) the synthesis of malonyl-CoA

Biotin carboxylase

$$ATP + HCO_3^- + BCCP \longrightarrow CO_2^- — BCCP + ADP + P_i$$

Carboxyl transferase

$$CO_2^- — BCCP + CH_3CO\text{-}CoA \longrightarrow BCCP + CO_2CH_2CO\text{-}CoA$$

BCCP = Biotin carboxylase carrier protein

b) the synthesis of saturated fatty acids

Acetyl-CoA-ACP transacylase

$$CH_3CO\text{-}CoA + ACP \longrightarrow CH_3CO\text{-}ACP + CoA$$

Malonyl-CoA-ACP transacylase

$$CO_2CH_2CO\text{-}CoA + ACP \longrightarrow CO_2CH_2CO\text{-}ACP + CoA$$

β-ketoacyl-ACP synthase

$$CH_3CO\text{-}ACP + CO_2CH_2CO\text{-}ACP \longrightarrow CO_2 + CH_3COCH_2CO\text{-}ACP + ACP$$

β-ketoacyl-ACP reductase

$$CH_3COCH_2CO\text{-}ACP + NADPH \longrightarrow CH_3CH(OH)CH_2CO\text{-}ACP + NADP^+$$

β-hydroxyacyl-ACP dehydrase

$$CH_3CH(OH)CH_2CO\text{-}ACP \longrightarrow CH_3CH{=}CHCO\text{-}ACP + H_2O$$

enoyl-ACP reductase

$$CH_3CHCHCO\text{-}ACP + NAD(P)H \longrightarrow CH_3CH_2CH_2CO\text{-}ACP + NAD(P)^+$$

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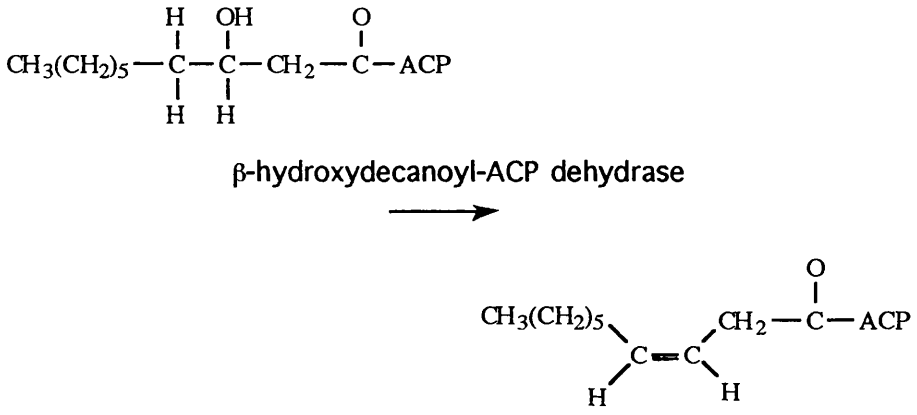
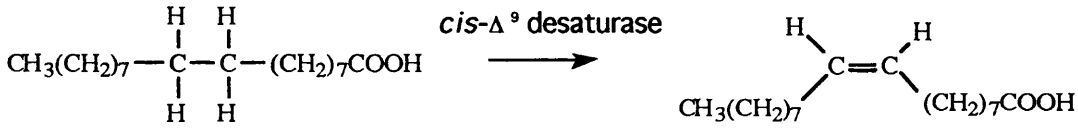
c) the specific step in the synthesis of unsaturated fatty acids



d) the desaturation of a fatty acid by an acyl desaturase



In the case of *E. coli* there are two distinct enzymes that can introduce a double bond into the acyl chain as it is synthesised. These iso-enzymes allow micro-organisms, such as *E. coli*, to synthesis both saturated and unsaturated fatty acids, under anaerobic conditions (e.g. de Mendoza & Cronan, 1983). The iso-enzyme β-hydroxydecanoyl-ACP dehydrase is specific for β-hydroxydecanoyl-ACP and produces a *cis*-Δ^3-decenoyl-ACP (Figure 1.4c) as apposed to the "normal" *trans*-Δ^2-enoyl-ACP that is produced by the other iso-enzyme, which is non-specific and can act on all chain lengths of β-hydroxyacyl-ACP, including β-hydroxydecanoyl-ACP (Figure 1.4b). Synthesis of the *cis*-Δ^3-decenoyl-ACP leads to the synthesis of *cis*-Δ^9-hexadecenoyl-ACP as the *cis*-Δ^3-decenoyl-ACP does not undergo immediate reduction, as would the *trans*-Δ^2-acyl-ACP, but is condensed with malonyl-ACP and therefore by-passes the normal reduction step (Rock & Cronan, 1985). The cycle of reactions then continues with the reduction of the β-keto group and after several more cycles of reduction, dehydration (with the non-specific dehydrase), reduction and condensation the synthesis of *cis*-Δ^9-hexadecenoyl-ACP results (de Mendoza & Cronan, 1983).

In *E. coli* the two enzymes, β-hydroxyacyl-ACP dehydrase and β-hydroxydecanoyl-ACP dehydrase, are present in similar quantities and have very similar K_m and V_{max} values for β-hydroxydecanoyl-ACP and therefore when *E. coli* is grown at, or near to, its optimum growth temperature *de novo* fatty acid synthesis results in the synthesis of equal proportions of saturated and unsaturated fatty acids (Volpe & Vagelos, 1976). However, when *E. coli* is grown at temperatures away from its optimum growth temperature there are marked changes in the ratio of the saturated and unsaturated fatty acids as a result of the increase or decrease in the activity of the temperature sensitive enzyme β-ketoacyl-ACP synthase II (de Mendoza & Cronan, 1983; Rock & Cronan, 1985). These changes in the unsaturated/saturated fatty acid ratio are brought about by post-synthetic modifications and are discussed in more detail in Section 1.3.2.

Obligate aerobes only possess the non-specific β-ketoacyl-ACP dehydrase and therefore the fatty acid synthase can only synthesise saturated fatty acids. The synthesis of unsaturated fatty acids is by post-synthetic modification of a saturated acyl chain. The enzyme responsible for this modification is called a desaturase and introduces a double bond into the acyl chain by the removal of hydrogens from two adjacent carbons (Figure 1.4d), typically resulting in the synthesis of a *cis*-Δ^9-mono-unsaturated fatty acid (Russell, 1978a). This type of unsaturated fatty acid synthesis can only occur during aerobic conditions as the cofactor used by the desaturase requires oxidising before it can be used again. This differs from the formation of the

double bond by facultative anaerobes as these organisms produce the double bond in the unsaturated fatty acid by the direct removal of water and therefore the enzyme is not dependent on a cofactor, which must be re-oxidised.

In bacteria the end product of fatty acid synthesis is usually a C_{16} fatty acid (de Mendoza & Cronan, 1983). Further elongation of this C_{16} fatty acid is possible and usually only involves the addition of one more C_2 unit to produce a C_{18} fatty acid. In *E. coli* the elongase is specific for *cis*-Δ^9-hexadecenoyl-ACP and results in the synthesis of *cis*-Δ^{11}-vaccenoyl-ACP (de Mendoza & Cronan, 1983), the elongation of a $C_{16:0}$ to a $C_{18:0}$ fatty acid is very rare (Baldassare *et al.*, 1976). Therefore, the fatty acid composition of *E. coli*, and other Gram-negative bacteria, consists predominantly of $C_{16:0}$, $C_{16:1}$ and $C_{18:1}$ fatty acids.

Although fatty acid synthases from a wide variety of organisms follow the same basic reactions described above, some organisms use different primer molecules to that of acetyl-ACP and/or malonyl-ACP and can, therefore, produce different end products. For example, *Bacillus subtilis*, a Gram-positive bacterium, use 2-methyl butyrate as a primer to produce branched chain saturated fatty acids and isobutyrate and isovalerate as primers to produce branched chain unsaturated fatty acids. Thus the major fatty acid present in bacilli is the anteiso-C_{15} fatty acid with smaller amounts of *n*-C_{16}, branched C_{17} and iso-C_{16} fatty acids (Fulco, 1984).

The synthesis of fatty acids discussed above is based largely on the Gram-negative bacterium *E. coli*. While the pathway in this bacterium has been used successfully as a model for other organisms, differences have been found both between obligate aerobes and facultative anaerobes and between Gram-positive and Gram-negative bacteria (Fulco, 1984). Although, it is presumed that the saturated fatty acid biosynthetic pathway of acinetobacters would resemble quite closely that of *E. coli* it is likely that the fine control of the enzymes of fatty acid biosynthesis and the synthesis of unsaturated fatty acids in *Acinetobacter* species would be different. For instance, in *E. coli* the condensation of acetyl-ACP with malonyl-ACP is the rate limiting step (Jackowski & Rock, 1987) and is therefore under strict control. As *E. coli* synthesises fatty acids almost exclusively for the synthesis of new membranes during growth (Rock & Cronan, 1985) and acinetobacters, during nitrogen-limited stationary phase continue to synthesis fatty acids for the synthesis of wax esters (Fixter *et al.*, 1986) it is likely that detailed examination of the acinetobacter enzymes would reveal interesting differences.

Indeed, in another central metabolic pathway, that of the Krebs cycle, very distinct differences in the control of the key enzymes have been reported (Weitzman, 1991).

In both *E. coli* and in *Acinetobacter* species citrate synthase is inhibited by NADH. Whereas in *E. coli* this inhibition is hyperbolic, in *Acinetobacter* species it is sigmodial. Also, the inhibition of the citrate synthase from *Acinetobacter* species could be relieved by the addition of AMP, whereas this was not true for the citrate synthase of *E. coli*. Weitzman and co-workers also found these kinds of differences between some of the other Krebs cycle enzymes of these two organisms. The differences in the control of these two sets of enzymes was rationalised on the basis that *Acinetobacter* species are obligate aerobes and are completely reliant on the energy produced from the Krebs cycle, whereas facultative anaerobes such as *E. coli*, are not. Therefore the re-activation of the Krebs cycle enzymes by AMP, in *Acinetobacter* species, makes some sense as AMP acts as a signal of depleted metabolic energy and thus serves as a positive effector on these enzymes (Weitzman, 1991).

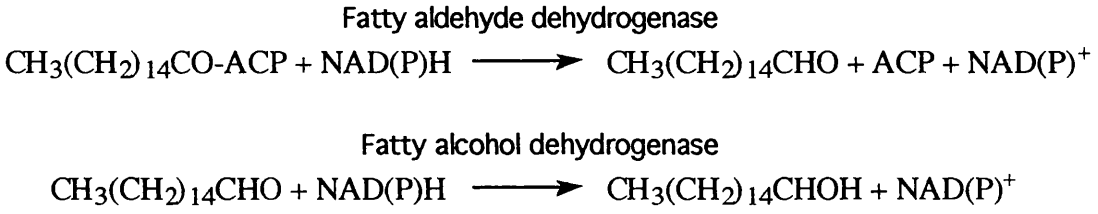
Although there has been very little detailed study on the fatty acid synthesis enzymes in *Acinetobacter* species, Sampson & Finnerty (1974) have reported on some aspects of the control of fatty acid biosynthesis in *Acinetobacter* sp. HO1-N. These workers grew *Acinetobacter* sp. HO1-N on *n*-hexadecane and showed that when these cultures were then incubated, under non-growing conditions, with [^{3}H]-acetate and [1-^{14}C]-hexadecanoic acid that there was significantly more radio-activity incorporated into the cellular lipids from [1-^{14}C]-hexadecanoic acid than from [^{3}H]-acetate. From these experiments Sampson & Finnerty (1974) concluded that when *Acinetobacter* sp. HO1-N was grown on *n*-hexadecane, that *de novo* fatty acid biosynthesis was significantly reduced, presumably because of repression of the fatty acid synthesis enzymes by the fatty acids produced from the oxidation of the *n*-alkane.

1.2.3.2 Biosynthesis of fatty alcohols

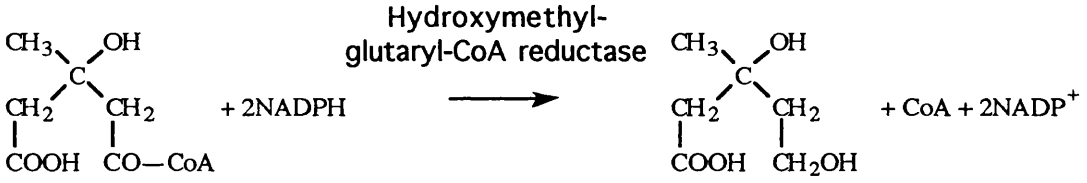
Primary fatty alcohols can be synthesised in bacteria by the reduction of fatty acids and by the terminal oxidation of hydrocarbons (Figure 1.5). The reduction of fatty acids to fatty alcohols proceeds via an aldehyde intermediate and therefore requires either two enzymes (Figure 1.5a), a fatty aldehyde dehydrogenase and a fatty alcohol dehydrogenase, or one enzyme with dual functionality. In *Acinetobacter* sp. HO1-N there have been reports of both fatty aldehyde dehydrogenase and fatty alcohol dehydrogenase activities (Singer & Finnerty, 1985a & b; Fox *et al.*, 1992). Indeed several fatty aldehyde and fatty alcohol dehydrogenase activities are reported by both authors but so far there have been no reports that both the reduction to the aldehyde and then to the alcohol is carried out, in bacteria, by the same enzyme. If such an enzyme did exist it might be analogous to the enzyme 3-hydroxy-3-

Figure 1.5 Biosynthesis of alcohols

a) from fatty acids



b) from 3-hydroxy-3-methylglutaryl-CoA



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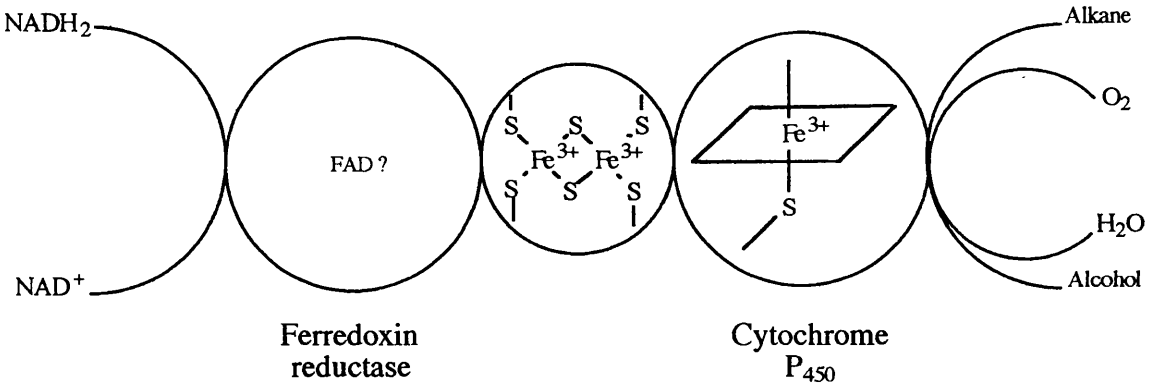
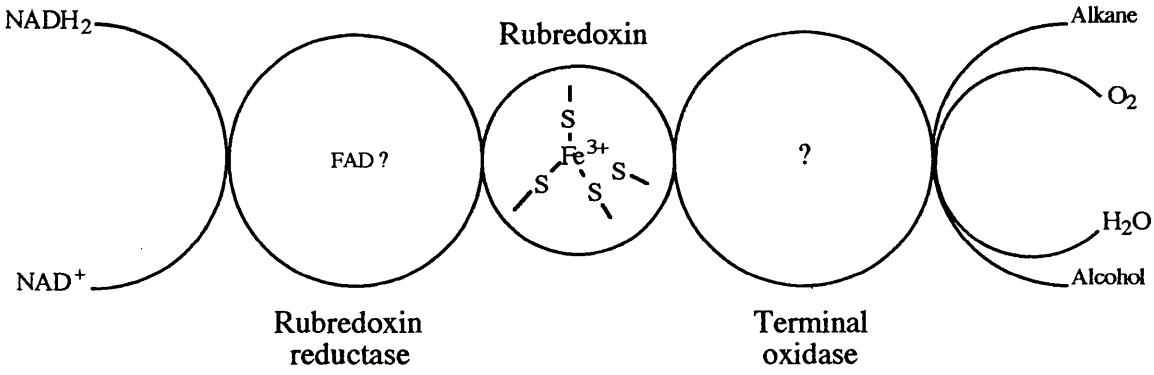
c) from *n*-alkanes

by *Acinetobacter calcoaceticus* EB104



by *Acinetobacter calcoaceticus* 69-V



The schematic representation of the proposed alkane monoxygenases in *Acinetobacter* strains EB104 and 69-V are taken from Asperger & Kleber (1991).

methylglutaryl-CoA reductase, which is found in animals (Lehninger, 1982). This enzyme is the controlling enzyme in the synthesis of cholesterol and catalyses the double reduction of 3-hydroxy-3-methylglutaryl-CoA to mevalonate, in a single step (Figure 1.5b).

Singer & Finnerty (1985a) report that there are both NADP- and NAD-dependent fatty aldehyde dehydrogenases present in *Acinetobacter* sp. HO1-N and that different iso-enzymes are present depending on the growth substrate used. Fox *et al.* (1992) confirm the findings of Singer & Finnerty (1985a) in that they also report both NADP- and NAD-dependent fatty aldehyde dehydrogenase activities. However, they also report the presence of a hexadecane inducible nucleotide independent fatty aldehyde dehydrogenase which they claim accounts for approximately 85 % of all the fatty aldehyde dehydrogenase activity (Fox *et al.*, 1992). Fox *et al.* (1992) also state, however, that it is the NADP-dependent fatty aldehyde dehydrogenase (which is also induced by growth on alkanes) that is associated with the hydrocarbon inclusions and that the nucleotide independent fatty aldehyde dehydrogenase is not associated with the membranes of the hydrocarbon inclusions.

As with the fatty aldehyde dehydrogenases there are NADP-dependent and NAD-dependent fatty alcohol dehydrogenases present in *Acinetobacter* sp. HO1-N (Singer & Finnerty, 1985b; Fox *et al.*, 1992). Singer & Finnerty (1985b) reported an NADP-dependent fatty alcohol dehydrogenase which is soluble and constitutively expressed as well as a membrane bound NAD-dependent fatty alcohol dehydrogenase that was induced by growth of *Acinetobacter* sp. HO1-N on *n*-hexadecane or *n*-hexadecan-1-ol. Fixter & Nagi (1984) have also reported the presence of a NADP-dependent fatty alcohol dehydrogenase from *Acinetobacter* sp. NCIB 8250. This enzyme was also soluble and constitutively expressed and has recently been purified to homogeneity and characterised (Wales, 1992). This enzyme has a subunit M_r of 40,000 Da and is probably active as a tetramer. Further characterisation showed that the enzyme was neither activated nor inhibited by metal ions, it did not possess a bound co-factor or prosthetic group, it was located in the cytoplasm and it did not oxidise hexadecan-1-ol but was optimally active on shorter chain length alcohols, e.g. C_5 - C_8 (Wales, 1992).

Fox *et al.* (1992) have reported on fatty alcohol dehydrogenases in *Acinetobacter* sp. HO1-N. These authors have identified three alcohol dehydrogenases, an NAD-dependent alcohol dehydrogenase and two NADP-dependent fatty alcohol dehydrogenases. The NAD-dependent alcohol dehydrogenase is probably an ethanol inducible enzyme that does, however, have residual activity when grown on

substrates other than ethanol (Fixter & Nagi, 1984; Singer & Finnerty, 1985b). The two NADP-dependent fatty alcohol dehydrogenases were both soluble enzymes and could be differentiated by thermal denaturation at 60 °C, non-denaturing PAGE stained for $NADP^+$ activity and by separation of these activities using a Sephacryl S-300 column. Fractionation of the cell free extract by passage down the Sephacryl S-300 column resulted in the loss of the more heat labile NADP-dependent fatty alcohol dehydrogenase activity. Wales (1992) has also purified an NADP-dependent fatty alcohol dehydrogenase from *Acinetobacter* sp. HO1-N using an almost identical purification scheme to that worked out for the NADP-dependent fatty alcohol dehydrogenase purified from *Acinetobacter* sp. NCIB 8250. Wales (1992) then showed that the enzyme from *Acinetobacter* sp. HO1-N was almost kinetically identical to that of the enzyme from *Acinetobacter* sp. NCIB 8250. However, Wales (1992) concluded that the enzyme from *Acinetobacter* sp. NCIB 8250 was not involved in wax ester metabolism and if this was correct perhaps the second enzyme observed by Fox *et al.* (1992) might be the alcohol dehydrogenase that is involved in wax ester metabolism.

The biosynthesis of fatty alcohols from *n*-alkanes has also been studied in *Acinetobacter* species and was first described by Stewart & Kallio (1959). Finnerty and co-workers (Finnerty, 1977; Singer & Finnerty, 1984) suggested that *n*-alkane oxidation in the strain *Acinetobacter* sp. HO1-N involved an *n*-alkyl hydroperoxide as the initial product. This would have required a di-oxygenase as the first enzyme followed by an alkyl hydroperoxide reductase. However, enzymic evidence for this pathway in *Acinetobacter* sp. HO1-N is lacking (Asperger & Kleber, 1991). There is, however, growing evidence for the existence of two different pathways for the terminal oxidation of *n*-alkanes to fatty alcohols by *Acinetobacter* species (Figure 1.5c). In strain *A. calcoaceticus* EB104 a cytochrome P_{450} has been shown to be involved in the oxidation of *n*-alkanes to fatty alcohols (Asperger *et al.*, 1981) whereas in *A. calcoaceticus* 69-V, a strain which can also grow on *n*-alkanes, no evidence for the presence of a cytochrome P_{450} has been found and as yet the identity of the terminal oxidase in this strain is unknown (Asperger & Kleber, 1991). Also, in strain EB104 ferredoxin reductase has been shown to be associated with the P_{450} activity but in the strain 69-V a rubredoxin and a NADH-dependent rubredoxin reductase (Figure 1.7b) have been found to be associated with *n*-alkane oxidation (Asperger & Kleber, 1991). It has also been shown that in these two strains the pathways for *n*-alkane utilization are inducible (Claus *et al.*, 1980; Asperger *et al.*, 1984) and that only those strains that possess the P_{450} system can utilize the middle chain length *n*-alkanes, i.e C_6 - C_9 (Asperger & Kleber, 1991).

Isolation of these two different *n*-alkane oxidizing pathways from *Acinetobacter* and

reconstitution experiments using purified components has provided evidence that *n*-alkanes are converted to the corresponding fatty alcohols by a mono-oxygenase (Asperger *et al.*, 1985). Although the cellular location of the enzymes in these pathways is still unknown it has been postulated that the enzymes are associated with the intracellular membranes that appear during growth on *n*-alkanes (Scott *et al.*, 1976 and Scott & Finnerty, 1976) as these workers have shown that when *Acinetobacter* sp. HO1-N was grown on *n*-hexadecane that intracellular membranes surrounded inclusion vesicles containing *n*-hexadecanol and wax esters.

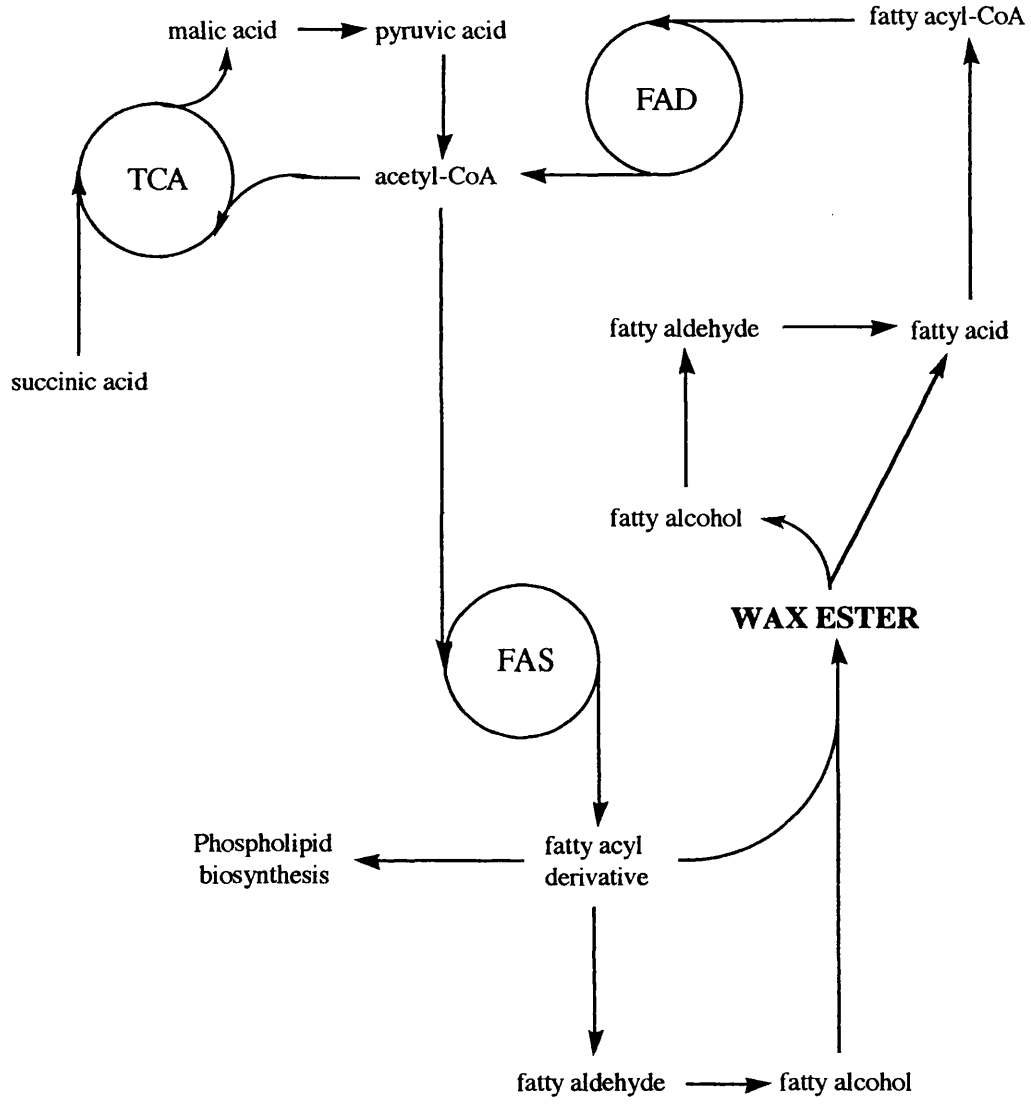
One aspect of *n*-alkane oxidation by *Acinetobacter* species that is still very unclear is the mechanism of uptake into intact cells. It has been shown that strains of *Acinetobacter* such as RAG-1, BD4 and BD413 can produce bio-emulsifiers (Rosenberg & Kaplan, 1987, Borneleit *et al.*, 1988; Gutnick *et al.*, 1991) which stabilize alkane/water emulsions but it also appears unlikely that these bio-emulsifiers are involved directly either in the mechanism of alkane transport across the cellular membranes or in the interaction of the *n*-alkanes with the enzymes of *n*-alkane oxidation (Asperger & Kleber, 1991).

1.2.3.3 Esterification of fatty acids and alcohols to form wax esters

Wax ester biosynthesis has been studied in a number of different organisms including *Brassica oleracea* (Kolattukudy, 1967); *Simmondsia chinensis* (Ohlrogge *et al.*, 1978); *Euglena gracilis* (Inui *et al.*, 1982); *Micrococcus cryophilus* (Lloyd & Russell, 1983) and in *Acinetobacter* species (Fixter & Fewson, 1974; Fixter & McCormack, 1976) and although the overall pathway for the synthesis of wax esters is well established (Figure 1.6) there have been very few reports of studies on the enzymes involved. Perhaps because of this it remains unclear whether the wax ester synthase uses a fatty acyl-CoA or a fatty acyl-ACP derivative as the donor for the fatty acid moiety. The evidence in the literature shows that cell free extracts are capable of synthesing wax esters given an exogenous supply of a fatty alcohol, fatty acid, ATP, CoA and Mg^{2+} or just a fatty alcohol and a fatty acyl-CoA (Nagi, 1981; Lloyd & Russell, 1983). However, as fatty acyl-ACP is the end product of fatty acid synthesis and that workers studying the enzymes of phospholipid biosynthesis found that *in vitro* acyl-CoA could be used as the acyl donor (Raetz, 1978) but that *in vivo* the acyl-ACP derivatives were used (Rock & Cronan, 1985) it is plausible that fatty acyl-ACP and not fatty acyl-CoA is the activated species used *in vivo* by the wax ester synthase and therefore it is only in cell free extracts that the fatty acyl-CoA derivative acts as an acyl chain donor.

With respect to the regulation of wax ester synthesis, it would seem unlikely that the

Figure 1.6 Schematic pathway for the biosynthesis of wax esters in *Acinetobacter* sp. NCIB8250 grown on succinic acid



TCA = Tricarboxylic acid cycle

FAS = Fatty acid synthesis enzymes

FAD = Fatty acid degradation enzymes

fatty acyl-CoA derivative would be used *in vivo* as it is also a substrate for fatty acid degradation, via β-oxidation. If fatty acyl-CoA were a substrate for the wax ester synthase then this could result in the futile cycling of fatty acids due to competition between the enzymes involved, i.e. those of β-oxidation and wax ester synthesis, unless these enzymes were either under very tight control or within different compartments of the cell. Eukaryotic organisms are well known for the separate compartmentalisation of synthetic and degradative pathways but in bacteria this is very problematic since there are no organelles (Wolfe, 1981). However, it has been shown that within *Acinetobacter* species, wax esters are stored in inclusions and that these vesicles are surrounded by a membrane (Fixter, 1976; Scott & Finnerty, 1976; Singer *et al.*, 1985). If the wax ester synthase was associated with the internal surface of the membrane of these vesicles then it would be possible for fatty acyl-CoA derivatives to act as the acyl donor for the fatty acids in wax ester synthesis as the enzymes of wax ester synthesis and β-oxidation would be spacially separated, the enzymes of β-oxidation being associated with the plasma membrane (Wolfe, 1981).

1.3 Phospholipids

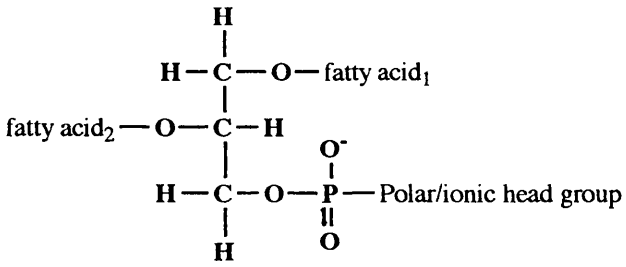
1.3.1 Biosynthesis of phospholipids

The major components of membranes are proteins and lipids. In bacteria the lipids in the plasma membrane are almost exclusively phospholipids. Phospholipids consist of an *sn*-glycero-3-phosphate back bone, two fatty acids and a polar or ionic head group (Figure 1.7a). Phospholipids are characterised by their amphipathic nature, that is, they have both hydrophobic and hydrophilic groups (Lehninger, 1982). The two fatty acyl chains esterified to the glycerol back bone give the phospholipids their hydrophobic character and the polar or ionic head group attached to the phosphate at the *sn*-3 position on the glycerol back bone gives the molecule its hydrophilic nature. There are a wide range of polar and ionic head groups known (Figure 1.7b), although, usually only a few are found in any one organism (Gennis, 1989). For instance, in Gram-negative bacteria the most commonly observed head groups are ethanolamine and glycerol. Thus, the phospholipids are known as phosphatidyl-ethanolamine and phosphatidylglycerol. A third phospholipid, diphosphatidyl-glycerol, is derived from phosphatidylglycerol and is also common in Gram-negative bacteria (Goldfine, 1984).

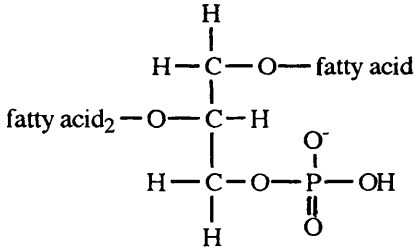
The pathway of phospholipid biosynthesis has been studied in a number of organisms and in considerable detail in *E. coli*. In this bacterium CDP-diglyceride is

Figure 1.7 Basic structure of a phospholipid with head groups

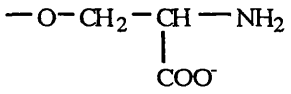
a) the basic structure of a phospholipid



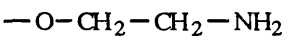
b) head groups commonly found in phospholipids



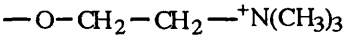
phosphatidic acid



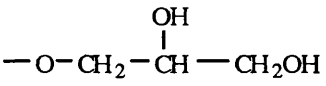
phosphatidylserine



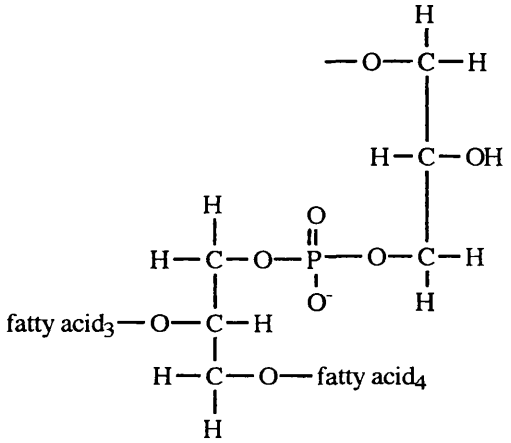
phosphatidylethanolamine



phosphatidylcholine



phosphatidylglycerol



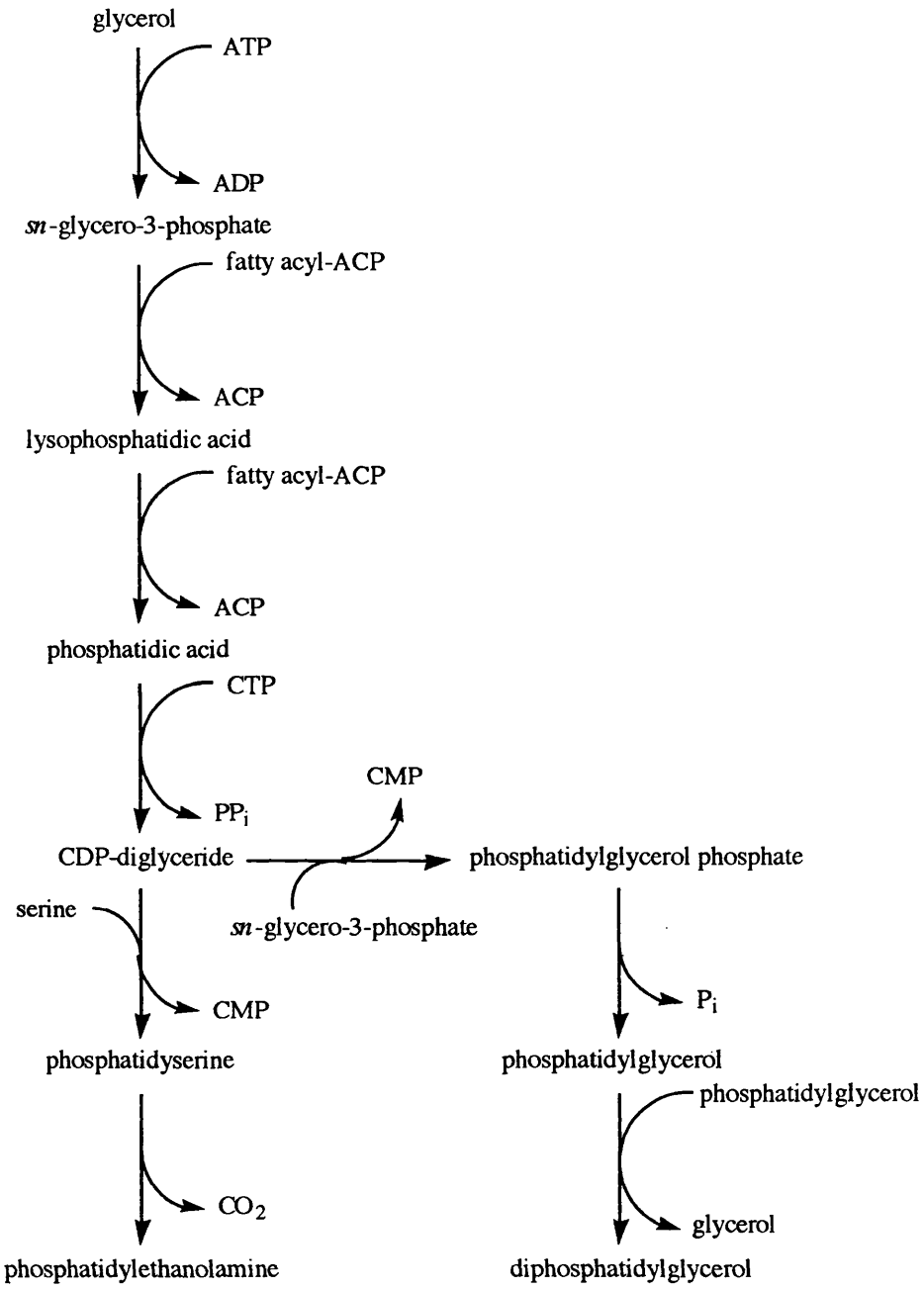
diphosphatidylglycerol

the common precursor for the synthesis of the three main phospholipids found in Gram-negative bacteria (Cronan & Vagelos, 1972; Raetz, 1978; Cronan & Rock, 1987). Synthesis of CDP-diglyceride starts with the activation of glycerol to *sn*-glycero-3-phosphate which is then acylated at both the *sn*-1 and *sn*-2 positions yielding phosphatidic acid. This intermediate is then activated by CTP to give CDP-diglyceride. Addition of serine with the concomitant loss of CMP followed by the decarboxylation of the serine head group, these reactions being catalysed by two separate enzymes, results in the synthesis of phosphatidylethanolamine (Figure 1.8). Alternatively, addition of *sn*-glycero-3-phosphate to CDP-diglyceride, again with the concomitant loss of CMP and followed by the hydrolysis of the phosphate group from the *sn*-glycero-3-phosphate yields phosphatidylglycerol. Synthesis of diphosphatidylglycerol (Figure 1.8) is by the condensation of two molecules of phosphatidylglycerol with the loss of one of the glycerol groups (Raetz, 1978; Cronan & Rock, 1987).

One area of phospholipid biosynthesis that has received attention is that of the relative proportions of the different phospholipid head groups. In wild type *E. coli* phosphatidylethanolamine represents 70 - 75 % of the total and phosphatidylglycerol and diphosphatidylglycerol represent 15 - 20 % and 5 - 10 % of the total, respectively (Cronan & Vagelos, 1972; Baldassare *et al.*, 1976). However, using mutants in phospholipid biosynthesis, e.g. a temperature sensitive mutant that is unable to decarboxylate the serine head group above the permissive temperature, it is possible to alter the phospholipid head group composition (Raetz, 1978). For instance, in the case of this type of mutant, synthesis of phosphatidylethanolamine is reduced by up to 80 % and is replaced by the accumulation of phosphatidylserine. Studies such as these have revealed that it is only after gross changes have been made to the phospholipid head group composition that any effect to the growth or phenotype of the organism can be detected. In mutants that accumulate phosphatidylserine (or are incapable of synthesizing it) cell division ceases and the cells become filamentous (Raetz, 1976; 1978). However, genetic lesions in which the phospholipid head group composition has been so altered that a change to the phenotype occurs, are rare and such mutations are difficult to obtain. More common, is the isolation of a mutant that is reduced in a particular enzyme activity, compared to the wild type, but which still displays the same phospholipid head group composition as the wild type (Raetz, 1978). It is, therefore, thought that the enzyme activity levels observed in wild type strains are in excess of that required for the synthesis of phospholipids.

The phospholipid compositions of *Acinetobacter* sp. NCIB 8250 and HO1-N have been determined (L.M. Fixter, unpublished results; Makula & Finnerty, 1970; Singer

Figure 1.8 Schematic pathway for the biosynthesis of phospholipids



et al., 1985). When *Acinetobacter* sp. NCIB 8250 was grown on glutamate phosphatidylethanolamine represented 51 % of the total with phosphatidylglycerol and diphosphatidylglycerol being 22 % and 16 % of the total respectively, 11% of the total being unidentified (L.M. Fixter, unpublished results). Finnerty and co-workers who grew *Acinetobacter* sp. HO1-N on acetate, *n*-hexadecane and *n*-hexadecane found a similar phospholipid composition, i.e. phosphatidyl-ethanolamine was 47 - 61 %; phosphatidylglycerol, 20 - 31 % and diphosphatidyl-glycerol, 7 - 15 % of the total (Makula & Finnerty, 1970; Singer *et al.*, 1985). These workers also report that tri-acyl-lysodiphosphatidylglycerol constituted up to 8 % of the total phospholipids.

1.3.2 Regulation of membrane fluidity

One of the purposes of the phospholipids in the plasma membrane is to act as a surface on to which or through which enzymes are attached or pass (Singer & Nicholson, 1972). Normal functioning of enzymes and therefore of the cell, dictates that enzymes must be able to alter their tertiary structure. Therefore, the environment in which enzymes are located must posses a certain amount of flexibility. As acyl chains provide the bulk of the phospholipid bilayer it follows that the fatty acid composition of the phospholipids have a major influence on the structure of the membrane, i.e. on its flexibility or alternatively, its degree of fluidity (Cronan & Gelman, 1975; Baldassare *et al.*, 1976; Goldfine, 1984).

The phenomenon of membrane fluidity has been studied for many years (Singer & Nicholson, 1972; Cronan & Gelman, 1975; Baldassare *et al.*, 1976; Nishihara *et al.*, 1976; de Mendoza & Cronan, 1983; Goldfine, 1984; Neidleman, 1987) and membrane lipid bilayers have been shown to undergo a reversible change, from a fluid, liquid crystalline environment to one of a more ordered hexagonal array. The ordered hexagonal array is also know as the gel state. The reversible change between these two states is temperature dependent and at low temperatures the ordered hexagonal array states dominate whereas at higher temperatures liquid crystalline states are dominant (Baldassare *et al.*, 1976). The temperature of the mid-point of the transition from the liquid crystalline state to the gel state is called the transition temperature. In bacteria this temperature is largely a function of the fatty acid composition of the membrane lipids as membrane modifiers, such as cholesterol, which are present in eukaryotic membranes, are not usually found in prokaryotes (Raetz, 1978). Therefore, for micro-organisms to be able to survive changes to the environmental temperature they must be able to alter the fatty acid composition of their phospholipids (Fulco, 1984; Russell, 1984; Neidleman, 1987) such that the gel

- liquid crystalline transition temperature prevents the fatty acyl chains from forming an ordered hexagonal array, thereby reducing the ability of the enzymes to function. In other words, the plasma membrane of bacteria must to a large extent be in the liquid crystalline state in order to allow normal cell functions to continue.

To achieve this bacteria, have developed a wide range of mechanisms that allow these poikilothermic organisms to maintain their plasma membrane lipid bilayer in a liquid crystalline state (Fulco, 1984; Russell, 1984). Central to these mechanisms is an alteration in the fatty acid composition of the phospholipids which may be achieved by altering the degree of unsaturation, chain length, proportion of branched chain or cyclopropane containing fatty acids. By increasing the degree of unsaturation or the proportion of branched chain or cyclopropane containing fatty acids as the growth temperature is decreased a greater degree of disorder is introduced into the membrane bilayer (Cronan & Gelman, 1975). This increase in the degree of disorder counteracts the effect of the decrease in growth temperature, which by a reduction in thermal motion would cause an the increase in the order of the acyl chains. Thus, as the degree of disorder amongst the acyl chains is increased the ability of the acyl chains to form a gel state, at the lowered growth temperatures is decreased and hence the liquid crystalline state of the membranes is maintained (Cronan & Vagelos, 1972; Baldassare *et al.*, 1976). An alternative but less frequently observed method of maintaining the liquid crystalline state of the membranes at lowered growth temperatures is by increasing the proportion of shorter chain fatty acids. Due to their physico-chemical properties, shorter chain length fatty acids have a lower melting point than that of the longer chain length fatty acids. Therefore, maintenance of membrane fluidity by this method is by directly decreasing the melting point of the fatty acids in the bilayer and thereby alters the degree of order/disorder of the acyl chains in the bulk bilayer to maintain the liquid crystalline state (Russell, 1984).

In general, bacteria only employ one particular type of fatty acid compositional change to regulate membrane fluidity. For instance, in *E. coli* a decrease in the growth temperature results in the increased activity of the enzyme β-ketoacyl-ACP synthase II (de Mendoza & Cronan, 1983; Rock & Cronan, 1985). This enzyme, which is also present at higher temperatures, together with β-ketoacyl-ACP synthase I, has a higher rate of reaction at lower growth temperatures. The increased activity of this enzyme at lower growth temperatures results in an increase in the proportion of the $C_{18:1}$ fatty acid with the concomitant decrease in the $C_{16:0}$ fatty acid, the proportion of the $C_{16:1}$ fatty acid remaining the same. $C_{16:1}$ fatty acids are incorporated into phospholipids exclusively at the *sn*-2 position due to the specificity of the acyltransferase. A second acyltransferase catalyses the acylation of the *sn*-1

position and this enzyme can use both $C_{16:0}$ and $C_{18:1}$ as substrates. Therefore the $C_{16:0}$ and $C_{18:1}$ fatty acids compete for the *sn*-1 position. At lower growth temperatures the alteration of the relative proportions of the $C_{16:0}$ and $C_{18:1}$ fatty acids results in the increased synthesis of di-unsaturated phospholipids and hence a decrease in the gel - liquid crystalline transition temperature (de Mendoza & Cronan, 1983; Rock & Cronan, 1985).

In contrast to the simple adaptation process of *E. coli*, *Bacillus* species show a much more complicated response. Growth of *B. licheniformis* 9259 at several growth temperatures showed that the average chain length of the fatty acids in the phospholipids decreased with a decrease in growth temperature, showing a marked increase in the C_{14} and shorter fatty acids at the expense of the C_{17} fatty acids. The proportion of the C_{15} and C_{16} fatty acids remained unchanged. However, there was a change in the ratio of the iso to anteiso C_{15} fatty acids. As the growth temperature was decreased the proportion of the anteiso C_{15} fatty acids increased (Quint & Fulco, 1973). Also, as the growth temperature was decreased there was an increase in the degree of unsaturation of the *n*-C_{16} fatty acids. All these changes, like those of *E. coli*, are dependent on *de novo* fatty acid and phospholipid biosynthesis but not necessarily *de novo* protein synthesis (Fulco, 1984). However, *B. megaterium* 14581 has a unique desaturase that acts directly on acyl chains in the phospholipid and the alteration of the fatty acid composition by this mechanism requires *de novo* protein synthesis. This desaturase is unique as it is hyper-induced when *B. megaterium* 14581 is shifted from 35 °C to 20 °C and as it introduces a *cis*-Δ^5 double bond into the acyl chain. This *cis*-Δ^5-desaturase was not observed in *B. licheniformis* 9259 (Fulco, 1984).

The psychrophilic bacterium *Micrococcus cryophilus* ATCC 15174 also relies on *de novo* fatty acid and phospholipid biosynthesis to adapt to a lower growth temperature. This bacterium has a very simple fatty acid composition profile in that, throughout its growth temperature range, *cis*-Δ^9-hexadecenoate and *cis*-Δ^9-octadecenoate represent 95 % of the total fatty acids found in the phospholipids (Russell, 1971). The mechanism by which this bacterium alters its fatty acid composition is by changing the ratio of these two mono-unsaturated fatty acids. Thus, at 0 °C there is a four-fold increase in the $C_{16:1}/C_{18:1}$ ratio compared to that at 20 °C (Russell, 1971). This change in the $C_{16:1}/C_{18:1}$ ratio is modulated through a temperature sensitive elongase which elongates $C_{16:0}$ to $C_{18:0}$ (Sandercock & Russell, 1980) and whose activity is reduced at lower growth temperatures. The percentage of mono-unsaturated fatty acids in the phospholipids is maintained by a temperature independent Δ^9-desaturase (Russell, 1978) which is membrane bound and uses saturated phospholipid as substrate (Foot *et al.*, 1983).

Although it is clear that mechanisms do exist for altering the fatty acid composition of phospholipids in response to changes in the growth temperature exactly how the bacterium "senses" the temperature change is not understood. It is possible, that, as desaturases require O_2 (for the re-oxidation of the cofactor) and that at lower temperatures O_2 is more soluble, that the increased concentration of O_2 would act as a signal although it is unlikely (Cullen *et al.*, 1971). However, its probable that the enzymes involved in the regulation of membrane fluidity are integral membrane proteins and therefore the changes in the gel-liquid crystalline states, induced by shifts in the growth temperature, would affect the enzyme's activity by altering their tertiary structure. For instance, an increase in the amount of the gel state would cause conformational changes to the tertiary structure that would activate a desaturase (or inhibit an elongase) and vice versa. Thus, modifications to the membrane fluidity would be auto-regulating. However, it is clear no single mechanism is universally employed and that a variety of mechanisms exist and that sometimes more than one mechanism is used in the same organism (Fulco, 1984).

1.4 Aims of this project

Fixter and co-workers have shown that *Acinetobacter* sp. NCIB 8250 accumulates wax esters and that the accumulation of wax esters is widespread throughout the genus (Fixter & Fewson, 1974; Fixter & McCormack, 1976). Fixter and co-workers also showed that in batch culture the greatest amount of wax ester accumulation was during stationary phase in nitrogen-limited cultures. During carbon-limited stationary phase there was very little wax ester accumulation. These workers also showed, using nitrogen-limited continuous culture, that the greatest amounts of wax esters accumulated was at low specific growth rates and that only small amounts of wax esters were accumulated at low specific growth rates in carbon-limited continuous culture (Fixter *et al.*, 1986).

Although, Fixter and co-workers (Fixter & Fewson, 1974; Fixter & McCormack, 1976; Fixter *et al.*, 1986) have studied in some detail the *amounts* of wax esters accumulated by a number of *Acinetobacter* strains they have not studied the wax ester composition in great detail. Therefore, the first aim of this project was to repeat and expand some of this earlier work and to analyse the compositions of the accumulated wax esters. The next aim was to study the effect of different growth temperatures on the amounts of wax esters accumulated and on their compositions. This work was carried out chiefly using continuous culture, as this technique allows the manipulation of one growth variable, e.g. growth temperature, whilst maintaining

other growth variables, e.g. specific growth rate, constant. A third aim was to analyse the fatty acid composition of the phospholipids extracted from the samples taken from these different culture conditions, i.e. those of different specific growth rate, growth temperature and nutrient limitation. It was of interest to do this because at the time of this work there were only a few reports in the literature on the fatty acid composition of the phospholipids in this genus and these workers had either used complex media or studied the effect of different growth substrates, such as acetate and hexadecane, on the fatty acid composition of the phospholipids (Makula & Finnerty, 1968; Nishimura *et al.*, 1979). As wax esters are synthesised, essentially, from two fatty acids a fourth aim was to analyse the fatty acid composition of the wax esters. Results could then be compared with those obtained for the fatty acid compositions of the phospholipids in the hope that they would reveal if there were different pools of fatty acids present in this organism. As expertise existed within the group to assay for the enzyme NADP-dependent fatty alcohol dehydrogenase, an alcohol dehydrogenase thought to be involved in wax ester metabolism, it was decided to investigate if there was any correlation of enzyme activity with wax ester content. Therefore, the activity of the enzyme was measured in samples taken from continuous culture over a range of specific growth rates and at different growth temperatures and nutrient limitation.

There are four chapters of results in this thesis. The first chapter details results of experiments carried out to investigate the validity of methods and procedures used during the course of the research. It also contains results from experiments that yielded interesting observations but which were not investigated further as they did not form part of the main thrust of the research. The second results chapter describes the determination of the maximum specific growth rates obtained with *Acinetobacter* sp. NCIB 8250 grown in batch and continuous culture at the different growth temperatures as well as the growth yields and other calculated growth parameters. Also, this chapter contains the results from the analysis of the wax esters at different specific growth rates, growth temperatures and nutrient limitations. The next chapter deals with the results obtained from the analysis of the fatty acids in the wax esters and phospholipids. The fourth results chapter discusses the experiments carried out on the enzyme NADP-dependent fatty alcohol dehydrogenase that was assayed in samples collected from continuous culture experiments.

The final chapter in this thesis draws together the conclusions made in each of the results chapters and contains some thoughts on what future work could be carried out.

Chapter Two

Methods

2.1 Materials

The bacteria used in this study were *Acinetobacter* sp. NCIB 8250 and *Pseudomonas putida* NCIB 9494. They were obtained from the National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland and were stored as described in Section 2.2.

The chemicals used were of reagent grade or better and were stored as directed on their labels. Dithiothreitol, NADP, Tris-base and Tris-HCl were obtained from BCL, Boehringer Mannhein House, Bells lane, Lewes, East Sussex, England. All compressed gases were obtained from BOC, Erskine House, North Avenue, Clydebank Business Park, Glasgow, Scotland. Ethanol was obtained from James Burrough (FAD) Ltd., 70, Eastways Industrial Park, Witham, Essex, England. Bacto-agar was obtained from Difco, Detroit, Michigan, USA. Ammonium sulphate and phenol were obtained from Formachem (Research International), 80, Kirk St., Strathaven, Scotland. Nitrilotriacetic acid, methanol and petroleum ether (b.p. 40 - 60 °C & 60 - 80 °C) were obtained from FSA Laboratory Supplies, Loughborough, Leicestershire, England. Silica gel H60 was obtained from Merck, Darmstadt, Germany. Ammonium hydroxide, sulphuric acid and toluene were obtained from M & B Ltd., Dagenham, London, England. Nutrient Broth (CM1) and Nutrient Agar (CM3) were obtained from Oxoid Ltd., Basingstoke, Hampshire, England. Florosil (60 -100 mesh), heptadecanoic acid and hexadecanol were obtained from Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, England. Browne's sterilizing tubes were obtained from A. Browne Ltd., Leicester, Leicestershire, England. Anprolene exposure indicator (AN85) and Steritest unit (AN80) were obtained from H.W. Anderson Products Ltd., Clacton-on-sea, Essex, England.

All other chemicals were obtained from BDH Laboratory supplies, Burnfield Avenue, Thornliebank, Glasgow, Scotland.

2.2 Culture Storage

The stock cultures and working cultures of *Acinetobacter* sp. NCIB 8250 and *P. putida* were stored at 4 °C in bottles containing 10 ml of nutrient broth (Section 2.3.1.1). The stock cultures were sub-cultured at approximately 6 month intervals and working cultures made from the stock cultures at that time. The working cultures were replaced either when there was very little remaining or at the 6 month interval. Culture purity was tested by streaking on nutrient agar plates (Section 2.3.1.2) and

incubating for 48 h at 30 °C for *Acinetobacter* sp. NCIB 8250 and 37 °C for *P. putida*, checking visually for colony homogeneity and then testing for the presence of oxidase (Section 2.15). The stock and working cultures of *Acinetobacter* sp. NCIB 8250 were also streaked on both L(+)-mandelate agar and D(-)-mandelate agar plates (Section 2.3.2.4). This test shows that the strain being sub-cultured was *Acinetobacter* sp. NCIB 8250 as this is the only strain of *Acinetobacter* sp. NCIB 8250 that is known to be L(+)-mandelate positive and D(-)-mandelate negative (Fewson, 1967).

2.3 Growth media

2.3.1 Complex media

2.3.1.1 Nutrient broth

One litre of this complex medium contained 13·0 g of Oxoid CM1 nutrient broth. The powder was dissolved in distilled water before dispensing into growth flasks and sterilizing (Section 2.4.1).

2.3.1.2 Nutrient agar

One litre of this medium contained 28·0 g Oxoid CM3 nutrient agar which was allowed to dissolve while being sterilized (Section 2.4.1). Immediately after sterilization the medium was placed in a 55 °C water bath. The medium was allowed to cool to this temperature, the plates poured in a laminar flow cabinet and after the agar had set, the plates were incubated for 48 h at 30 °C. After that time any plates that had visible signs of growth were sterilized (Section 2.4.1) and then discarded. The remaining plates were stored at 4 °C until required.

2.3.2 Minimal media

2.3.2.1 Standard salts medium

In one litre the standard salts medium contained 2·00 g KH_2PO_4, 1·00 g $(NH_4)_2SO_4$ and 1·18 g succinic acid, titrated to pH 7·0 with NaOH. The medium was sterilized (Section 2.4.1) and at the time of inoculation 20 ml sterile 2% ($^{w}/_{v}$) $MgSO_4.7H_20$ was added.

2.3.2.2 Medium containing a low concentration of succinic acid

This medium was used in both batch and continuous culture experiments. One litre

contained 2·00 g KH_2PO_4, 1·00 g $(NH_4)_2SO_4$ and 0·42 g succinic acid titrated to pH 7·0 with NaOH. The medium was sterilized (Section 2.4.1) and at the time of inoculation 20 ml sterile 2% ($^w/_v$) $MgSO_4.7H_20$ was added. To ensure nutrient limitation in the stationary phase of batch culture experiments, 250 ml of a sterile medium supplement containing 2·00 g KH_2PO_4 and 1·00 g $(NH_4)_2SO_4$ titrated to pH 7·0 with NaOH was added per litre of culture 2 h before harvesting. For continuous culture experiments a trace metal supplement (Section 2.3.3) was added to the medium at the same time as the $MgSO_4$ solution.

2.3.2.3 Medium containing a low concentration of $(NH_4)_2SO_4$

This medium was used in both batch and continuous culture experiments. One litre contained 2·00 g KH_2PO_4, 0·10 g $(NH_4)_2SO_4$ and 1·18 g succinic acid, titrated to pH 7·0 with NaOH. The medium was sterilized (Section 2.4.1) and at the time of inoculation 20 ml sterile 2% ($^w/_v$) $MgSO_4.7H_20$ was added. To ensure nutrient limitation in the stationary phase of batch culture experiments, 250 ml of a sterile medium supplement containing 2·00 g KH_2PO_4 and 1·18 g succinic acid titrated to pH 7·0 with NaOH was added per litre of culture 2 h before harvesting. For continuous culture experiments a trace metal supplement (Section 2.3.3) was added to the medium at the same time as the $MgSO_4$ solution.

2.3.2.4 Mandelate/salts agar

One litre of this medium contained 2·00 g KH_2PO_4, 1·00 g $(NH_4)_2SO_4$ and either 0·38 g L(+)-mandelic acid or 0·38 g D(-)-mandelic acid and titrated to pH 7·0 with NaOH before the addition of 15·0 g of Difco Bacto-agar and sterilizing (Section 2.4.1). Immediately after sterilization the medium was placed in a water bath at 55 °C and after cooling, 20 ml sterile 2% ($^w/_v$) $MgSO_4.7H_20$ was added and mixed. After the medium was poured and had set, the plates were incubated for 48 h at 30 °C and any that had visible signs of growth were sterilized (Section 2.4.1) and then discarded. The remaining plates were stored at 4 °C until required.

2.3.3 Trace metal supplement

The trace metal supplement of 2 ml l^{-1} was used only in the continuous culture experiments (Beggs & Fewson, 1977). This supplement was made by dissolving 50·0 g of nitrilotriacetic acid in 625 ml of 1·0 M NaOH and titrating the solution to pH 7·0 with HCl. Each of the following were then added in the order stated and allowed to dissolve before the addition of the next component: 1·10 g $FeSO_4.7H_2O$, 50 mg $Na_2MoO_4.7H_2O$, 50 mg $MnSO_4.4H_2O$, 50 mg $ZnSO_4.7H_2O$, 25 mg $CuSO_4.5H_2O$ and 25 mg $CoCl_2.6H_2O$. The solution was then made to a final volume

of one litre and stored at 4 °C until required.

2.4 Sterilization

2.4.1 Steam

Growth media and components were steam sterilized at 109 °C (5 psi) in an autoclave (Manlove, Alliott & Co. Ltd., Nottingham, England) for a predetermined fixed time set by the largest volume being sterilized (C.A. Fewson, unpublished results). The steam used in the autoclave was generated by an electrode boiler attached to the autoclave solely for this purpose. Gilson pipette tips and Eppendorf tubes (in sealed containers) and cultures that were to be discarded were also sterilized by this method. Sterilization was confirmed by having a Browne's tube in a container filled with water equal to the largest volume being sterilized.

2.4.2 Dry heat

This method was used to sterilize glass pipettes. The pipettes in a metal canister were placed in an oven, heated to 160 °C for 2 h and then cooled slowly back to room temperature. The heating and cooling cycle was carried out overnight. Sterilization was confirmed by having a Browne's tube in the lid of the metal canister.

2.4.3 Ethylene oxide

Plastic pipettes were sealed in polythene bags and placed inside an AN 74 sterilization box (H.W. Anderson products Ltd., Clacton-on-sea, Essex, England). An ampoule containing the ethylene oxide was broken and the door to the box closed immediately and time-locked for 24 h. After this time the door was opened and the contents aired for a further 24 h to aid the removal of any residual gas. Sterilization was verified by an Anprolene exposure indicator or by a Steritest unit. The whole procedure was carried out in a fume cupboard, usually over a weekend.

2.5 Culture growth conditions

2.5.1 Preparation of the inocula

The inocula for batch culture experiments were prepared by 3 successive cultures in the standard salts medium (Section 2.3.2.1). For the first culture 0·1 ml of the

working culture was transferred into a 250 ml Erlenmeyer flask containing 50 ml of the sterile medium and incubated overnight on a rotary shaker operating at approximately 180 rpm (LH Engineering Co. Ltd., Bells Hill, Stoke Poges, Buckinghamshire, England) in a thermostatically controlled room maintained at 30 °C. The second culture was set up the following morning by transferring 0·5 ml of the first culture into a second 250 ml Erlenmeyer flask and incubating as above for at least 8 hours; 0·1 ml of this culture was then used to inoculate the third culture which was incubated overnight and contained 100 ml of medium in a 500 ml Erlenmeyer flask.

For continuous culture experiments the inocula were prepared by transferring 0·1 ml of the working culture into 10 ml of nutrient broth in a bottle fitted with a septum. This was grown for 36 h without shaking in a thermostatically controlled room maintained at 30 °C.

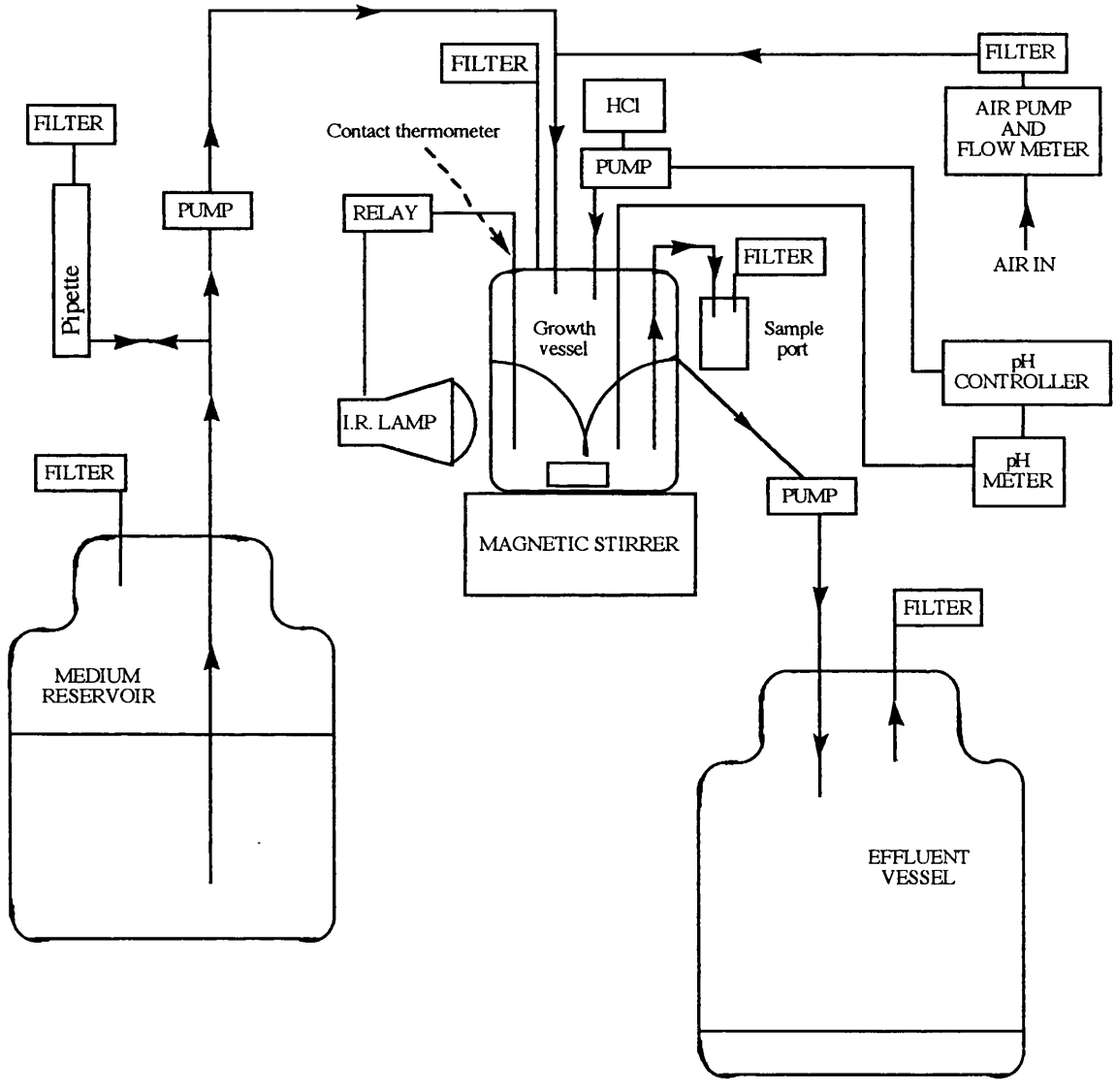
2.5.2 Batch culture

Acinetobacter sp. NCIB 8250 was grown at 30 °C in 2 l stirred growth flasks. A 2 l flask contained 800 ml of minimal medium (Section 2.3.2.2 or 2.3.2.3) and was inoculated with 20 ml of the inoculum culture (Section 2.5.1). Growth flasks were clamped securely to a stirring unit (Harvey *et al.*, 1968) and the culture vigorously aerated using an appropriately sized polypropylene coated magnetic bar in the bottom of the growth flask. Air, regulated by a Gapmeter fitted in-line, was delivered at 250 - 300 ml min^{-1} into the head space of the flask via a 10 ml glass pipette plugged with non-absorbent cotton wool. The growth of the culture was followed by aseptically removing a small sample of the culture through a side arm of the growth flask and determining the dry weight of the culture (Section 2.6). In this way the timing of harvesting exponential phase cultures, the addition of the medium supplement (Section 2.3.2.2 or 2.3.2.3) to stationary phase cultures and the harvesting of these cultures could be determined.

2.5.3 Continuous culture

The continuous culture apparatus used (Figure 2.1) was based on that described by Baker (1968). The culture volume was approximately 650 ml. A sterile syringe and needle was used to aseptically transfer 5 ml of the inoculum (Section 2.5.1) into the continuous culture vessel through a septum in one of the ports in the lid. Cultures were grown initially as a batch and then as a chemostat using either the medium containing a low concentration of succinic acid (Section 2.3.2.2) or low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). Steady states were achieved over a specific growth rate range of 0·14 to 0·80 h^{-1} and over the growth temperature range

Figure 2.1 Schematic diagram of the continuous culture apparatus



of 15 to 33 °C. At least ten culture volumes were passed through the vessel, after changing the growth conditions of the culture, before aseptically harvesting a sample of the culture directly from the vessel into ice-chilled centrifuge tubes (Section 2.7). A sample of this was plated on to nutrient agar, L(+)-mandelate/salts agar and D(-)-mandelate/salts agar plates to test for culture purity (Section 2.2). Another sample was used for the determination of the optical density of the culture at the time of harvesting (Section 2.6). After harvesting, the pellets and culture supernatants were separated. The pellets were resuspended in chilled 10 mM KH_2PO_4, pH 7·0, centrifuged (Section 2.7) and stored at minus 20 °C until the lipids could be extracted and analysed (Section 2.8 & 2.10). A sample of each of the supernatants were filtered through a Millex®-GS filter [Millipore (UK) Ltd., 11 - 15 Peterborough road, Harrow, Middlesex, England] into sterile Eppendorf tubes and either analysed immediately for organic carbon (Section 2.11) and NH_3 (Section 2.12) or stored at minus 20 °C until the analysis could be done, which was usually within 24 h. The supernatants were assayed to prove that the desired limitation had been achieved by confirming the absence of significant amounts of either organic carbon or NH_3. When significant amounts (i.e. greater than 5 % of the initial amount) of both organic carbon and NH_3 were present in the culture supernatants the culture was taken as being oxygen-limited.

The maximum specific growth rate (μ_{max}) of *Acinetobacter* sp. NCIB 8250 was determined at each of the temperatures studied. This was done by the method of Tempest (1970) and involved adjusting the dilution rate so that it was greater than μ_{max} and over a period of time measuring the decrease in the optical density (Section 2.6) of the culture as it was washed out. The μ_{max} could then be calculated using the equation:

$$\mu_{max} = D + \frac{\ln\left(\frac{x_2}{x_1}\right)}{t_2 - t_1} \text{ where;}$$

μ_{max} = maximum specific growth rate (h^{-1})
D = dilution rate (h^{-1})
t_1 and t_2 = time points 1 and 2 (h)
x_1 and x_2 = biomass (mg ml^{-1}) at time points 1 and 2 respectively.

2.6 Optical density measurement

The optical density of a liquid minimal medium culture was determined using a PU

8470 spectrophotometer (Pye-Unicam, Cambridge, England) at a wavelength of 500 nm (OD_{500}). Using a calibration curve of OD_{500} against dry weight (see Section 3.3), the dry weight of the culture could be determined by removing small volumes of the culture from the growth vessel. To ensure that the OD_{500} was within the range of the calibration curve the culture was diluted with 10 mM KH_2PO_4, pH 7·0, when required. For batch culture experiments a plot of $\log_{10}$ (dry weight) against time were constructed to show the growth of the culture with time. In the continuous culture experiments the calculation of the growth yields ($Y_{x/s}$) were determined for each of the steady states achieved under either succinate or ammonium limitation. The $Y_{x/s}$ is a measurement of the dry weight of the culture produced per g of succinic acid, for those cultures grown under succinate limitation and the dry weight of the culture produced per g of $(NH_4)_2SO_4$, for those cultures grown under ammonium limitation. The $Y_{x/s}$ values were calculated using the equation:

$$Y_{x/s} = \frac{\text{g dry weight per litre of culture}}{\text{g limiting nutrient per litre of medium}}.$$

2.7 Harvesting of cultures

Different procedures were used depending on the volume of culture to be harvested. For volumes greater than 250 ml an MSE 6L refrigerated centrifuge (fixed angle rotor type 59116, 5000 rpm, 15 min, 4 °C) was used. For volumes less than 250 ml a Beckman J2-21 refrigerated centrifuge was used (8000 rpm, 10 min, 4 °C). The rotors use with this centrifuge were the fixed angle JA20 (8 x 50 ml) and the fixed angle JA14 (6 x 250 ml). For volumes of 1 ml or less an MSE Microcentaur microfuge was used (13000 rpm, 2 min).

2.8 Extraction of bacterial lipids for total wax ester content and fatty acid and wax ester composition analysis

The method of Folch *et al.* (1957) was used to extract the lipids from samples that were to be analysed for either fatty acids or wax esters. To 1·0 ml of a bacterial suspension (equivalent OD_{500} of 20 - 25) 20 ml of 2:1 ($^{v}/_{v}$) $CHCl_3$:MeOH was added followed by the internal standard wax ester (Section 2.10.1). The amount of internal standard added to the sample was one sixth that of the total amount of wax esters expected. This amount was based on both personal experience and on previous batch and continuous culture experiments (Fixter *et al.*, 1986). After addition of the internal standard the extraction tube was stoppered, mixed thoroughly by vortexing

and then 5·0 ml of 0·87 % ($^{w}/_{v}$) KCl added. After separation of the phases the aqueous layer was removed and anhydrous Na_2SO_4 added. The organic phase was then filtered through a sintered glass filter and the Na_2SO_4 washed with 3 x 3 ml of $CHCl_3$. Each wash was filtered through the sintered glass filter and collected. The $CHCl_3$ was then evaporated in a conical tube concentrating the lipid sample into a smaller volume. The evaporation of the $CHCl_3$ from the conical tube was aided by a constant stream of O_2-free N_2 and by having the tube in a heating block at 40 - 45 °C. To aid the concentration of the lipid sample the sides of the tube were washed with a further 2 x 50 µl of $CHCl_3$ which was then removed as above.

2.9 Separation and localization of the different classes of lipids by thin layer chromatography

2.9.1 Preparation of the thin layer chromatography plates

The glass plates (20 x 20 x 0·5 cm) were washed with scouring powder and copious amounts of tap water, rinsed with distilled water and then dried using laboratory tissue paper. Five thin layer chromatography (tlc) plates were prepared at any one time using a Quick fit tlc clamp and spreader. In a fume cupboard, 40 g of silica gel H60 was weighed into a 500 ml Erlenmeyer flask, 100 ml of distilled water added and the flask stoppered. A slurry was made by shaking the flask for 1 min, and was then poured into a spreader with a 0·5 mm thickness slot. The spreader was then moved quickly but smoothly along the length of the apparatus and the plates left to dry at room temperature, usually overnight.

2.9.2 Separation and localization of the different classes of lipids

The silica on a plate was scored along the vertical length at 4 cm intervals to provide four separate tracks on the plate numbered from left to right, tracks 1 - 4. The samples, dissolved in $CHCl_3$, were loaded on to the plate across the middle of tracks 2 - 4 (1 sample per track) at the origin (approximately 1·5 - 2·0 cm from the bottom) using a 25 or 100 µl Hamilton syringe, with the appropriate standards being loaded on to track 1. Evaporation of the solvent was aided by blowing cold air from a hair dryer across the plate. After loading, the plate was placed vertically into a tlc tank containing the appropriate solvent system (Section 2.10) and removed when the solvent reached within 1 - 2 cm of the top of the plate. The plate was kept vertical in a fume cupboard while the solvent evaporated and was then sprayed with 0·1 % ($^{w}/_{v}$) 2',7'-dichlorofluorescein in ethanol. This reagent allows the localization of the lipid spots but does not alter the unsaturation of the acyl chains (Skipski & Barclay,

1969). After the ethanol had evaporated the plate was placed horizontally under a UV source and the circumference of the spots marked. The area within the marked circumference was later scraped off the plate and the lipids recovered (Section 2.10).

2.10 Estimation of wax ester content and fatty acid and wax ester compositions

2.10.1 Chemical synthesis and purification of the internal standard

The wax ester internal standard, hexadecanoyl heptadecanoate, used to quantify the wax esters found in the bacterial samples was synthesised by the method of Selinger & Lapidot (1966). N,N-Dicyclohexylcarbodiimide (10 mmol) was dissolved in 50 ml of dry CCl_4 and was added to 10 mmol of heptadecanoic acid and 10 mmol of hexadecanol both dissolved in a total of 150 ml of dry CCl_4. The mixture was left at room temperature overnight and the precipitate removed by filtration through a sintered glass filter under reduced pressure. The wax esters were recovered from the CCl_4 fraction by the evaporation of the CCl_4 using a rotary evaporator at 45 °C.

The initial purification of the synthesised wax ester was by the method described by Carroll (1976). A 40 x 2 cm column was filled with hydrated Florosil (7 ml water:100 g dry Florosil) in petroleum spirit (b.p. 60 - 80 °C). The synthesised wax ester, dissolved in 2 ml of petroleum spirit (b.p. 40 - 60 °C) was loaded on to the column at 30 mg wax ester (g hydrated Florosil)$^{-1}$ and eluted with 100 ml of 5 % ($^{v}/_{v}$) diethyl ether in petroleum spirit (b.p. 40 - 60 °C). The eluate was collected and the solvent removed in a rotary evaporator as described above. Further purification was achieved by dissolving the wax ester in warm petroleum spirit (b.p. 60 - 80 °C) and then, to remove any insoluble material, filtering through a warm sintered glass filter under reduced pressure. The filtrate was then chilled to minus 20 °C and filtered through a clean chilled sintered glass filter. The crystals on the filter were redissolved in warm petroleum spirit (b.p. 60 - 80 °C) and the "chill then filter" cycle repeated twice. The crystals were then placed in a clean porcelain dish, covered with pricked aluminium foil and any remaining petroleum spirit allowed to evaporate. Analysis of the purified wax ester by gas liquid chromatography (glc) (Section 2.10.2) showed the synthesised wax ester to be 92 % pure based on the area occupied by the major peak.

2.10.2 Gas liquid chromatographic analysis of wax esters

The wax esters were separated from the other lipid components in the extract using

toluene as the tlc solvent and after location (Section 2.9.3) of the spot corresponding to the wax esters, they were scraped from the plate into a sintered glass filter. The isolated wax esters were extracted from the silica with 3 x 3 ml of $CHCl_3$. The $CHCl_3$ extract was evaporated in a conical tube as described in Section 2.8. The sample was re-dissolved in 5 - 10 µl toluene and 0·2 - 0·4 µl injected into a Perkin-Elmer 8420 capillary glc operated in the split mode. The column:split ratio was 1:25 or 1:50 depending on the amount of wax ester expected in the sample. The temperature programme used was an initial oven temperature of 250 °C increasing at 5 °C min^{-1} to a final oven temperature of 300 °C, which was held for 20 min. The injection and detector temperatures were 350 °C. A 12 m SGE BP-1 column was used. The instrument's integrator produced retention times and peak areas which were used to identify the wax esters by comparison with authentic wax ester standards (Nagi, 1981) and to quantify the identified wax esters by comparing the peak areas obtained with that of the internal standard. The amount of each wax ester component present in the sample was calculated using the equation:

$$\text{mg wax ester (g dry weight)}^{-1} = \frac{\frac{\text{area of component}}{\text{area of standard}} \cdot \text{mg standard added}}{\text{g dry weight of bacteria used in the extraction}}.$$

Each component was then calculated as a percentage of the total and chain length (cli) and unsaturation (ui) indices calculated using the equations:

$$\text{cli} = \frac{[2 \times (\%\ C_{36:2} + \%\ C_{36:1} + \%\ C_{36:0})] + (\%\ C_{34:2} + \%\ C_{34:1} + \%\ C_{34:0})}{100} \text{ and}$$

$$\text{ui} = \frac{[2 \times (\%\ C_{36:2} + \%\ C_{34:2} + \%\ C_{32:2})] + (\%\ C_{36:1} + \%\ C_{34:1} + \%\ C_{32:1})}{100}$$

respectively. These indices were used to give an indication of the variation of overall chain length and the degree of unsaturation of the wax ester composition in a sample. The values of these indices could range from 0·00 to 2·00. For instance, if the wax ester composition of a sample was 100 % C_{32} (where the % C_{32} = % $C_{32:2}$ + % $C_{32:1}$ + % $C_{32:0}$) then the chain length index would be 0·00, whereas if the composition was 100 % C_{36} (where the % C_{36} = % $C_{36:2}$ + % $C_{36:1}$ + % $C_{36:0}$), then the chain length index would be 2·00. Similarly, values of 0·00 or 2·00 would be obtained if the wax ester composition consisted of 100 % saturated wax esters (where the % saturated wax esters = % $C_{36:0}$ + % $C_{34:0}$ + % $C_{32:0}$) or 100 % di-unsaturated wax esters (where the % di-unsaturated wax esters = % $C_{36:2}$ + % $C_{34:2}$ + % $C_{32:2}$) respectively. However, the wax ester compositions usually observed are a complex mixture, and therefore, the values for the chain length index or unsaturation index would normally be between 0·00 and 2·00.

2.10.3 Gas liquid chromatographic analysis of the fatty acid methyl esters

2.10.3.1 Preparation of the fatty acid methyl esters

The fatty acid moieties of both the phospholipids and wax esters were prepared for analysis by glc by trans-methylation. After analysis of the isolated wax esters by glc the wax esters remaining were dissolved in $CHCl_3$, transferred to 15 ml pyrex screw cap tubes and the $CHCl_3$ removed by evaporation as described in Section 2.8. The phospholipids, located at the origin of the tlc plate used to separate the wax esters from the other lipid components, were scraped off the plate into 15 ml pyrex screw cap tubes. To each of the tubes, 2·0 ml of 2 % ($^{v}/_{v}$) H_2SO_4 in dry methanol was added and the tubes heated in a dry block for 3 h at 60 °C. After cooling to room temperature, 4·0 ml of diethyl ether was added followed by 5·0 ml of distilled water. The phases were allowed to separate and the organic phase removed and added to 5·0 ml of 0·05 % ($^{w}/_{v}$) Na_2CO_3. Again the phases were allowed to separate, the organic phase removed and passed through anhydrous Na_2SO_4 in a sinter funnel. The aqueous layer was washed with 2 x 2·0 ml diethyl ether and after the phases had separated, the diethyl ether was passed through anhydrous Na_2SO_4 which was then washed with a further 2 x 2·0 ml of diethyl ether.

The pooled diethyl ether filtrates were evaporated to dryness (Section 2.8) and the fatty acid methyl esters (FAME) were then purified by tlc (Section 2.9) using two solvents. The first solvent, 150:50:1 ($^{v}/v/_{v}$) petroleum spirit (b.p. 60 - 80 °C):diethyl ether:formic acid, was used to half way up the tlc plate. The plate was then removed from the solvent, allowed to dry and then placed in the second solvent, toluene, which was run to the top of the plate. The FAME were visualized (Section 2.9) and recovered from the tlc plate as described for the wax esters in Section 2.10.2.

2.10.3.2 Analysis of the fatty acid methyl esters by gas liquid chromatography

The analysis of the FAME samples were as described in Section 2.10.2 except that the temperature programme was an initial oven temperature of 200 °C, holding for 20 min then ramping to 250 °C at 10 °C min^{-1} with a final hold of 5 min. The injection and detector temperatures were 300 °C. The programme eluted all the FAME isothermally and then rapidly eluted other peaks, thus shortening the time between injections. No internal standard was present in the phospholipid samples hence the data obtained from the glc analysis was used only to determine the fatty acid compositions of the wax esters and phospholipids. Each component was then

calculated as a percentage of the total and chain length (cli) and unsaturation indices (ui) calculated using the equations:

$$cli = \frac{[2 \times (\% C_{18:1} + \% C_{18:0})] + (\% C_{16:1} + \% C_{16:0})}{100} \text{ and}$$

$$ui = \frac{(\% C_{18:1} + \% C_{16:1})}{100} \text{ respectively. These indices were used to give an indication}$$

of the overall chain length and the degree of unsaturation of the fatty acid composition in the sample. The values for the chain length index could be between 0·00 and 2·00 and for the unsaturation index between 0·00 and 1·00. Generally, the lower the index value then the shorter the overall chain length or the more saturated the composition, and vice versa.

2.10.4 Gas liquid-mass spectrometry

Total lipid extracts were analysed on a VG 70-250 S mass spectrometer (VG Ltd, Crewe Rd., Wythenshawe, Manchester, England) operated at 70 eV in the electron impact, positive ion mode. The mass spectrometer was connected via a direct line interface at 280 °C to a Hewlett Packard 5890 glc equiped with a Chrompack CP-Sil 5 fused silica capillary column. The samples were dissolved in 10 µl toluene and 0·2 µl injected into the instrument. The oven temperature programme was an initial temperature of 50 °C increasing at 3 °C min^{-1} to a final temperature of 250 °C. The injector temperature was 300 °C. The mass spectra of interest were then identified by comparison with the computer's library of spectra. The interpretation of fragment patterns and the assignment of individual mass peaks was then carried out.

2.11 Estimation of organic carbon and identification of the organic acids in culture supernatants

2.11.1 Estimation of the organic carbon concentration

The total carbon and the inorganic carbon in the supernatants of continuous culture samples were determined using a Shimadzu TOC 500 total carbon analyser (Dyson Instruments Ltd., Houghton-le-Spring, Tyne & Weir, DH5 0RH, England) and the organic carbon present was then obtained by difference. Both the total carbon channel and the inorganic carbon channel were calibrated by injecting, at least four times, 10 µl of the appropriate standard. The samples were injected at least three times and from the calibration of the instrument the concentration of the carbon in the samples was determined. The total carbon channel was operated at 680 °C and the inorganic carbon channel at 150 °C. The flow rate of carbon free air used was

150 ml min^{-1}. The standards used were, for the total carbon channel, 0·85 g potassium hydrogen phthalate per litre and for the inorganic carbon channel, a mixture of 1·77 g Na_2CO_3 and 1·40 g $NaHCO_3$ per litre.

2.11.2 Identification of the organic acids and estimation of the concentrations

The organic acids present in the supernatants of continuous culture samples were analysed by high pressure liquid chromatography. The organic acids were separated using an Aminex HPX-87-H organic acids column (Bio-rad Laboratories, 1414 Harbour Way South, Richmond, California) with 10 mM H_2SO_4 (filtered and de-gassed through a 0·22 μm filter) as the mobile phase flowing at 0·5 ml min^{-1}. The organic acids were detected at 210 nm using a UV monitor and the peaks recorded on the chart recorder. The peaks obtained were identified by comparison of their retention times with those of authentic standards. The standards, used to produce calibration curves, were injected four times and the continuous culture samples three times.

2.12 Estimation of the ammonia concentration

The estimation of the NH_3 concentration in culture supernatants was by the method of Chaney & Marbach (1962).

Reagents:

A 50·0 g phenol and 0·25 g sodium nitroprusside per litre

B 25·0 g NaOH and 10·0 ml sodium hypochlorite (14 % available chlorine) per litre

Procedure:

To 1·0 ml of reagent A was added 200 μl of the culture supernatant followed by the addition of 1·0 ml of reagent B with immediate mixing. The solution was then incubated for 20 min at 37 °C and the absorbance at 630 nm measured using a PU 8470 spectrophotometer. A calibration curve with an NH_3 concentration range of 0·0 - 2·0 mM was prepared each time the assay was used. The concentration of the NH_3 in the culture supernatant was then determined directly from the calibration curve. The stock standard $(NH_4)_2SO_4$ was titrated with H_2SO_4 to pH 2·0 to reduce the loss of NH_3 from solution during storage at minus 20 °C.

2.13 Collection of *Acinetobacter* sp. NCIB 8250 from continuous culture, the preparation of cell free extracts and assaying for NADP-dependent alcohol dehydrogenase activity

2.13.1 Collection and preparation of the cell free extracts used in the NADP-dependent alcohol dehydrogenase assay

The samples of *Acinetobacter* sp. NCIB 8250 used in this assay were collected from the effluent line of the continuous culture vessel (Section 2.5.3) into centrifuge tubes kept on ice. The samples collected from any one steady state were pooled and the cell pellet stored at minus 80 °C until samples had been collected from all the steady states being studied. Immediately before assaying these samples for NADP-dependent alcohol dehydrogenase activity the cell pellets were resuspended in 50 mM Tris-HCl, pH 8·0, containing 2 mM DTT so that the equivalent OD_{500} was greater than 20. The cells were then broken by ultrasonic disruption (Lucas-Dawes, London, England) with an ice/water slurry being used to cool the soniprobe and the cell suspensions. Three by 30 s periods of exposure to 80 W was used to disrupt the bacteria, with each period of disruption being followed by a cooling period of 30 s. After disruption the samples were microfuged in an MSE Microcentaur centrifuge (13000 rpm, 10 min, 4 °C), the supernatant separated from the pellet and used in the NADP-dependent alcohol dehydrogenase assay.

2.13.2 NADP-dependent alcohol dehydrogenase assay

One unit of enzyme activity was defined as 1 μmol of substrate converted per min. The molar absorption coefficient of NADPH was assumed to be $6{\cdot}3 \times 10^3$ $M^{-1}.cm^{-1}$ at 340 nm (Bergmeyer, 1985). The initial concentration of hexan-1-ol in the assay buffer was calculated from its M_r, density and purity, the appropriate volume being dispensed using a Hamilton syringe. The oxidation of hexan-1-ol was then measured in an assay mixture containing:

800 μl of 100 mM glycine/NaOH, pH 10·2 (assay concentration 80 mM), containing 25 mM hexan-1-ol (assay concentration 20 mM);

100 μl of 10 mM $NADP^+$ (assay concentration 1 mM);

Cell free extract;

Distilled water to 1·0 ml.

The assay was initiated by addition of the cell free extract. All the assays were carried out in duplicate and at a constant temperature of 27 °C. The rate of $NADP^+$ reduction was measured at 340 nm in 1 ml plastic cuvettes using a Pye-Unicam SP8-100 spectrophotometer. From the rate of reduction of $NADP^+$ the specific activity of the NADP-dependent alcohol dehydrogenase present in the cell free extracts was

calculated.

2.14 Estimation of the protein concentration

Protein concentrations were estimated by the method of Bradford (1976) using bovine serum albumin (Fraction V) to construct standard curves. The sample or standard (100 µl) was added to 5·0 ml of the protein reagent and after 5 min the absorbance of the solution measured at 595 nm using a PU 8470 spectrophotometer.

2.15 Oxidase test

This test was used in conjunction with other tests (Section 2.2) to determine the purity of a bacterial culture streaked on a nutrient agar plate (Section 2.3.1.2). A positive reaction produced a dark purple colour in the colonies on the agar surface within 10 - 15 s when 5 - 10 ml of a 1 % ($^{w}/_{v}$) tetramethyl-*p*-phenylenediamine dichloride solution was poured on to the agar plate containing the culture to be tested. *Acinetobacter* strains are negative for this reaction and *P. putida* was used as a positive control.

2.16 Statistical analysis of data

The results presented in the Tables and Figures in chapters 3 to 6 are the mean ± one standard deviation. The number of analyses used in these calculations was three, unless otherwise stated. The wax ester compositions and the fatty acid compositions of the wax esters and phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in both batch and continuous culture were analysed using analysis of variance techniques to determine significant relationships within the data. This work was carried out by Mr. T.C. Aitchison and Mr. D. Watt in the Department of Statistics, University of Glasgow using software packages available in that department.

Statistical analyses of the specific activity of the enzyme NADP-dependent alcohol dehydrogenase with either wax ester content, growth temperature or specific growth rate were carried out by Mr. D Buchanan of Paisley College, Paisley, Scotland using the suite of programs at the Department of Statistics, University of Glasgow. Analysis of variance was used to determine significant relationships and where appropriate, simple linear regression analysis was used to show relationships.

The results of all statistical analyses are quoted as being either significant ($P < 0{\cdot}05$) or not significant ($P > 0{\cdot}05$).

2.17 Cleaning of glassware

2.17.1 General purpose glassware

This type of glassware (beakers, measuring cylinders, test tubes, etc) was soaked overnight in a 2 % ($^{w}/_{v}$) Haemosol solution and then rinsed with copious amounts of tap water followed by distilled water before being placed in an oven to dry.

2.17.2 Growth flasks

All flasks used for the growth of micro-organisms were placed in a solution of 2 % ($^{w}/_{v}$) Haemosol and then heated in the autoclave to 109 °C (5 psi) for 90 min. The glassware was then rinsed with copious amounts of tap water, followed by distilled water before being placed in an oven to dry.

2.17.3 Glassware used for the extraction of lipids

All glassware used to extract lipids were soaked overnight in 5 % ($^{v}/_{v}$) Decon 90, rinsed with copious amounts of tap water and distilled water before being dried in an oven.

2.17.4 Pipettes

The non-absorbent cotton wool plug was first removed from the pipettes before they were soaked overnight in a solution of 2 % ($^{w}/_{v}$) Haemosol. The pipettes were rinsed twice in tap water and then in distilled water using an automatic rinsing apparatus before being placed in an oven to dry. The pipettes were re-plugged with non-absorbent cotton wool before being re-used.

2.18 Distilled Water

Glass distilled water was used for making all media components and assay reagents and for rinsing all glassware after washing in tap water.

2.19 Safety

Bacterial cultures were killed by autoclaving and all spillages of live bacteria were

swabbed with 10 % ($^{v}/_{v}$) propan-1-ol. Silica gel used in preparing the tlc plates was dispensed into an Erlemeyer flask in a fume cupboard. The distilled water was added and the flask stoppered before removing the flask from the fume cupboard. Any spilt silica gel was wiped up with damp laboratory tissue paper which were then wrapped in dry laboratory tissue paper before depositing in a bin. All other safety precautions followed were as described in the University of Glasgow Safety Handbook (1987) and the Department of Biochemistry's Safety Code and Radiation Rules (1989). From 1990 COSHH assessments were completed for all procedures.

Chapter Three

Validation of Experimental Methods

3.1 Introduction

The results and discussions presented in this chapter deal with some of the routine analyses carried out to support the main line of research. The results of experiments designed to test the validity of the methods and procedures that were used to obtain the data that are presented in the later chapters are also detailed here. Finally, this chapter includes results from experiments that yielded interesting observations but which were not investigated further as they did not form part of the main thrust of the research.

3.2 Results and Discussion

3.2.1 Calibration curves of optical density against dry weight and against the carbon content of cells

Calibration curves of OD_{500} against dry weight (Figure 3.1a) and against the carbon content of the cells (Figure 3.1b) were constructed for *Acinetobacter* sp. NCIB 8250 grown to stationary phase on both the media with a low concentration of succinate and with a low concentration of $(NH_4)_2SO_4$. The cells obtained were resuspended in distilled water to an equivalent OD_{500} of approximately 200 and the dry weight and organic carbon content in these stock suspensions determined (Table 3.1). From these determinations the carbon content of the cells as a percentage of the dry weight was 37·9 - 41·5. These values were lower than that obtained by Fewson (1985) who determined the the carbon content of *Acinetobacter* sp. NCIB 8250 to be 47·64 ± 1·40 % when *Acinetobacter* sp. NCIB 8250 was grown to carbon limitation in batch culture.

From each stock cell suspension a duplicate series of dilutions were made and the OD_{500} determined for each dilution. The average OD_{500} was then plotted against the corresponding value for the dry weight or carbon content (Figure 3.1). The OD_{500} against dry weight curves were the same for either limitation and were linear up to an OD_{500} of approximately 0·55 (Figure 3.1a). Above this optical density the cultures were diluted so that the measurements fell within the range of the calibration curve. This curve was used to follow the increase in the dry weight when *Acinetobacter* sp. NCIB 8250 was grown in batch culture. It was also used in helping to calculate the amount of wax ester internal standard to be added to samples when extracting the lipids for wax ester analysis and in the calculation of the amount of wax esters accumulated per g dry weight, as determined by glc analysis.

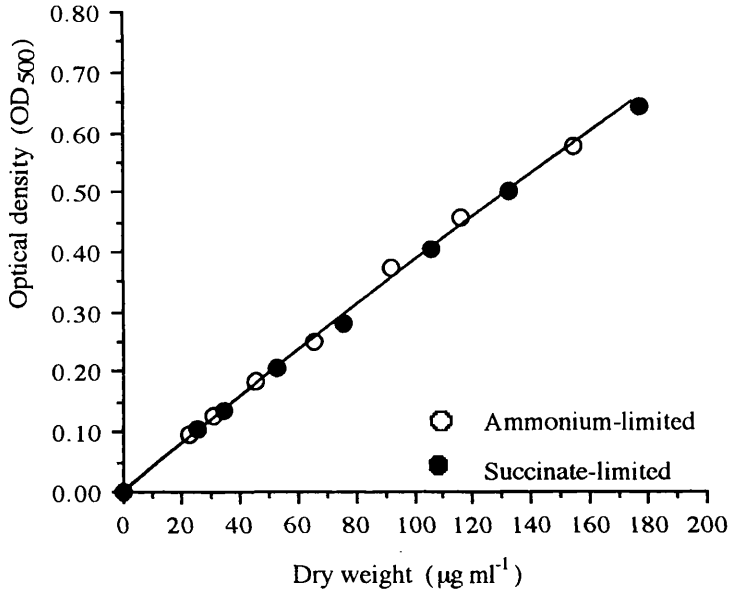
Figure 3.1

Calibration curves of optical density against dry weight and cell total carbon content

Acinetobacter sp. NCIB 8250 was grown in batch culture at 30 °C in 2 l flasks on the medium with a low concentration of succinic acid (Section 2.3.2.2) or on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in stationary phase (Section 2.5.2), washed twice with chilled distilled water and the pellets resuspended in distilled water to an equivalent OD_{500} of approximately 200. Three 1 ml aliquots from each of the two stock cell suspensions were then dried to constant weight in aluminium weigh boats and in using an oven at 105 °C to determine the dry weight of the stock cell suspensions. The value for the dry weight for each of the stock cell suspensions was taken as the mean of the three values. The total carbon content of the cells in the stock cell suspensions was determined using a total carbon analyser (Section 2.11.1). From each stock cell suspension a duplicate series of dilutions were made and the OD_{500} determined for each dilution. The average OD_{500} was then plotted against the corresponding value for the dry weight or carbon content.

Figure 3.1a shows the calibration curve for optical density against dry weight and Figure 3.1b shows the calibration curve for optical density against total carbon content.

a) calibration curve of optical density against dry weight



b) calibration curve of optical density against carbon concentration

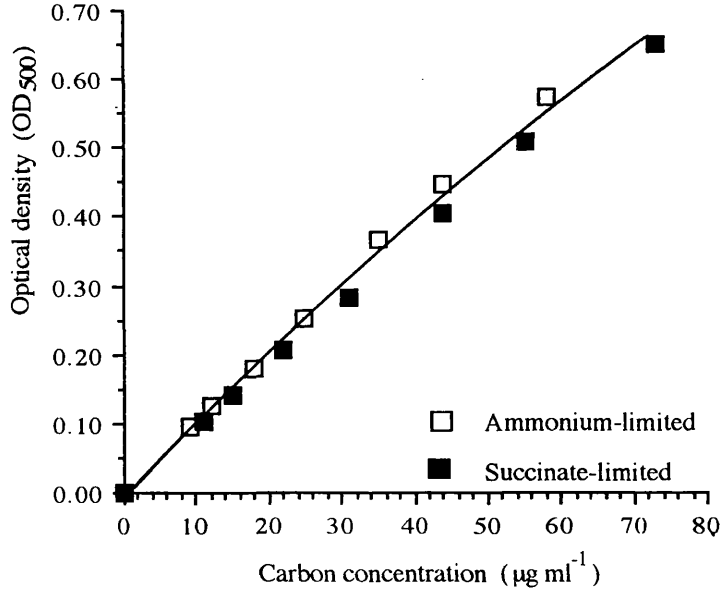


Table 3.1

Determination of the percentage carbon content of *Acinetobacter* sp. NCIB 8250 grown in batch culture

Acinetobacter sp. NCIB 8250 was grown in batch culture at 30 °C in 2 l flasks on the medium with a low concentration of succinic acid (Section 2.3.2.2) and on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in stationary phase (Section 2.5.2), washed twice with chilled distilled water and the pellets resuspended in distilled water to an equivalent OD_{500} of approximately 200. Three 1 ml aliquots from each of the two stock cell suspensions were then dried to constant weight in aluminium weigh boats and in using an oven at 105 °C to determine the dry weight of the stock cell suspensions. The value for the dry weight for each of the stock cell suspensions was taken as the mean of the three value. The total carbon content of the cells in the stock cell suspensions was determined using a total carbon analyser (Section 2.11.1). The results for the dry weight and total carbon content are the mean ± standard deviation of three determinants. The percentage carbon content was then derived form these data.

Limitation	Dry weight ($g\ l^{-1}$)	Carbon content ($g\ l^{-1}$)	% carbon
Ammonium	4·62 ± 0·05	1·75 ± 0·08	37·9
Succinate	5·30 ± 0·17	2·20 ± 0·03	41·5

In the calibration curve of OD_{500} against carbon content (Figure 3.1b) there was no discernible difference, by eye, between the data for the carbon content of the ammonium-limited sample and that of the succinate-limited sample. These data suggests that although *Acinetobacter* sp. NCIB 8250 accumulates wax esters under ammonium limitation (Nagi, 1981; Fixter *et al.*, 1986; this thesis) the amount accumulated does not significantly influence the carbon content of the cells. An explanation for this might be that under ammonium limitation the protein content of the cells decreases and that the loss of carbon from the protein was countered by the increase in the carbon from the wax esters.

3.2.2 Determination of the nutrient limitation in culture supernatants

In continuous culture, the rate of growth of a micro-organism is limited by the concentration of one of the medium components, the other components being present in excess (Tempest, 1970). In the experiments described in this thesis, proof that the desired limitation had been achieved was obtained by assaying culture supernatants and confirming the absence of significant amounts of either organic carbon or NH_3 (Table 3.2). In the medium with a low concentration of succinic acid the initial utilisable organic carbon concentration was 168 mg l^{-1}. Using a total carbon analyser to determine the organic carbon concentration in culture supernatants the minimum amount of carbon detectable was 4 - 6 mg l^{-1}. The initial NH_3 concentration in the medium designed to give ammonium-limited growth was 1·5 mM and using the method of Chaney & Marbach (1962) it was not possible to detect less than 0·08 mM NH_3 i.e. less than 5 % of the initial NH_3 concentration (Figure 3.2). Supernatants of cultures growing on the medium with a low concentration of succinic acid were also assayed for NH_3 and supernatants of cultures growing on the medium with a low concentration of $(NH_4)_2SO_4$ were assayed for organic carbon. These tests were done to prove the presence of significant amounts of the non-limiting substrate. Therefore cultures were designated carbon or ammonium-limited only if there was less than 5 % of the initial concentration of the growth limiting substrate present and an excess of the non-limiting substrate in the culture supernatant.

Occasionally cultures were found to have significant amounts of both organic carbon and NH_3 present in the culture supernatants (Table 3.2c). Under these conditions the cultures were designated as being oxygen-limited. Proof that the oxygen transfer rate had been limiting growth was obtained by increasing the stirrer speed, thereby making more oxygen available to the culture. Under these increased oxygen transfer conditions the biomass of the culture increased and the concentration of the

Table 3.2

Confirmation of the nutrient limitation when *Acinetobacter* sp. NCIB 8250 was grown in continuous culture

Acinetobacter sp. NCIB 8250 was grown in continuous culture at 30 °C (Section 2.5.3) on either the medium with a low concentration of succinic acid (Section 2.3.2.2) or on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). During steady state conditions a 30 ml sample of culture was harvested (Section 2.7) and the supernatant assayed for organic carbon (Section 2.11.1) and NH_3 (Section 2.12).

The data presented in this Table shows a selection of the assay results on the analysis of culture supernatants taken from succinate-limited (Table 3.2a), ammonium-limited (Table 3.2b) and oxygen-limited (Table 3.2c) continuous cultures.

a) succinate-limited

Dilution rate (h^{-1})	Biomass ($\mu g.ml^{-1}$)	NH_3 concentration (mM)	Carbon concentration ($\mu g.ml^{-1}$)
0·14	104	nd	< 8
0·31	120	nd	< 8
0·49	130	nd	< 8

b) ammonium-limited

Dilution rate (h^{-1})	Biomass ($\mu g.ml^{-1}$)	NH_3 concentration (mM)	Carbon concentration ($\mu g.ml^{-1}$)
0·14	116	< 0·08	202
0·48	110	< 0·08	283
0·62	150	< 0·08	259

c) oxygen-limited

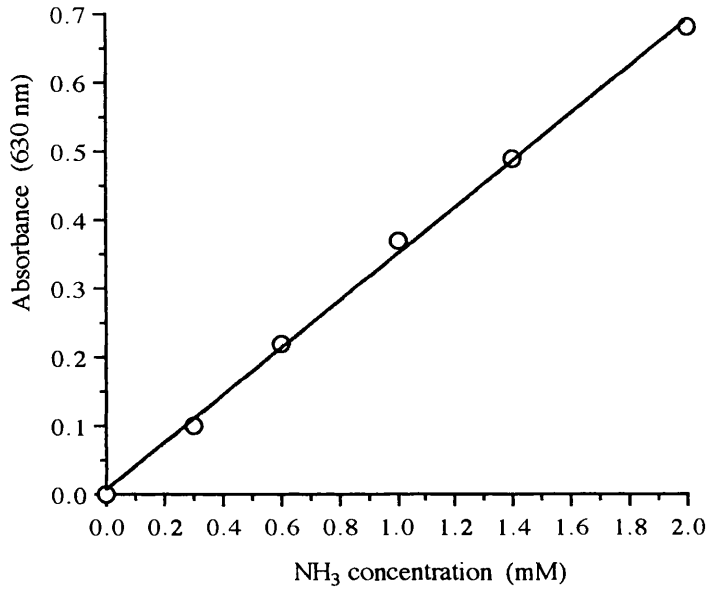
Dilution rate (h^{-1})	Biomass ($\mu g.ml^{-1}$)	NH_3 concentration (mM)	Carbon concentration ($\mu g.ml^{-1}$)
0·30	82	0·27	350
0·48	55	0·44	394
0·58	63	0·32	307
0·61	127	0·10	206

nd = not determined

Figure 3.2

An example of a typical calibration curve used for the determination the NH_3 concentration in culture supernatants

This Figure shows a typical calibration curve obtained when the NH_3 concentration of culture supernatants was determined using the method of Chaney & Marbach (1962) (Section 2.12).


Absorbance (630 nm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
NH3 concentration (mM)

remaining growth limiting nutrient decreased still further. This point is illustrated in a comparison of the samples taken at a specific growth rate of 0·48 h^{-1} from a culture growing on the medium with a low concentration of $(NH_4)_2SO_4$ but under oxygen limitation (Table 3.2c). Under these conditions the biomass was only 55 mg dry weight l^{-1} and the NH_3 concentration was 0·44 mM. However, when the stirrer rate was increased and ammonium-limited conditions were achieved (Table 3.2b) the biomass was 110 mg dry weight l^{-1} with no detectable NH_3 present in the culture supernatant. The carbon concentration remaining in the culture supernatant was also higher under the oxygen-limited conditions than under the ammonium-limited conditions (Tables 3.2 b & c).

3.2.3 Extraction and analysis of the lipids in *Acinetobacter* sp. NCIB 8250

In the initial experiments on the separation and analysis of the wax esters by glc, total lipid extracts were used. During these analyses it was noted that there were peaks present that could not be assigned as wax esters. These peaks eluted after the wax esters and to simplify the chromatogram and reduce the analysis time the wax esters were purified from the other lipid components by tlc.

To test that the procedure for the extraction and analysis of wax esters was reproducible, *Acinetobacter* sp. NCIB 8250 was grown in batch culture using the medium with a low concentration of $(NH_4)_2SO_4$ and harvested in stationary phase. The cells obtained were resuspended and divided into four equal samples, A - D. The lipids in each sample were extracted, the wax esters purified by tlc and then analysed four times by glc. The results (Table 3.3) show the wax ester compositions and the total amount of wax esters present for each of the four samples. Comparison of the wax ester compositions found in this experiment with that reported by Fixter *et al.* (1986), for the same strain grown and harvested under the same conditions, showed that the wax ester compositions were very similar.

Analysis of variance on the data, summarised in Table 3.3a, revealed that there was no significant difference ($P \gg 0{\cdot}05$) in the total amount of wax esters accumulated for the samples A, C and D. However, the amount of wax esters present in sample B was shown to be significantly different from the amount of wax esters present in samples A, C and D (Table 3.3b). Further statistical analysis based on Student's t-test and using the results from extractions B and C [these extractions show the largest difference in total wax ester content, i.e. 1·28 mg (g dry weight)$^{-1}$], showed that the 95 % confidence limits were 0·29 - 2·65 mg (g dry weight)$^{-1}$ for the difference in the amounts of wax esters present in each sample. However, for the

Table 3.3

Reproducibility of the extraction and analysis of the wax esters extracted from *Acinetobacter* sp. NCIB 8250

Acinetobacter sp. NCIB 8250 was grown in batch culture at 30 °C in 2 l flasks containing the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The culture was harvested (Section 2.7) in stationary phase (Section 2.5.2), washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0, resuspended in 4 ml of the buffer and divided into four 1 ml aliquots. The lipids from each aliquot were then extracted (Section 2.8), the wax esters separated from the other lipid components in the sample by tlc (Section 2.9) and then analysed by glc (Section 2.10). Each of the four extracted aliquots were analysed four times.

Table 3.3a shows the results for each wax ester component (mean ± standard deviation for the four analyses) in the four extractions and the total amount of wax esters accumulated in mg wax ester (g dry weight)$^{-1}$. Table 3.3b shows the range for the value of P in the analysis of variance that was carried out on the total amount of wax esters accumulated in each of the aliquots.

a) wax ester composition

	mg wax esters (g dry weight)$^{-1}$			
Wax	extraction A	extraction B	extraction C	extraction D
32:1	0·91 ± 0·12	0·95 ± 0·10	0·90 ± 0·14	0·98 ± 0·09
32:0	11·10 ± 0·17	11·64 ± 0·09	11·01 ± 0·20	11·32 ± 0·16
34:2	0·35 ± 0·03	0·40 ± 0·03	0·34 ± 0·05	0·30 ± 0·13
34:1	2·06 ± 0·06	2·18 ± 0·04	2·09 ± 0·06	2·03 ± 0·12
34:0	7·99 ± 0·05	8·18 ± 0·14	7·81 ± 0·18	7·96 ± 0·18
36:2	0·52 ± 0·05	0·51 ± 0·14	0·54 ± 0·13	0·50 ± 0·09
36:1	0·91 ± 0·15	0·97 ± 1·11	0·95 ± 0·11	0·93 ± 0·08
36:0	1·82 ± 0·03	1·78 ± 0·12	1·71 ± 0·10	1·75 ± 0·07
Total	25·64 ± 0·27	26·62 ± 0·50	25·34 ± 0·72	25·77 ± 0·40

b) determination of P values in the comparison of the different extractions

	extraction A	extraction C	extraction D
extraction B	$0{\cdot}01 > P > 0{\cdot}002$	$0{\cdot}02 > P > 0{\cdot}01$	$0{\cdot}05 > P > 0{\cdot}02$

upper limit, the difference at the 95 % confidence limit represents a maximum difference from the mean of only 10·5 %.

From this experiment it was concluded that the extraction and analysis of the wax esters from samples of *Acinetobacter* sp. NCIB 8250 was reproducible from sample to sample and that multiple analyses on a single sample, rather than multiple analyses of several samples, would be sufficient in the experiments designed to examine the effect of growth temperature, specific growth rate and nutrient limitation on the wax ester content and composition.

3.2.4 Evidence for the presence of diglycerides in the lipids extracted from *Acinetobacter* sp. NCIB 8250

During the preliminary glc analysis of the wax esters, peaks appeared 5 - 10 min after the $C_{36:0}$ wax ester (Figure 3.3) that did not correspond with authentic wax ester standards. Using glc-ms these later eluting peaks were unambiguously identified as being mono-unsaturated $C_{16:0}$ - $C_{18:1}$, di-unsaturated $C_{16:1}$ - $C_{18:1}$ and di-unsaturated $C_{18:1}$ - $C_{18:1}$ diglycerides (Figure 3.4). In Figure 3.4a the fragment at $^{m}/_{z}$ = 576 corresponds to the mass ion ($^{m}/_{z}$= 594, i.e. the molecular weight of the whole molecule minus an electron) minus H_2O and the fragments at $^{m}/_{z}$ = 339 and 313 correspond to a glycerol back bone with a $C_{18:1}$ fatty acid attached and a glycerol back bone with a saturated C_{16} fatty acid attached, respectively. The fragments at $^{m}/_{z}$ = 264 and 239 correspond to the $C_{18:1}$ and $C_{16:0}$ fatty acid radicals, respectively. In Figure 3.4b the fragment at $^{m}/_{z}$ = 574 corresponds to the mass ion ($^{m}/_{z}$= 592) minus H_2O and the fragments at $^{m}/_{z}$ = 311 and 237 corresponded to a mono-unsaturated version of the fragments observed at $^{m}/_{z}$ = 313 and 239 in Figure 3.4a.

There was no evidence using tlc or glc-ms for the presence of triglycerides although there are reports that some strains of *Acinetobacter* can accumulate triglycerides and wax esters (Fixter & McCormack, 1976) and that *Acinetobacter* sp. HO1-N can accumulate mono, di and triglycerides as well as wax esters (Makula *et al.*, 1975). *Acinetobacter* sp. HO1-N and the two strains reported by Fixter & McCormack (1976) to be capable of producing triglcerides are classified as being in group A1, according to the scheme of Baumann *et al.* (1968). The strain NCIB 8250, included in the survey by Fixter & McCormack (1976) and classified as being in the group A2, was not reported as being a triglyceride producer. It therefore leaves some doubt as to whether *Acinetobacter* sp. NCIB 8250 accumulates diglycerides (and perhaps triglycerides) or whether the diglycerides found in this present work were an artifact of the extraction procedure. For instance, bacteria have the enzyme phospholipase C

Figure 3.3

A gas chromatogram of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture at 30 °C under ammonium limitation prior to preparative thin layer chromatography

Acinetobacter sp. NCIB 8250 was grown at 0·28 h^{-1} in continuous culture at 30 °C under ammonium limitation (Section 2.5.3) and a 30 ml sample was harvested (Section 2.7) during steady state. The pellet was washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0, the lipids extracted (Section 2.8) and the sample analysed by glc (Section 2.10.2).

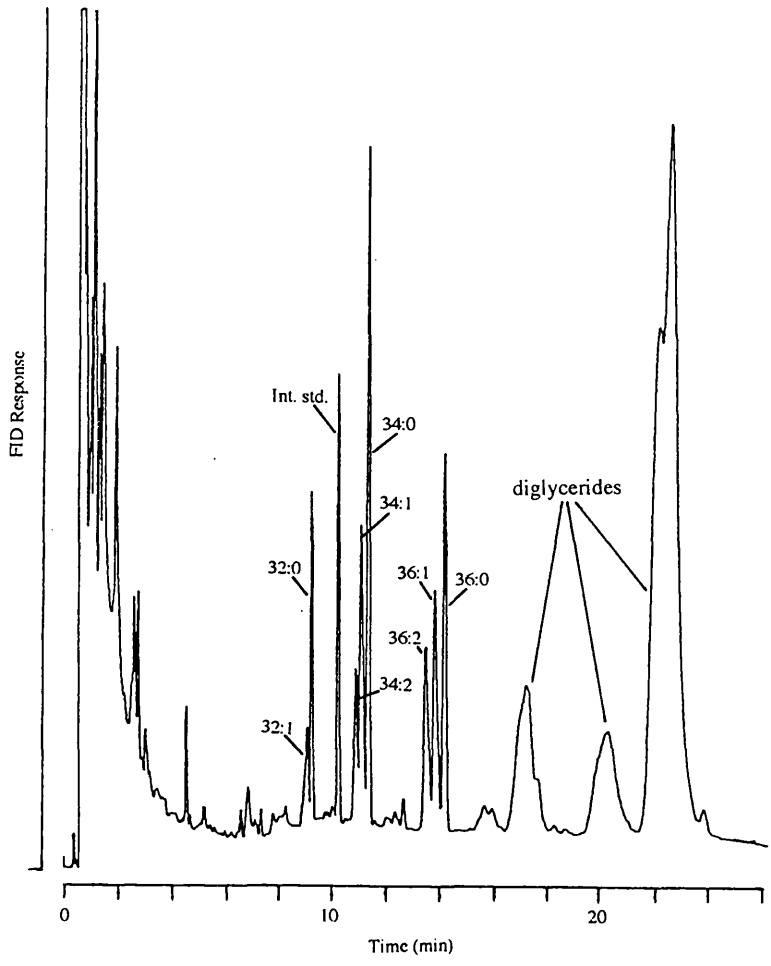

FID Response
Int. std.
34:0
34:1
32:0
36:1
36:0
36:2
34:2
32:1
diglycerides
0
10
20
Time (min)

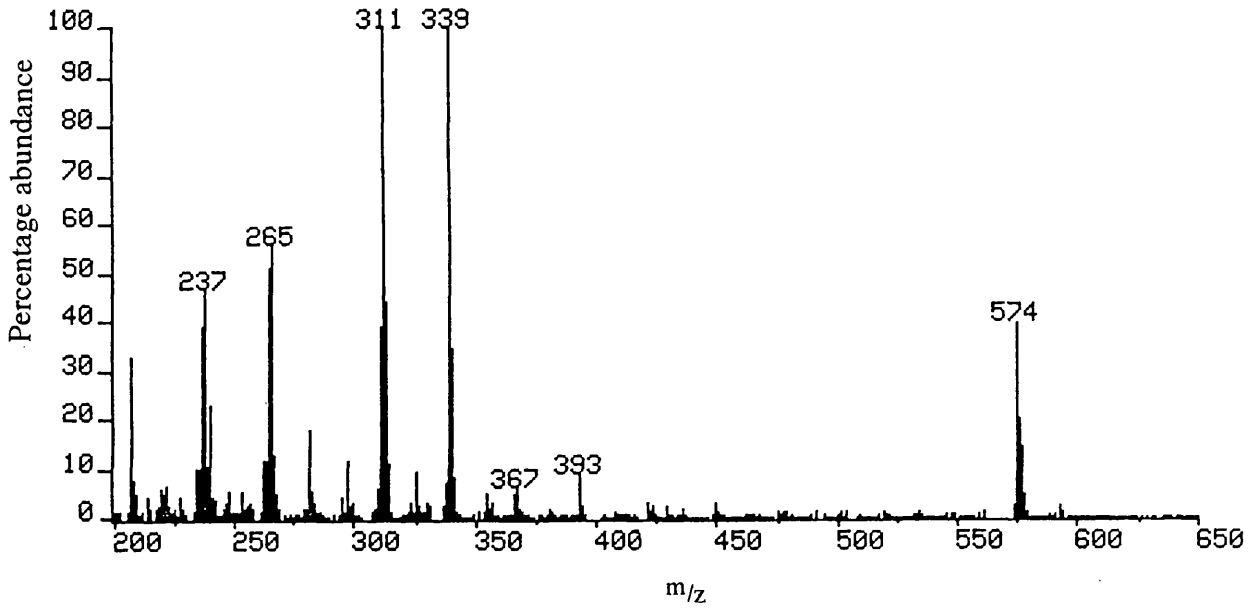
Figure 3.4

Mass spectra of diglycerides found in the lipids extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under ammonium limitation

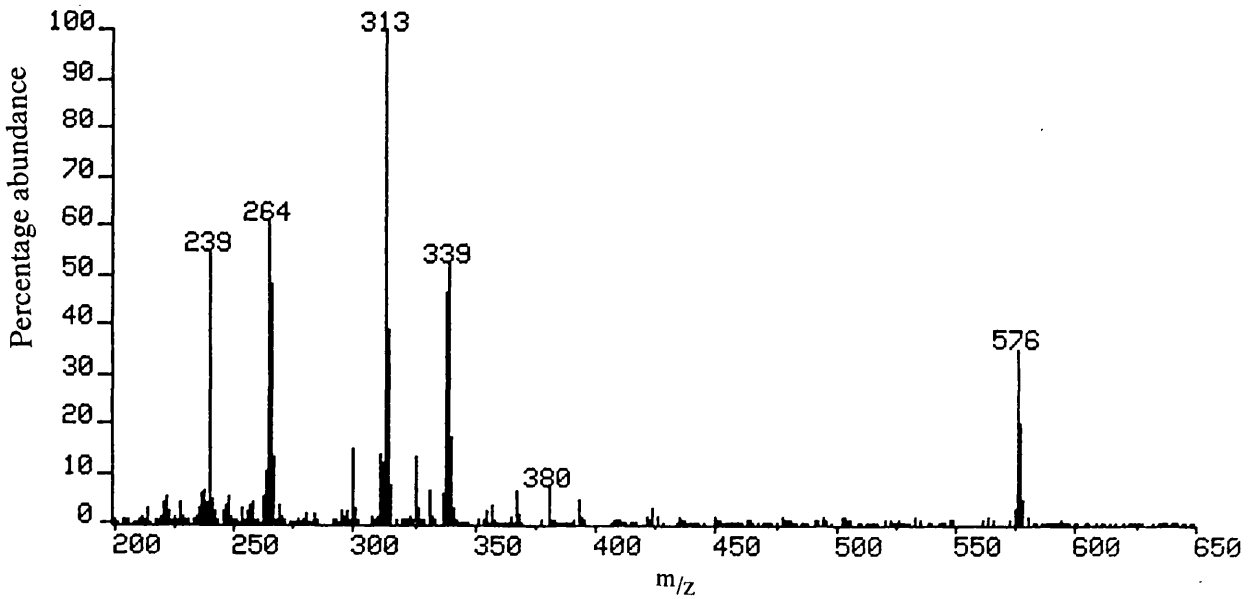
Acinetobacter sp. NCIB 8250 was grown at 0·28 h^{-1} in continuous culture at 30 °C under ammonium limitation (Section 2.5.3) and a 30 ml sample of culture was harvested (Section 2.7) during steady state. The pellet was washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0, the lipids extracted (Section 2.8) and the sample analysed by glc-ms (Section 2.10.4).

Figure 3.4a shows the mass spectrum of a compound eluting from the glc and identified by ms as a mono-unsaturated diglyceride containing a $C_{16:0}$ fatty acid and a $C_{18:1}$ fatty acid. Figure 3.4b shows the mass spectrum of a compound eluting from the glc and identified by ms as a di-unsaturated diglyceride containing a $C_{16:1}$ fatty acid and a $C_{18:1}$ fatty acid.

a) Mass spectrum of a $C_{16:0}$ - $C_{18:1}$ mono-unsaturated diglyceride



b) Mass spectrum of a $C_{16:1}$ - $C_{18:1}$ di-unsaturated diglyceride



in the periplasm. This enzyme catalyses the hydrolysis of the head group of the phospholipid from the glycerol back bone (Gennis, 1989), producing a diglyceride. Therefore, it may be that the diglycerides observed in this present work originated from the phospholipids in the membrane and that *Acinetobacter* sp. NCIB 8250 does not accumulate diglycerides.

3.2.5 High pressure liquid chromatographic analysis of the culture supernatants

Supernatants from both succinate- and ammonium-limited continuous culture experiments were analysed by HPLC for the presence of organic acids. It was of interest to see what compounds, if any, *Acinetobacter* sp. NCIB 8250 was excreting into the culture supernatant as this might help to give a more complete understanding of the physiology of the organism and the biochemistry of wax ester synthesis.

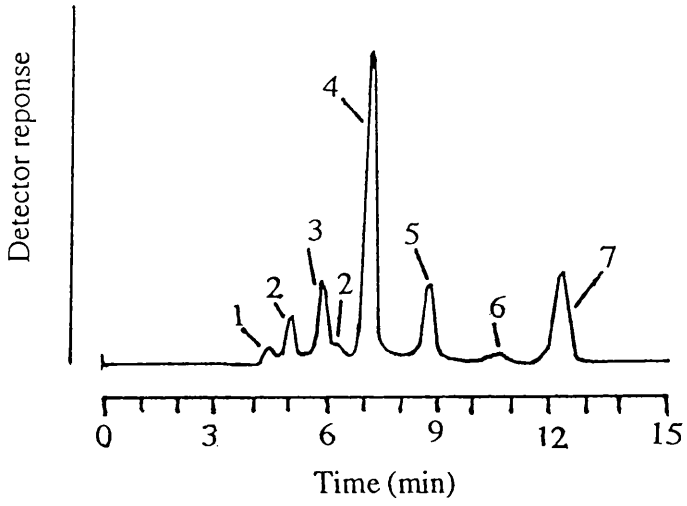
Under succinate limitation there were no peaks detected in the supernatants from cultures grown at low specific growth rates, although at higher specific growth rates, e.g. above 0·5 h^{-1}, succinate could be detected. However, under ammonium limitation numerous organic acids were detected at low specific growth rates (Figure 3.5) but as the specific growth rate was increased the concentration of these organic acids decreased except in the case of succinate, which increased. Some of these organic acids were identified on the basis of similar retention times with authentic standards, although only three of the peaks, succinate, acetate and fumarate, could be assigned uniquely. The concentration of these organic acids at specific growth rates less than 0·2 h^{-1} were approximately 4 mM, 300 μM and 15 μM respectively. At higher specific growth rates only fumarate (due to its high molar extinction coefficient) and succinate could be detected, the concentration of fumarate at these specific growth rates was less than 10 μM. The concentration of succinate observed in the culture supernatant increased as the specific growth rate increased, i.e. the amount of succinate taken up by the culture decreased although the actual rate of succinate consumption increased with increased specific growth rate.

The other peaks observed were only assigned as being either pyruvate or malate, which co-eluted under the conditions used and either oxaloacetate or 2-oxoglutarate, which also co-eluted. If this latter peak was oxaloacetate then the former peak would probably be pyruvate, as it is known that oxaloacetate spontaneously decarboxylates to pyruvate, the half-life of oxaloacetate being approximately 3·5 h (Figure 3.6). Determination, using a lactate dehydrogenase assay, showed that pyruvate was present in the supernatant samples at concentrations of approximately 0·2 mM.

Figure 3.5

A high pressure liquid chromatogram of the organic acids from an ammonium-limited continuous culture supernatant

Acinetobacter sp. NCIB 8250 was grown at 0·14 h^{-1} in continuous culture at 30 °C under ammonium limitation (Section 2.5.3) and a 30 ml sample of the culture was harvested (Section 2.7) during steady state conditions and the supernatant analysed by HPLC (Section 2.11.2).

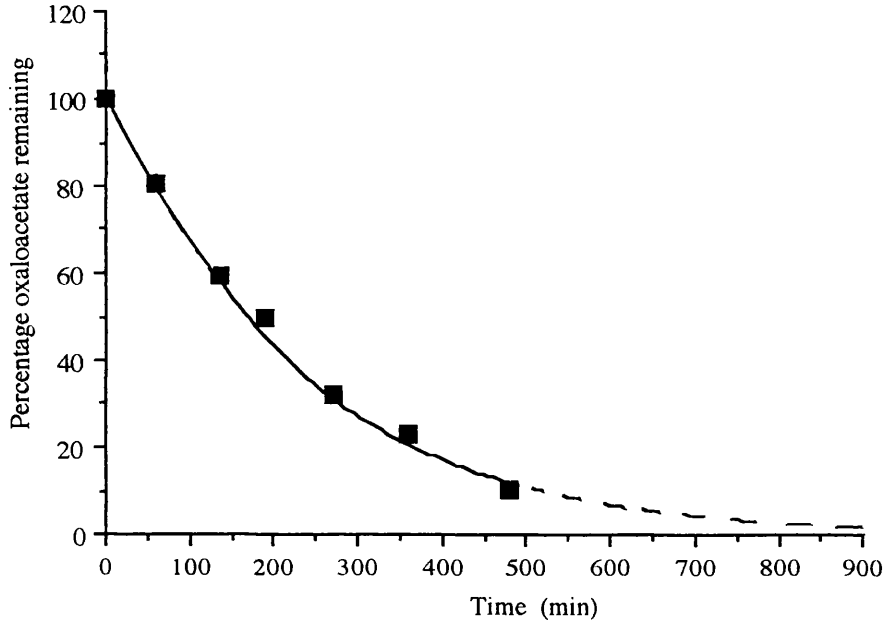


1 solvent front
2 unknown
3 ?oxaloacetate/2-oxoglutarate
4 ?pyruvate/malate
5 succinate
6 acetate
7 fumarate

Figure 3.6

Determination of the half-life of oxaloacetate under the growth conditions normally used in the continuous culture of *Acinetobacter* sp. NCIB 8250

The continuous culture vessel containing 500 ml of the standard salts medium (Section 2.3.2.1) together with 1 ml of the trace metal supplement (Section 2.3.3) and 1 mM oxaloacetate was used to monitor the decrease in the concentration of oxaloacetate over an eight hour period. Samples were removed from the vessel at approximately 60 min intervals and assayed by HPLC (Section 2.11.2) for the presence of oxaloacetate.


Percentage oxaloacetate remaining
120
100
80
60
40
20
0
0
100
200
300
400
500
600
700
800
900
Time (min)

However, the peak observed on HPLC chromatograms, that corresponded with the authentic pyruvate standard, could be not solely pyruvate as the concentration determined from the peak area using a pyruvate calibration curve was over 4·0 mM. Therefore, it would appear that when grown on succinic acid *Acinetobacter* sp. NCIB 8250 produces a very complicated mixture of overflow metabolites.

The remaining peaks observed on the HPLC chromatograms did not correspond with any of the other standards tried, such as isocitrate, citrate and glyoxylate. Therefore, this line of research, although interesting in itself, was not pursued further.

Chapter Four

Effect of Specific Growth Rate, Growth Temperature and Nutrient Limitation on the Wax Ester Content and Composition in *Acinetobacter* sp. NCIB 8250

4.1 Introduction

There have been numerous studies on the metabolic versatility of *Acinetobacter* strains and in this respect *Acinetobacter* sp. NCIB 8250 has been studied, in batch culture at least, in considerable detail (Fewson, 1967; Fixter *et al.*, 1986; Fewson, 1991). Fixter and co-workers have studied the wax esters in this strain (Fixter & Fewson, 1974; Fixter & McCormack, 1976; Fixter *et al.*, 1986; Sherwani & Fixter, 1989) and several other workers (Gallagher, 1971; Makula *et al.*, 1975; Bryn, 1977; de Witt, 1982; Geigert, 1984) have studied the wax ester content and composition in other strains of *Acinetobacter* although most of this work has only been to show that those strains produced wax esters. Also, there has been very little enzymic or genetic work carried out on the actual pathway of wax ester biosynthesis.

The work described in this chapter details a more in depth study on what effect the growth medium, growth temperature and specific growth rate had on the growth of *Acinetobacter* sp. NCIB 8250 and on the wax ester content and composition of that strain. This work expands on the results of Nagi (1981) and should serve to underpin any future study on the enzymes in this metabolic pathway.

4.2 Results

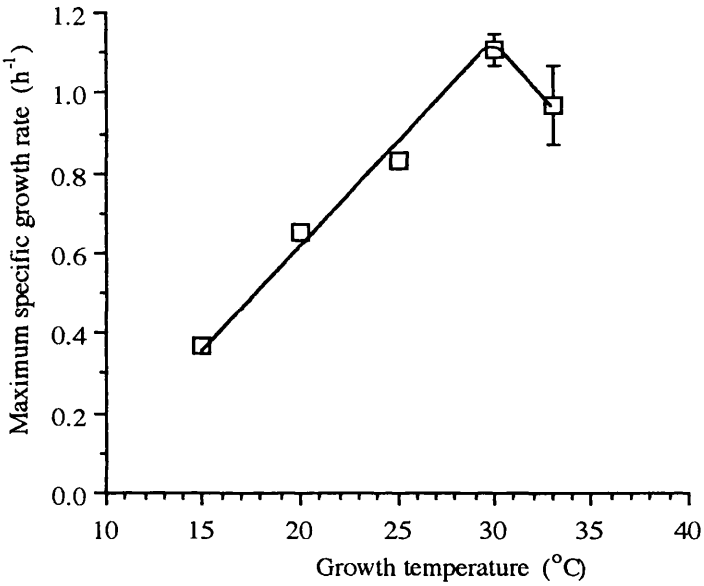
4.2.1 Growth rates and growth yields of *Acinetobacter* sp. NCIB 8250 grown in batch and continuous culture

Acinetobacter sp. NCIB 8250 was grown in batch culture at 15 °C and 30 °C using both the medium with a low concentration of succinic acid and the medium with a low concentration of $(NH_4)_2SO_4$ and at 25 °C and 33 °C in the standard salts medium. Under these growth conditions the mean generation times, regardless of the medium composition, were 210 min ($\mu = 0{\cdot}20\ h^{-1}$) at 15 °C and 60 min ($\mu = 0{\cdot}69\ h^{-1}$) at 30 °C. At 25 °C and 33 °C the mean generation times were approximately 70 min ($\mu = 0{\cdot}59\ h^{-1}$) and 75 min ($\mu = 0{\cdot}55\ h^{-1}$) respectively. Fewson (1967) had shown that in batch culture *Acinetobacter* sp. NCIB 8250 grows very slowly above 33 °C, therefore, in this present work 33 °C was the highest growth temperature used. When *Acinetobacter* sp. NCIB 8250 was grown in continuous culture under ammonium limitation and the maximum specific growth rate determined at growth temperatures ranging from 15 - 33 °C the optimum growth temperature, with a μ_{max} of $1{\cdot}11\ h^{-1}$, was 30 °C. At 15 °C the μ_{max} was only $0{\cdot}37\ h^{-1}$ and at 33 °C it was approximately $0{\cdot}97\ h^{-1}$ (Figure 4.1).

Figure 4.1

Effect of the growth temperature on the maximum specific growth rates of *Acinetobacter* sp. NCIB 8250 grown in continuous culture on the medium with a low concentration of $(NH_4)_2SO_4$

Acinetobacter sp. NCIB 8250 was grown in continuous culture at 15, 20, 25, 30 and 33 ^{o}C under ammonium limitation and the maximum specific growth rate at each of the temperatures was determined (Section 2.5.3).


Maximum specific growth rate (h^{-1})
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
15
20
25
30
35
40
Growth temperature (°C)

Under batch culture conditions both the succinate and NH_3 growth yields of *Acinetobacter* sp. NCIB 8250 were lower at 15 °C than at 30 °C (Table 4.1). This was also true under ammonium limitation in continuous culture where the growth yields increased as the growth temperature was increased (Table 4.2). Also in continuous culture at 30 °C it was more noticeable that the growth yields were affected by the specific growth rate (Table 4.2a). Under succinate limitation the growth yields increased from 26·0 g dry weight (mol succinate)$^{-1}$ at 0·14 h^{-1} to 34·2 g dry weight (mol succinate)$^{-1}$ at 0·31 h^{-1} with only a slight further increase to 36·6 g dry weight (mol succinate)$^{-1}$ at 0·80 h^{-1} (Table 4.2b). Under ammonium limitation a similar trend was observed. At 0·14 h^{-1} the growth yield was 151·9 g dry weight (mol NH_3)$^{-1}$ and increased to 179·7 g dry weight (mol NH_3)$^{-1}$ at 0·28 h^{-1} with only a slight increase to 190·3 g dry weight (mol NH_3)$^{-1}$ at 0·77 h^{-1} (Table 4.2a).

Using the data obtained from continuous culture under succinate limitation (Table 4.2b) the theoretical maximum growth yield and maintenance coefficient were determined by plotting the reciprocal of the growth yields against the reciprocal of the specific growth rates (Figure 4.2). These values were 42·9 g dry weight (mol succinate)$^{-1}$ and 2·18 mmol succinate (g dry weight)$^{-1}$ h^{-1} for the maximum growth yield and maintenance coefficient, respectively.

4.2.2 Wax ester content of *Acinetobacter* sp. NCIB 8250 grown in batch and continuous culture

When *Acinetobacter* sp. NCIB 8250 was grown at 30 °C on the medium with a low concentration of $(NH_4)_2SO_4$ the amount of wax esters accumulated was greater when harvested during stationary phase in batch culture and at low specific growth rates in continuous culture than when harvested during exponential phase in batch culture, at high specific growth rates in continuous culture under ammonium limitation or under any growth conditions when grown on the medium with a low concentration of succinic acid (Tables 4.3 & 4.4 and Figure 4.3). Under conditions of greatest wax ester accumulation, 10 - 15 mg (g dry weight)$^{-1}$ were achieved in batch culture (Table 4.3b) and 40 - 45 mg (g dry weight)$^{-1}$ in continuous culture (Table 4.4a). Whereas during exponential growth on either media and during succinate-limited growth in continuous culture the amounts of wax esters accumulated were only 0·2 - 1·0 mg (g dry weight)$^{-1}$ (Tables 4.3 & 4.4b). During growth at 30 °C under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$ the amounts of wax esters accumulated were comparable with that achieved under ammonium limitation (Tables 4.4a & c).

The amounts of wax esters accumulated by *Acinetobacter* sp. NCIB 8250 grown

Table 4.1

Effect of growth temperature on the growth yields of *Acinetobacter* sp. NCIB 8250 grown in batch culture on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$

Acinetobacter sp. NCIB 8250 was grown in batch culture at 15 °C and 30 °C in a 2 l flask on the medium with a low concentration of succinic acid (Section 2.3.2.2) and on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in stationary phase (Section 2.5.2), the biomass of the culture determined and the growth yields for $(NH_4)_2SO_4$ and succinic acid calculated (Section 2.6).

Growth temperature °C	Growth yield	
	g dry weight (mol succinate)$^{-1}$	g dry weight [mol $(NH_4)_2SO_4$]$^{-1}$
15	23·6	55·6
30	28·4	92·7

Table 4.2

Effect of the specific growth rate and growth temperature on the growth yields of *Acinetobacter* sp. NCIB 8250 grown in continuous culture under ammonium and succinate limitation

Acinetobacter sp. NCIB 8250 was grown in continuous culture at 15, 20, 25, 30 and 33 °C under ammonium limitation and at 30 °C under succinate limitation (Section 2.5.3) with steady states achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states, the biomass of the culture determined and the growth yields for $(NH_4)_2SO_4$ and succinic acid calculated (Section 2.6).

Table 4.2a shows the growth yields for *Acinetobacter* sp. NCIB 8250 grown under ammonium imitation and Table 4.2b shows the growth yields for *Acinetobacter* sp. NCIB 8250 grown under succinate limitation.

a) growth yields for *Acinetobacter* sp. NCIB 8250 grown under ammonium limitation

Growth temperature (°C)	Specific growth rate (h^{-1})	Growth yield [g dry weight (mol $(NH_4)_2SO_4)^{-1}$]
15	0·22	124·2
	0·28	118·9
20	0·27	179·7
25	0·18	171·8
	0·60	175·7
30	0·14	151·9
	0·28	179·9
	0·48	178·4
	0·62	194·2
	0·77	190·3
33	0·44	198·2
	0·66	198·2

b) growth yields for *Acinetobacter* sp. NCIB 8250 grown under succinate limitation

Growth temperature (°C)	Specific growth rate (h^{-1})	Growth yield [g dry weight (mol succinic acid)$^{-1}$]
30	0·14	26·0
	0·31	34·2
	0·49	35·4
	0·56	37·8
	0·80	36·6

Figure 4.2

Determination of the maintenance coefficient for *Acinetobacter* sp. NCIB 8250 grown in continuous culture at 30 °C under succinate limitation

Acinetobacter sp. NCIB 8250 was grown in continuous culture at 30 °C under succinate limitation (Section 2.5.3) and steady states were achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states, the biomass of the cultures determined and the growth yields for succinic acid calculated (Section 2.6). The true molar growth yield and maintenance coefficient were then determined by plotting the reciprocal of the growth yields against the reciprocal of the specific growth rate at which the samples had been taken. The value for the true molar growth yield was taken as being the intercept with the y-axis and the maintenance coefficient was taken as the gradient of the line of best fit through the points.

The data used in this Figure are taken from Table 4.2b.

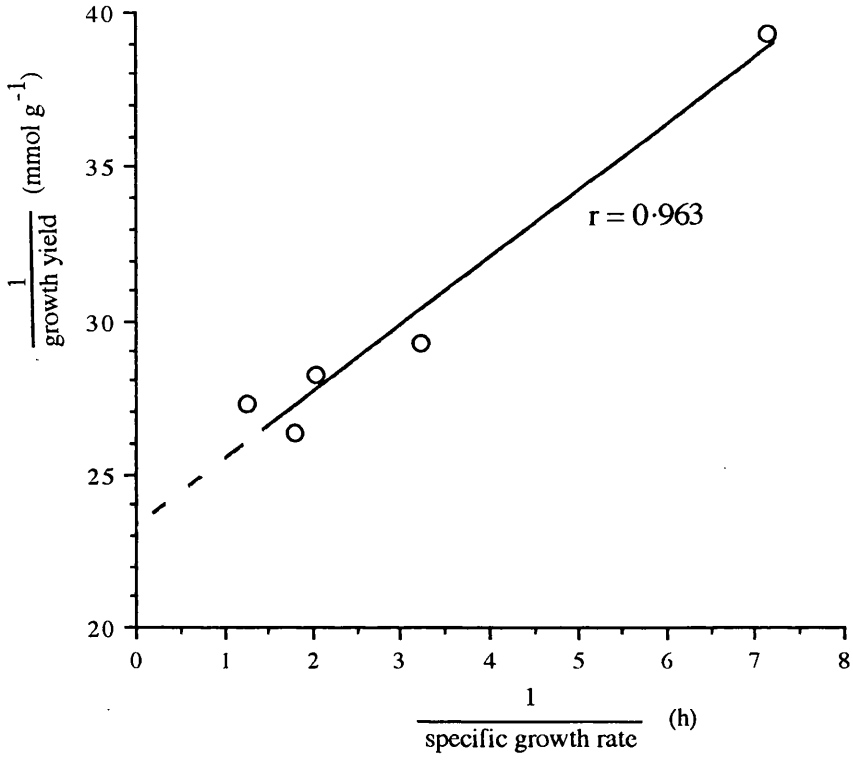

40
35
30
25
20
0
1
2
3
4
5
6
7
8
r = 0·963
1 / growth yield (mmol g-1)
1 / specific growth rate (h)

Table 4.3

Total amount of wax esters accumulated by *Acinetobacter* sp. NCIB 8250 grown in batch culture at 15 °C and 30 °C on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during either the exponential phase of growth or in stationary phase

Acinetobacter sp. NCIB 8250 was grown at 15 °C and 30 °C in batch culture in 2 l flasks on the medium with a low concentration of succinic acid (Section 2.3.2.2) and on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in either the exponential phase of growth or in stationary phase (Section 2.5.2), the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9) and then analysed by glc (Section 2.10.2). Each sample was analysed in triplicate and the total amount of wax esters accumulated determined. The results are shown in the table as the mean ± standard deviation.

Two separate experiments were carried out at 30 °C for *Acinetobacter* sp. NCIB 8250 grown in batch culture and harvested during the exponential phase of growth and in stationary phase. These are designated as (1) and (2).

a) medium with a low concentration of succinic acid

Growth temperature (°C)	Growth phase	Wax ester content [mg (g dry weight)$^{-1}$]
15	Exponential	3·25 ± 0·04
30	Exponential (1)	0·42 ± 0·06
	Exponential (2)	0·52 ± 0·06
	Stationary (1)	0·38 ± 0·05
	Staationary (2)	0·26 ± 0·04

b) medium with a low concentration of $(NH_4)_2SO_4$

Growth temperature (°C)	Growth phase	Wax ester content [mg (g dry weight)$^{-1}$]
15	Exponential	3·08 ± 0·03
30	Exponential (1)	0·69 ± 0·11
	Exponential (2)	0·92 ± 0·06
	Stationary (1)	10·40 ± 0·05
	Stationary (2)	15·40 ± 0·12

Table 4.4

Total amount of wax esters accumulated by *Acinetobacter* sp. NCIB 8250 grown in continuous culture over a range of growth temperatures and specific growth rates and under succinate, ammonium and oxygen limitation

Acinetobacter sp. NCIB 8250 was grown at 15, 20, 25, 30 and 33 °C in continuous culture under ammonium and succinate limitation and under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.5.3). Steady states were achieved over a wide range of specific growth rates and a 30 ml sample of culture was harvested (Section 2.7) at each of the steady states, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9) and then analysed by glc (Section 2.10.2). Each sample was analysed in triplicate and the total amount of wax esters accumulated determined. The results are shown in the table as the mean ± standard deviation.

Tables 4.4a, b & c show the total amount of wax esters accumulated by *Acinetobacter* sp. NCIB 8250 under ammonium, succinate and oxygen limitation respectively.

a) ammonium limitation

Growth temperature (°C)	Specific growth rate (h^{-1})	Wax ester content [mg (g dry weight)$^{-1}$]
15	0·22	6·34 ± 0·20
	0·28	3.29 ± 0.20
20	0·27	7·88 ± 0·27
25	0·18	43·45 ± 1·33
	0·60	2·28 ± 0.09
30	0·14	41·10 ± 0·63
	0·28	27·10 ± 0·46
	0·48	12·30 ± 0·86
	0·62	3·41 ± 0·06
	0·77	1·80 ± 0·05
33	0·44	6·58 ± 0·46
	0·66	3·65 ± 0·07

b) succinate limitation

Growth temperature (°C)	Growth phase	Wax ester content [mg (g dry weight)$^{-1}$]
30	0·14	0·23 ± 0·05
	0·31	0·18 ± 0·02
	0·49	0·27 ± 0·02
	0·56	0·26 ± 0·08
	0·80	0·53 ± 0·13

c) oxygen limitation

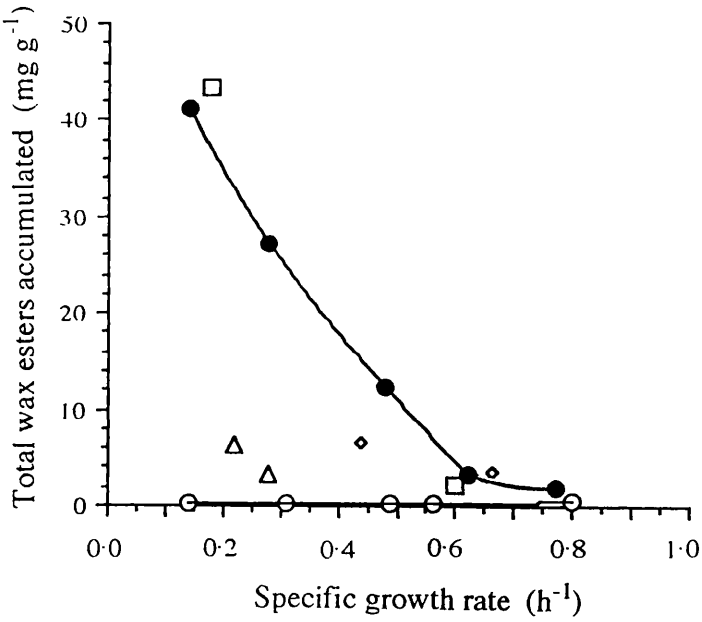
Growth temperature (°C)	Growth phase	Wax ester content [mg (g dry weight)$^{-1}$]
15	0·22	3·79 ± 0·22
	0·28	4·98 ± 0·12
30	0·58	4·67 ± 0·04
	0·61	2·42 ± 0·04

Figure 4.3

The effect of the growth temperature and the specific growth rate on the total amount of wax esters accumulated by *Acinetobacter* sp. NCIB 8250 grown in continuous culture under succinate and ammonium limitation

Acinetobacter sp. NCIB 8250 was grown at 30 °C in continuous culture under succinate limitation and at 15, 25, 30 and 33 °C in continuous culture under ammonium limitation (Section 2.5.3) with steady states achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states achieved, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9), analysed in triplicate by glc (Section 2.10.2) and the total amount of wax esters accumulated in each sample was then calculated and plotted against the specific growth rate at which the sample had been taken.

The data used in this Figure are taken from Table 4.4



○ Succinate-limited at 30 °C
△ Ammonium-limited at 15 °C
□ Ammonium-limited at 25 °C
● Ammonium-limited at 30 °C
◇ Ammonium-limited at 33 °C

under ammonium limitation in continuous culture showed the same trends at all growth temperatures studied, i.e. at each growth temperature the lower the specific growth rate the more wax esters that were accumulated (Table 4.4a and Figure 4.3). This was also true in batch culture where *Acinetobacter* sp. NCIB 8250 was grown at 15 °C and 30 °C. During exponential growth at 15 °C there was approximately five times more wax esters accumulated than during exponential growth at 30 °C (Table 4.3).

4.2.3 Wax ester composition of *Acinetobacter* sp. NCIB 8250 grown in batch and continuous culture

Saturated, mono- and di-unsaturated C_{32}, C_{34} and C_{36} wax esters were the major components observed when *Acinetobacter* sp. NCIB 8250 was grown in batch and continuous culture using either the medium with a low concentration of succinic acid or the medium with a low concentration of $(NH_4)_2SO_4$. Other saturated and unsaturated wax esters (C_{30}, C_{31}, C_{33}, C_{35}, C_{37} and C_{38}) were also observed but only as trace components. Figure 4.4 shows examples of gas chromatograms of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 after growth at 15 °C and 33 °C.

4.2.3.1 Wax ester composition of *Acinetobacter* sp. NCIB 8250 grown in batch culture at 15 °C and harvested during exponential phase

When *Acinetobacter* sp. NCIB 8250 was grown at 15 °C and harvested during the exponential phase, the medium had little effect on the composition of the wax esters (Table 4.5 and Figure 4.5a), as indicated by the chain length and unsaturation indices (cli = 1·32 - 1·40, ui = 1·40 - 1·42). The unsaturated wax esters accounted for nearly 90 % of the total, with the values for the $C_{32:2}$ and $C_{36:2}$ wax esters being double that of the corresponding mono-unsaturated wax esters (Table 4.5 & Figure 4.5a). The $C_{34:2}$ and $C_{34:1}$ wax esters were present in the same amounts, approximately 16 %. The saturated wax esters were minor components totalling only approximately 10 % (Table 4.5 & Figure 4.5a).

4.2.3.2 Wax ester composition of *Acinetobacter* sp. NCIB 8250 grown in batch culture at 30 °C and harvested during exponential phase

The compositions of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C and harvested during exponential phase from either the medium with a low concentration of succinic acid or the medium with a low concentration of

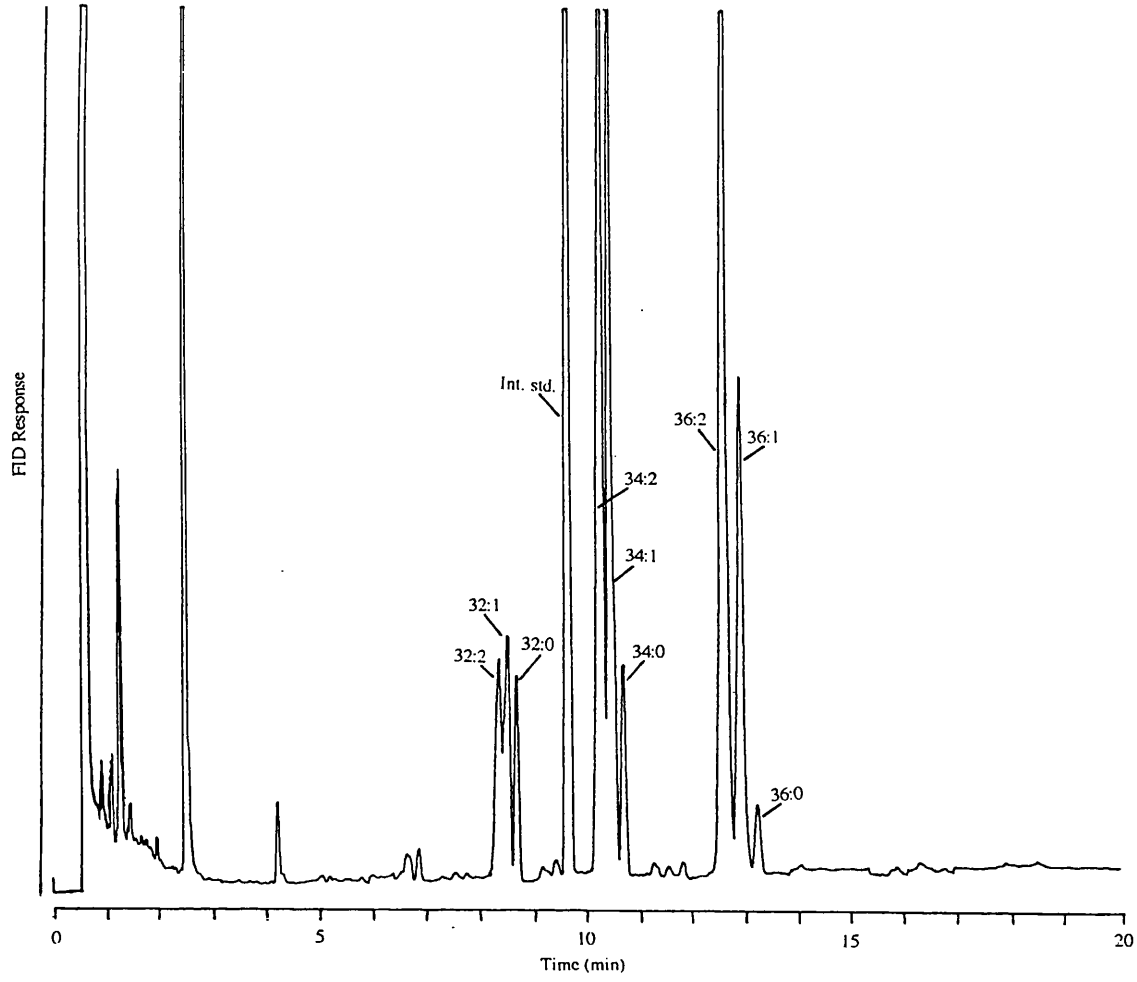
Figure 4.4

Gas chromatograms of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 15 °C on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase of growth and in continuous culture at 33 °C under ammonium limitation

Acinetobacter sp. NCIB 8250 was grown in batch culture at 15 °C in 2 l flasks on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3) and in continuous culture at 33 °C under ammonium limitation (Section 2.5.3). The batch culture was harvested in the exponential phase of growth and a 30 ml sample was harvested during steady state from the continuous culture (Section 2.7). The pellets were washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0, the lipids extracted (Section 2.8) and the wax esters separated from the other lipid components in the sample by tlc (Section 2.9) and then analysed by glc (Section 2.10.2).

Figure 4.4a shows a gas chromatogram of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 15 °C on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase of growth and Figure 4.4b shows a gas chromatogram of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture at 33 °C under ammonium limitation.

a) gas chromatogram of wax esters extracted *Acinetobacter* sp. NCIB 8250 grown at 15 °C



b) gas chromatogram of wax esters extracted *Acinetobacter* sp. NCIB 8250 grown at 33 °C

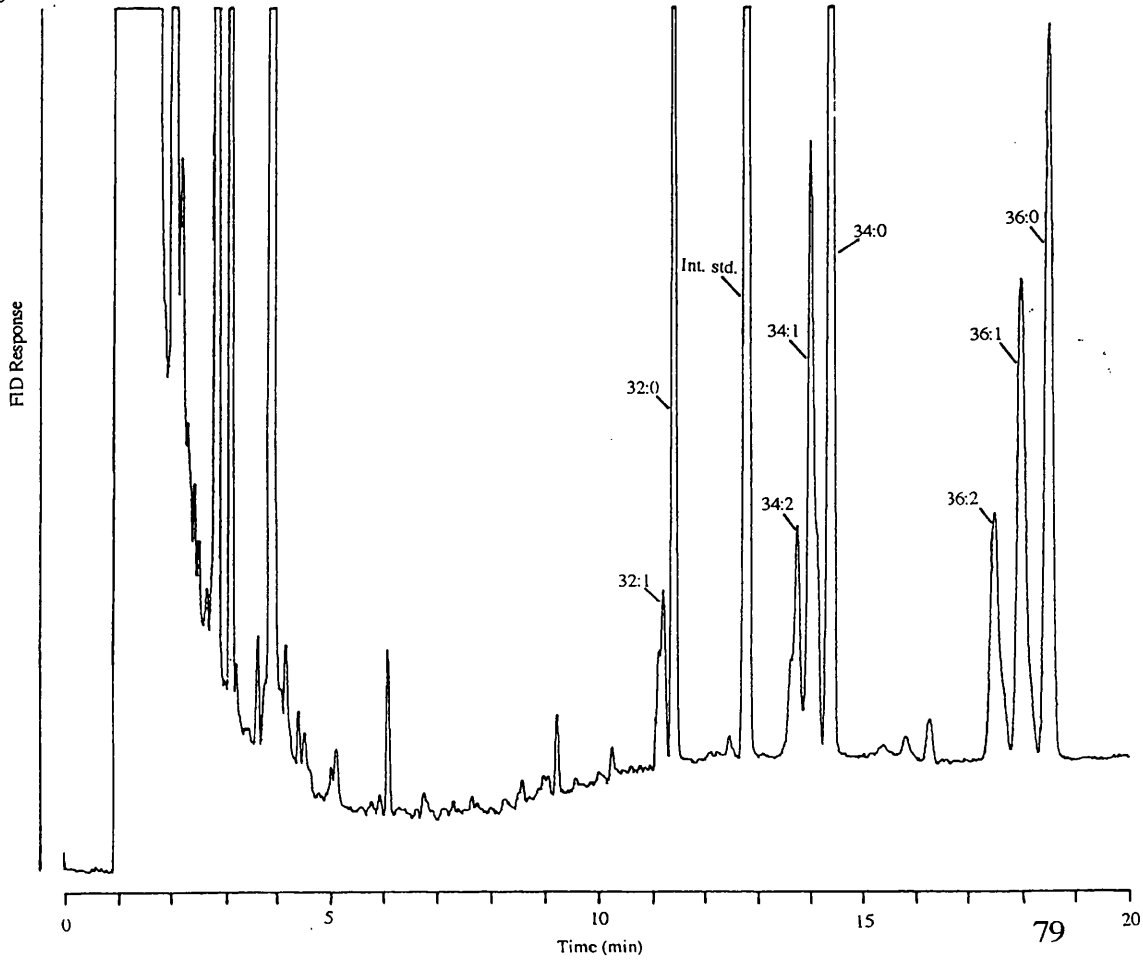


Table 4.5

Wax ester compositions extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 15 °C on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase of growth

Acinetobacter sp. NCIB 8250 was grown at 15 °C in batch culture in 2 l flasks on the medium with a low concentration of succinic acid (Section 2.3.2.2) and on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in the exponential phase of growth (Section 2.5.2), the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9) and then analysed by glc (Section 2.10.2). Each sample was analysed in triplicate and the amount of each wax ester component determined. Each component was then calculated as a percentage of the total and are shown as the mean ± standard deviation together with the chain length (cli) and unsaturation indices (ui).

*Denotes that $P < 0{\cdot}05$ for a comparison of the wax ester values between the two media. None of the others were significantly different, i.e. $P > 0{\cdot}05$.

Wax ester	Percentage of the total Low succinate	Low ammonium
32:2	5·5 ± 0·55	8·1 ± 0·78
32:1	2·4 ± 0·06	3·0 ± 0·15
32:0	3·0 ± 0·51	3·9 ± 0·30
34:2	14·6 ± 0·06	16·5 ± 0·32
34:1	18·1 ± 0·06	16·7 ± 0·25
34:0	4·9 ± 0·06	4·9 ± 0·06
36:2	31·5 ± 0·70	29·8 ± 0·15
36:1	16·2 ± 0·26	13·9 ± 0·12
36:0	3·6 ± 0·15	3·3 ± 0·23
cli	1·40	1·32
ui	1·40	1·42

Figure 4.5

Comparison of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 15 °C and 30 °C on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase of growth or in stationary phase

Acinetobacter sp. NCIB 8250 was grown in batch culture at 15 °C and 30 °C in 2 l flasks on either the medium with a low concentration of succinic acid (Section 2.3.2.2) and on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in exponential phase or stationary phase (Section 2.5.2) and the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0. The lipids were extracted (Section 2.8), the wax esters separated from the other lipid components in the sample by tlc (Section 2.9) and then analysed by glc (Section 2.10.2). Each sample was analysed in triplicate and each wax ester component in the sample was then calculated as a percentage of the total.

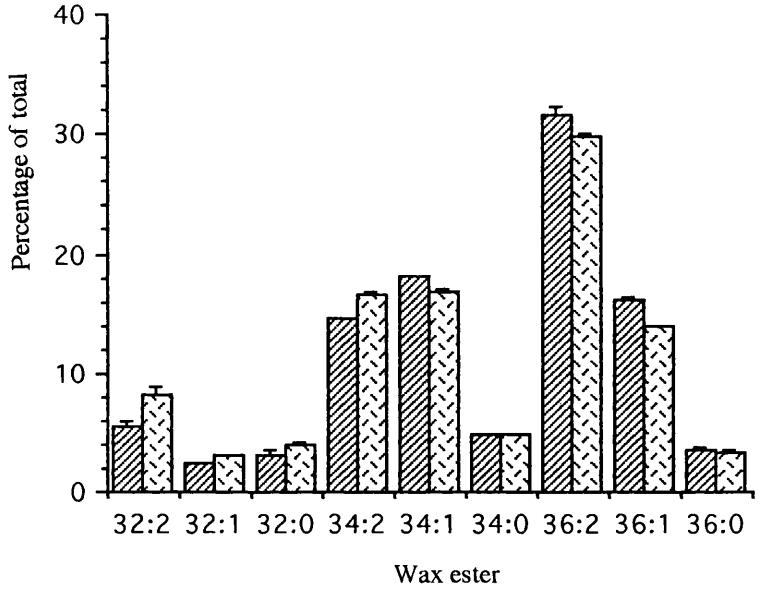
Figure 4.5a compares the compositions of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 15 °C on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase of growth. Figure 4.5b compares the compositions of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase of growth. Figure 4.5c (next page) compares the compositions of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during stationary phase.

The data used in this Figure are taken from Tables 4.5, 4.6 & 4.7.

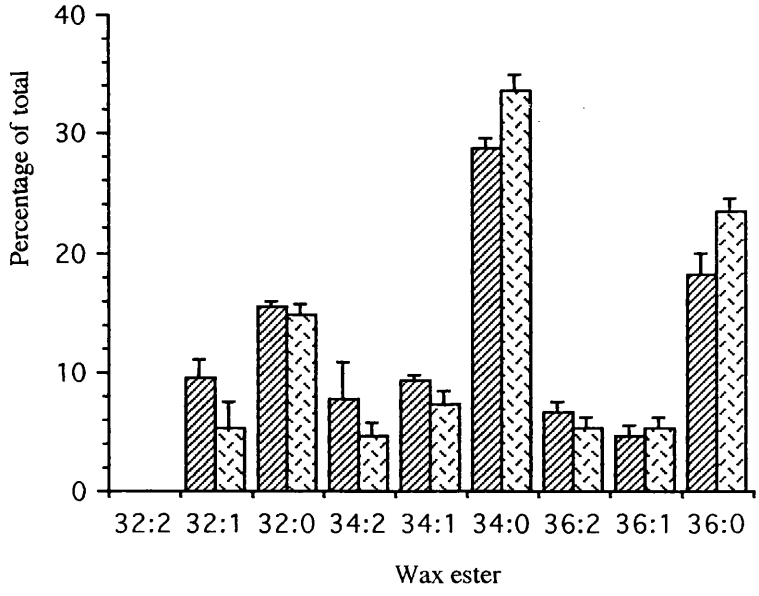

Succinate-limited


Ammonium-limited

a) wax ester composition of *Acinetobacter* sp. NCIB 8250 harvested from exponential phase at 15 °C



b) wax ester composition of *Acinetobacter* sp. NCIB 8250 harvested from exponential phase at 30 °C



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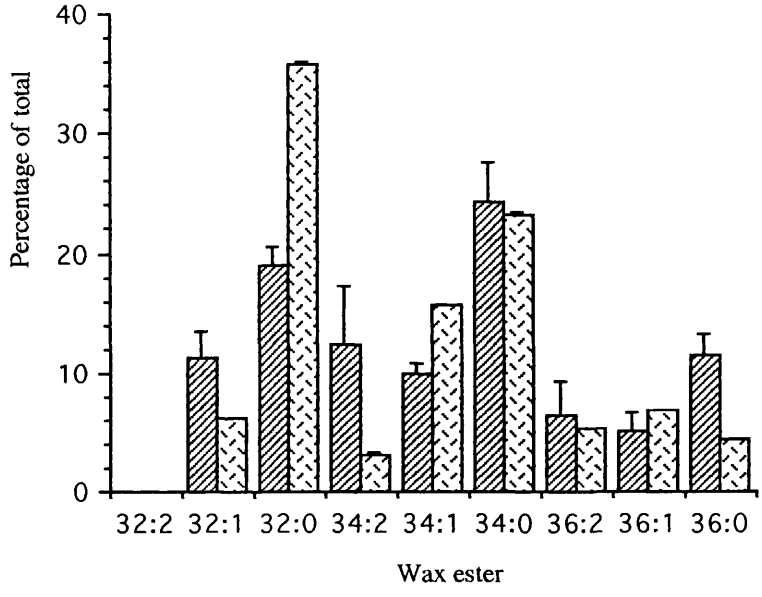
c) wax ester composition of *Acinetobacter* sp. NCIB 8250 harvested from stationary phase at 30 °C



$(NH_4)_2SO_4$ were very similar, with only the values for the $C_{36:1}$ and $C_{36:0}$ wax esters being significantly different ($P < 0{\cdot}05$) between the two media (Table 4.6). The major wax esters found in the samples extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C were the saturated C_{32}, C_{34} and C_{36}, which between them accounted for 60 - 80 % of the total (Table 4.6 and Figure 4.5b). The $C_{32:2}$ wax ester was not observed and the other di-unsaturated wax esters were present only as minor components (Table 4.6 and Figure 4.5b). The major difference in the compositions of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 15 °C and 30 °C and harvested during the exponential phase from either media was in the degree of unsaturation. At 30 °C the wax esters were much more saturated (ui = 0·25 - 0·45) than at 15 °C (ui = 1·40 - 1·42) and there was an increase in the proportion of the shorter chain length wax esters at 30 °C (cli = 1·05 - 1·14) compared to that at 15 °C (cli = 1·32 - 1·40) (Tables 4.5 & 4.6 and Figures 4.5a & b).

4.2.3.3 Wax ester composition of *Acinetobacter* sp. NCIB 8250 grown in batch culture at 30 °C and harvested during stationary phase

Acinetobacter sp. NCIB 8250 was grown at 30 °C on both the medium with a low concentration of succinic acid and the medium with a low concentration of $(NH_4)_2SO_4$ and harvested in stationary phase (Table 4.7). Although the saturated wax esters were predominant in both compositions, the wax esters extracted from *Acinetobacter* sp. NCIB 8250 harvested from ammonium-limited batch culture contained a greater proportion of the $C_{32:0}$ wax ester than the $C_{36:0}$ wax ester and therefore, overall, had a shorter chain length index (cli = 0·68 - 0·75) than the wax esters extracted from *Acinetobacter* sp. NCIB 8250 harvested from succinate-limited batch culture (cli = 0·90 - 0·93) (Table 4.7 and Figure 4.5c). Also, there was a significant difference ($P < 0{\cdot}05$) in the values for the $C_{32:0}$, $C_{34:1}$, $C_{36:1}$ and $C_{36:0}$ wax esters between the two limitations (Table 4.7).

There was very little difference in the wax ester compositions extracted from *Acinetobacter* sp. NCIB 8250 harvested during stationary phase from succinate-limited batch culture with those wax ester compositions extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C and harvested during exponential phase from either media (Tables 4.6 & 4.7). There was however, considerable difference between the wax ester compositions extracted from *Acinetobacter* sp. NCIB 8250 harvested during stationary phase from ammonium-limited batch culture with the wax ester compositions extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C and harvested from exponential phase from either media. The major differences were that the wax esters extracted from *Acinetobacter* sp. NCIB 8250

Table 4.6

Wax ester compositions extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 30 °C on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase of growth

Acinetobacter sp. NCIB 8250 was grown at 30 °C in batch culture in 2 l flasks on the medium with a low concentration of succinic acid (Section 2.3.2.2) or on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in the exponential phase of growth (Section 2.5.2), the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9) and then analysed by glc (Section 2.10.2). Each sample was analysed in triplicate and the amount of each wax ester component determined. Each component was then calculated as a percentage of the total and are shown as the mean ± standard deviation together with the chain length (cli) and unsaturation indices (ui).

Two separate experiments were carried out at 30 °C for *Acinetobacter* sp. NCIB 8250 grown in batch culture and harvested during exponential phase. These are designated as experiment 1 and experiment 2.

*Denotes that $P < 0{\cdot}05$ for a comparison of the wax ester values between the two media. None of the others were significantly different, i.e. $P > 0{\cdot}05$.

a) medium with a low concentration of succinic acid

Wax ester	Percentage of the total Experiment 1	Experiment 2
32:1	ND	9·4 ± 1·65
32:0	20·7 ± 3·63	15·4 ± 0·49
34:2	1·7 ± 1·70	7·8 ± 2·98
34:1	5·2 ± 4·54	9·3 ± 0·45
34:0	41·0 ± 2·62	28·7 ± 0·82
36:2	6·9 ± 1·46	6·7 ± 0·75
*36:1	3·1 ± 0·96	4·6 ± 0·87
*36:0	21·4 ± 2·51	18·1 ± 1·71
cli	1·11	1·05
ui	0·25	0·43

b) medium with a low concentration of $(NH_4)_2SO_4$

Wax ester	Percentage of the total Experiment 1	Experiment 2
32:1	7·4 ± 2·47	5·4 ± 2·04
32:0	16·7 ± 2·24	14·8 ± 0·91
34:2	4·5 ± 3·89	4·7 ± 1·01
34:1	9·6 ± 3·85	7·2 ± 1·11
34:0	27·0 ± 2·90	33·6 ± 1·42
36:2	6·5 ± 2·75	5·4 ± 0·76
*36:1	6·2 ± 1·23	5·4 ± 0·76
*36:0	22·3 ± 2·10	23·5 ± 1·04
cli	1·11	1·14
ui	0·45	0·38

Table 4.7

Wax ester compositions extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 30 °C on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during stationary phase

Acinetobacter sp. NCIB 8250 was grown at 30 °C in batch culture in 2 l flasks on the medium with a low concentration of succinic acid (Section 2.3.2.2) and on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in stationary phase (Section 2.5.2), the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9) and then analysed by glc (Section 2.10.2). Each sample was analysed in triplicate and the amount of each wax ester component determined. Each component was then calculated as a percentage of the total and are shown as the mean ± standard deviation together with the chain length (cli) and unsaturation indices (ui).

Two separate experiments were carried out at 30 °C for *Acinetobacter* sp. NCIB 8250 grown in batch culture and harvested in stationary phase. These are designated as experiment 1 and experiment 2.

*Denotes that $P < 0{\cdot}05$ for a comparison of the wax ester values between the two media. None of the others were significantly different, i.e. $P > 0{\cdot}05$.

a) medium with a low concentration of succinic acid

Wax ester	Percentage of the total Experiment 1	Experiment 2
32:1	1·8 ± 1·59	11·2 ± 2·39
*32:0	24·6 ± 2·97	18·9 ± 1·56
34:2	1·0 ± 1·73	12·4 ± 4·77
*34:1	6·8 ± 5·05	10·0 ± 0·83
34:0	35·2 ± 5·77	24·3 ± 3·35
36:2	3·5 ± 1·36	6·5 ± 2·77
*36:1	2·6 ± 0·32	5·0 ± 1·71
36:0	17·5 ± 0·75	11·5 ± 1·80
cli	0·90	0·93
ui	0·20	0·64

b) medium with a low concentration of $(NH_4)_2SO_4$

Wax ester	Percentage of the total Experiment 1	Experiment 2
32:1	3·7 ± 0·12	6·1 ± 0·10
*32:0	36·3 ± 0·35	35·7 ± 0·23
34:2	1·3 ± 0·10	3·0 ± 0·21
*34:1	12·6 ± 0·21	15·6 ± 0·10
34:0	28·8 ± 1·10	23·2 ± 0·30
36:2	4·4 ± 0·06	5·2 ± 0·10
*36:1	5·7 ± 0·10	6·8 ± 0·06
36:0	7·8 ± 0·17	4·4 ± 0·10
cli	0·68	0·75
ui	0·33	0·45

harvested in stationary phase from ammonium-limited batch cultures were of a shorter chain length than those extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C but harvested during exponential phase from either media (Tables 4.6 & 4.7 and Figures 4.5b & c).

4.2.3.4 Wax ester composition of *Acinetobacter* sp. NCIB 8250 grown in continuous culture under succinate limitation

When *Acinetobacter* sp. NCIB 8250 was grown in continuous culture under succinate limitation the specific growth rate did not significantly affect ($P > 0.05$) the composition of the wax esters (Table 4.8). However, there was a slight increase in the overall chain length (cli = 1·04 at 0·14 h^{-1} and cli = 1·14 at 0·56 h^{-1}) and a slight decrease in the degree of unsaturation (ui = 0·76 at 0·14 h^{-1} and ui = 0·65 at 0·80 h^{-1}) as the specific growth rate was increased (Table 4.8). Throughout the range of specific growth rates studied the saturated wax esters were predominant, accounting for 45 - 50 % of the total, with the di-unsaturated wax esters accounting for approximately 20 %. This result was similar to that found with the composition of wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture on the medium with a low concentration of succinic acid and harvested during exponential phase (Table 4.6a) except for the proportion of the $C_{34:0}$ wax ester. This was greater in the batch culture than in the continuous culture, with a concomitant decrease in each of the mono-unsaturated wax esters. Hence the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under succinate limitation had a higher unsaturation index (ui = 0·65 - 0·76) than the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture (ui = 0·25 and 0·43) (Tables 4.6a & 4.8).

4.2.3.5 Wax ester composition of *Acinetobacter* sp. NCIB 8250 grown in continuous culture under ammonium limitation

The composition of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under ammonium limitation was significantly affected ($P < 0.05$) by both the growth temperature and specific growth rate (Table 4.9). The major effect of increasing the growth temperature on the composition of the wax esters was to decrease the degree of unsaturation (Figure 4.6a). For instance, at 15 °C, more than 90 % of the wax esters were unsaturated (ui = 1·45 - 1·53), with the majority being di-unsaturated wax esters (Table 4.9), whereas at 33 °C only

50 - 55 % of the wax esters were unsaturated (ui = 0·62 - 0·67), with the majority of these being the mono-unsaturated (Table 4.9 and Figure 4.4b). This decrease in the degree of unsaturation resulted in the complete disappearance of the $C_{32:2}$ wax ester

Table 4.8

Wax ester compositions extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under succinate limitation

Acinetobacter sp. NCIB 8250 was grown at 30 °C in continuous culture under succinate limitation (Section 2.5.3) and steady states were achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and the amount of each wax ester component determined. Each component was then calculated as a percentage of the total and are shown in the table as the mean percentage ± standard deviation together the chain length (cli) and unsaturation indices (ui).

*Denotes that the specific growth rate had a significant effect ($P < 0{\cdot}05$) on the composition of those wax esters.

	Specific growth rate				
	0·14 h^{-1}	0·31 h^{-1}	0·49 h^{-1}	0·56 h^{-1}	0·80 h^{-1}
Wax ester					
32:1	7·5 ± 4·74	11·6 ± 4·69	6·4 ± 3·16	13·1 ± 3·89	10·8 ± 1·31
32:0	21·4 ± 12·12	16·0 ± 7·14	15·0 ± 0·63	11·7 ± 1·79	15·5 ± 1·37
34:2	9·4 ± 2·60	9·5 ± 3·13	12·0 ± 0·35	9·7 ± 2·36	6·9 ± 1·71
34:1	18·5 ± 3·92	14·3 ± 0·80	23·4 ± 2·01	10·7 ± 0·63	11·7 ± 1·08
34:0	10·3 ± 2·81	11·4 ± 3·37	7·5 ± 2·65	16·4 ± 1·88	19·8 ± 2·42
36:2	11·1 ± 9·88	9·3 ± 4·58	4·6 ± 1·33	10·9 ± 0·59	9·1 ± 2·29
36:1	8·7 ± 4·58	8·2 ± 2·88	4·9 ± 1·08	8·5 ± 1·89	10·2 ± 1·30
36:0	13·0 ± 6·21	19·7 ± 5·37	26·1 ± 1·42	19·1 ± 4·78	16·0 ± 1·37
cli	1·04	1·10	1·14	1·14	1·09
ui	0·76	0·72	0·68	0·73	0·65

Table 4.9

Wax ester compositions extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under ammonium limitation

Acinetobacter sp. NCIB 8250 was grown in continuous culture at 15, 20, 25, 30 and 33 ^{o}C under ammonium limitation (Section 2.5.3) with steady states achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and the amount of each wax ester component determined. Each component was then calculated as a percentage of the total and are shown in the table as the mean percentage ± standard deviation together with the chain length (cli) and unsaturation indices (ui).

*Denotes that the growth temperature and the specific growth rate had a significant effect ($P < 0{\cdot}05$) on the composition of those wax esters.

ND = not detected

Growth temperature	15 °C		20 °C	25 °C	
Specific growth rate	0·22 h^{-1}	0·28 h^{-1}	0·27 h^{-1}	0·18 h^{-1}	0·60 h^{-1}
Wax ester					
32:2	6·0 ± 0·38	10·6 ± 0·70	1·4 ± 0·26	1·4 ± 0·57	14·5 ± 5·22
*32:1	5·8 ± 0·13	6·5 ± 1·10	3·1 ± 0·07	1·6 ± 0·21	5·7 ± 0·62
*32:0	3·3 ± 0·15	4·1 ± 0·32	2·5 ± 0·05	3·1 ± 0·24	8·3 ± 2·95
*34:2	22·4 ± 0·59	27·0 ± 0·87	9·4 ± 0·29	5·8 ± 0·28	12·7 ± 0·99
*34:1	18·1 ± 0·37	13·8 ± 0·39	15·3 ± 0·30	13·6 ± 0·18	14·1 ± 1·04
*34:0	4·6 ± 0·93	3·9 ± 0·48	6·7 ± 0·51	11·1 ± 0·14	9·4 ± 0·97
*36:2	26·4 ± 0·74	25·7 ± 0·86	28·9 ± 0·25	16·5 ± 0·26	10·2 ± 3·16
*36:1	11·4 ± 0·18	6·7 ± 0·40	24·9 ± 0·45	30·4 ± 1·16	16·2 ± 1·16
*36:0	2·0 ± 0·41	1·8 ± 0·77	7·8 ± 0·11	16·4 ± 0·37	8·8 ± 1·70
cli	1·25	1·13	1·55	1·57	1·07
ui	1·45	1·53	1·23	0·93	1·11

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Growth temperature	30 °C				33 °C		
Specific growth rate	0·14 h^{-1}	0·28 h^{-1}	0·48 h^{-1}	0·62 h^{-1}	0·77 h^{-1}	0·44 h^{-1}	0·66 h^{-1}
Wax ester							
32:2	ND	ND	ND	ND	ND	ND	ND
*32:1	3·6 ± 0·27	2·4 ± 0·69	2·7 ± 0·36	7·2 ± 0·09	6·9 ± 0·12	5·0 ± 1·31	5·5 ± 0·17
*32:0	10·2 ± 0·18	6·5 ± 1·28	6·2 ± 0·38	10·3 ± 0·10	11·3 ± 0·50	12·8 ± 1·08	16·8 ± 0·24
*34:2	4·8 ± 0·33	5·4 ± 1·31	8·4 ± 1·51	10·0 ± 0·01	10·6 ± 1·30	7·2 ± 0·90	8·1 ± 0·63
*34:1	14·4 ± 0·32	9·8 ± 0·30	12·7 ± 0·55	24·5 ± 0·23	23·2 ± 0·49	13·4 ± 0·70	17·1 ± 1·29
*34:0	23·6 ± 0·65	21·0 ± 0·95	19·1 ± 0·98	13·9 ± 0·15	14·6 ± 0·09	23·0 ± 1·76	21·8 ± 0·54
*36:2	10·4 ± 0·37	9·0 ± 0·18	8·6 ± 1·60	11·4 ± 0·05	9·4 ± 0·79	8·2 ± 0·91	7·9 ± 1·41
*36:1	14·8 ± 0·34	19·7 ± 1·77	20·4 ± 1·56	16·2 ± 0·11	16·4 ± 0·32	12·8 ± 1·13	12·5 ± 0·54
*36:0	18·2 ± 0·46	26·1 ± 2·55	22·0 ± 1·40	6·4 ± 0·09	7·7 ± 0·17	17·5 1·34	10·3 ± 0·32
cli	1·30	1·46	1·42	1·17	1·15	1·21	1·08
ui	0·63	0·61	0·70	0·91	0·86	0·62	0·67

Figure 4.6

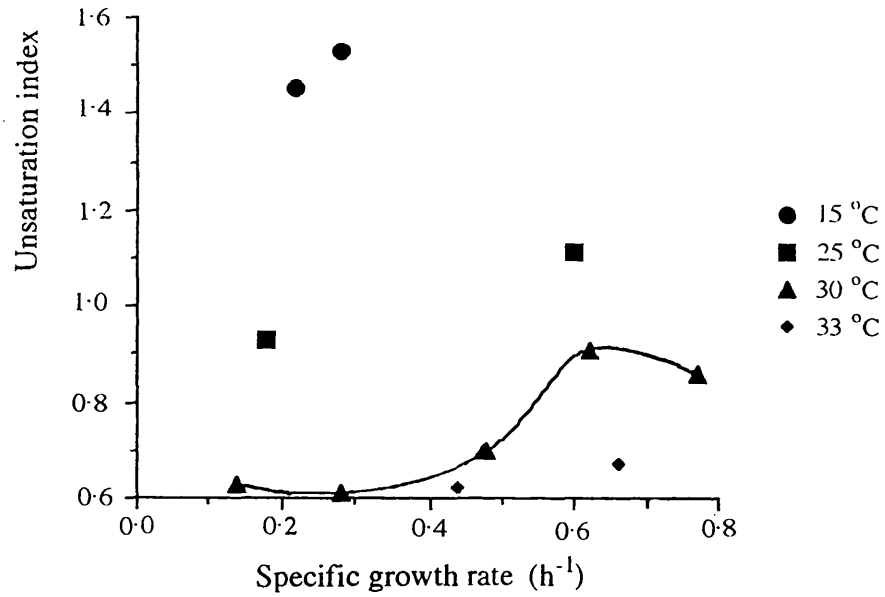
The effect of the growth temperature and the specific growth rate on the chain length and unsaturation indices of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under ammonium limitation

Acinetobacter sp. NCIB 8250 was grown at 15, 25, 30 and 33 °C in continuous culture under ammonium limitation (Section 2.5.3) and steady states were achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states achieved, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9) and then analysed by glc (Section 2.10.2). Each sample was analysed in triplicate and each wax ester component in that sample was then calculated as a percentage of the total.

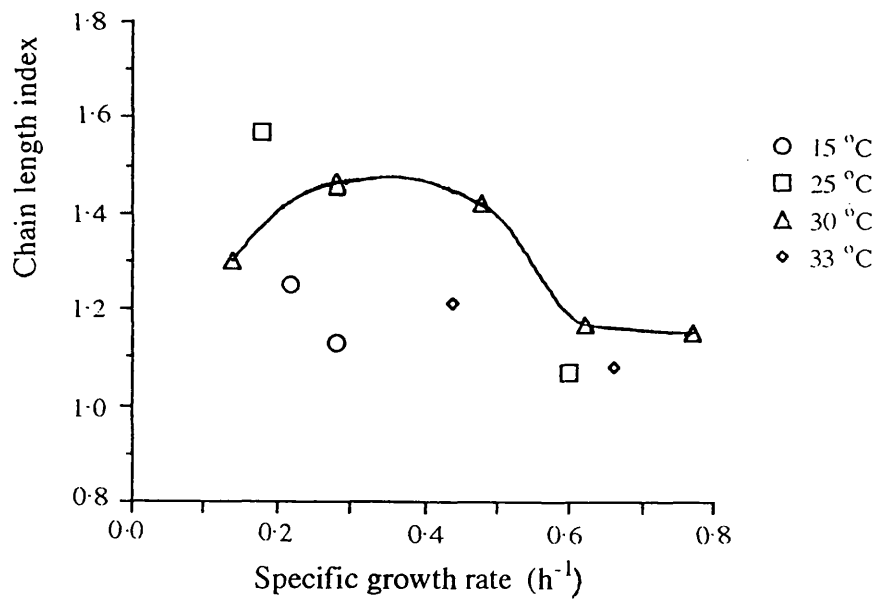
Figure 4.6a shows the effect of the specific growth rate on the unsaturation index (ui) at each of the growth temperatures studied and Figure 4.6b shows the effect of the specific growth rate on the chain length index (cli) at each of the growth temperatures studied.

The data used in this Figure are taken from Table 4.9

a) the trend in the unsaturation index with increasing specific growth rate



b) the trend in the chain length indexwith increasing specific growth rate



at 30 °C and 33 °C (Tables 4.8 & 4.9 and Figure 4.4b). At 30 °C as the specific growth rate was increased from 0·28 h^{-1} to 0·77 h^{-1} the overall chain length of the wax esters decreased from 1·46 to 1·15 (Table 4.9 and Figure 4.6b). Also, as the specific growth rate was increased the proportion of the $C_{32:1}$ and $C_{34:1}$ wax esters increased resulting in an increase in the degree of unsaturation (Figure 4.6a).

These effects of the specific growth rate on the chain lengths and degree of unsaturation were observed at each of the growth temperatures studied under ammonium limitation (Table 4.9 & Figure 4.6). They were also opposite to that which was observed with the composition of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown under succinate limitation (Tables 4.8 & 4.9).

The wax esters of *Acinetobacter* sp. NCIB 8250 grown at 30 °C in continuous culture under ammonium limitation and at specific growth rates of 0·62 h^{-1} and 0·77 h^{-1} were more unsaturated (ui = 0·86 - 0·91) (Table 4.9) than the wax esters of *Acinetobacter* sp. NCIB 8250 harvested during exponential phase in batch culture (ui = 0·38) (Table 4.7b), although there was little difference in the overall chain length indices (cli = 1·15 - 1·17 in continuous culture and cli = 1·14 in batch culture). However, there were differences in both the chain length and unsaturation indices when the wax ester compositions from lower specific growth rates (i.e. 0·14 h^{-1} and 0·28 h^{-1}) (Table 4.9) were compared with those for *Acinetobacter* sp. NCIB 8250 grown in batch culture on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during exponential phase (Table 4.6b).

When *Acinetobacter* sp. NCIB 8250 was grown at 15 °C the wax ester compositions were similar regardless of whether *Acinetobacter* sp. NCIB 8250 was grown in batch culture and harvested during exponential phase or grown in continuous culture under ammonium limitation (Tables 4.6 & 4.9 and Figure 4.7). The similarities were reflected in the chain length indices (cli = 1·13 - 1·25 in continuous culture and cli = 1·32 in batch culture) although an increased proportion in the $C_{34:2}$ wax ester of the continuous culture samples had a slight effect on the unsaturation index (ui = 1·45 - 1·53 in continuous culture and ui = 1·42 in batch culture) (Tables 4.5 & 4.9).

4.2.3.6 Wax ester composition of *Acinetobacter* sp. NCIB 8250 grown in continuous culture under oxygen limitation

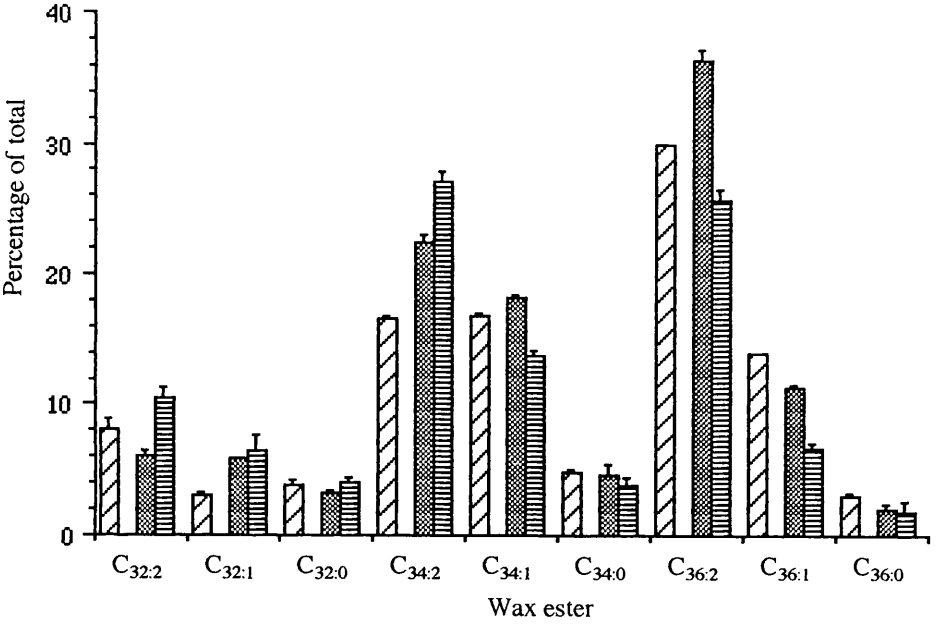
When *Acinetobacter* sp. NCIB 8250 was grown under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$ (Table 4.10) the composition of the wax esters was significantly effected ($P < 0·05$) by the growth temperature. The effect of increasing the growth temperature was to decrease the degree of unsaturation from 1·49 - 1·53 at 15 °C to 0·63 - 0·70 at 30 °C (Table 4.10 and Figure

Figure 4.7

Comparison of the wax ester compositions extracted from *Acinetobacter* sp. NCIB 8250 grown at 15 °C in batch culture on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase of growth and at 15 °C in continuous culture under ammonium limitation

Acinetobacter sp. NCIB 8250 was grown in batch culture at 15 °C in 2 l flasks on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3) and in continuous culture at 15 °C under ammonium limitation (Section 2.5.3). The batch culture was harvested (Section 2.7) in the exponential phase of growth (Section 2.5.2) and a 30 ml sample was taken during steady states from the continuous culture (Section 2.7). The pellets were washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0, the lipids extracted (Section 2.8) and the wax esters separated the from the other lipid components in the sample by tlc (Section 2.9) and then analysed by glc (Section 2.10.2). Each sample was analysed in triplicate and each wax ester component in that sample was then calculated as a percentage of the total.

The data used in this Figure are taken from Tables 4.5 & 4.9.



Batch culture - exponential phase

Continuous culture - 0·22 h^{-1}

Continuous culture - 0·28 h^{-1}

Table 4.10

Wax ester compositions extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$

Acinetobacter sp. NCIB 8250 was grown at 15 °C and 30 °C in continuous culture under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.5.3). A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states achieved, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9) and analysed by glc (Section 2.10.2). Each sample was analysed in triplicate and the amount of each wax ester component determined. Each component was then calculated as a percentage of the total and are shown as the mean percentage ± standard deviation together with the chain length (cli) and unsaturation indices (ui).

*Denotes that the growth temperature and the specific growth rate had a significant effect ($P < 0·05$) on the composition of those wax esters.

ND = not detected

Growth temperature	15 °C		30 °C	
Specific growth rate	0·22 h^{-1}	0·28 h^{-1}	0·58 h^{-1}	0·61 h^{-1}
Wax ester				
32:2	12·7 ± 0·04	11·4 ± 0·01	ND	ND
*32:1	3·4 ± 0·02	5·2 ± 0·01	6·0 ± 0·06	5·8 ± 0·02
*32:0	3·7 ± 0·03	4·4 ± 0·01	12·8 ± 0·01	15·3 ± 0·01
*34:2	27·7 ± 0·02	27·5 ± 0·02	4·9 ± 0·03	5·4 ± 0·01
*34:1	15·0 ± 0·01	16·7 ± 0·02	18·0 ± 0·02	21·1 ± 0·01
*34:0	3·4 ± 0·02	3·6 ± 0·01	22·5 ± 0·02	19·4 ± 0·01
*36:2	22·4 ± 0·06	20·3 ± 0·05	5·6 ± 0·04	6·6 ± 0·01
*36:1	9·2 ± 0·02	9·0 ± 0·01	16·1 ± 0·02	16·1 ± 0·02
*36:0	2·4 ± 0·04	2·0 ± 0·01	14·3 ± 0·01	11·2 ± 0·01
cli	1·14	1·10	1·17	1·13
ui	1·53	1·49	0·63	0·70

Figure 4.8

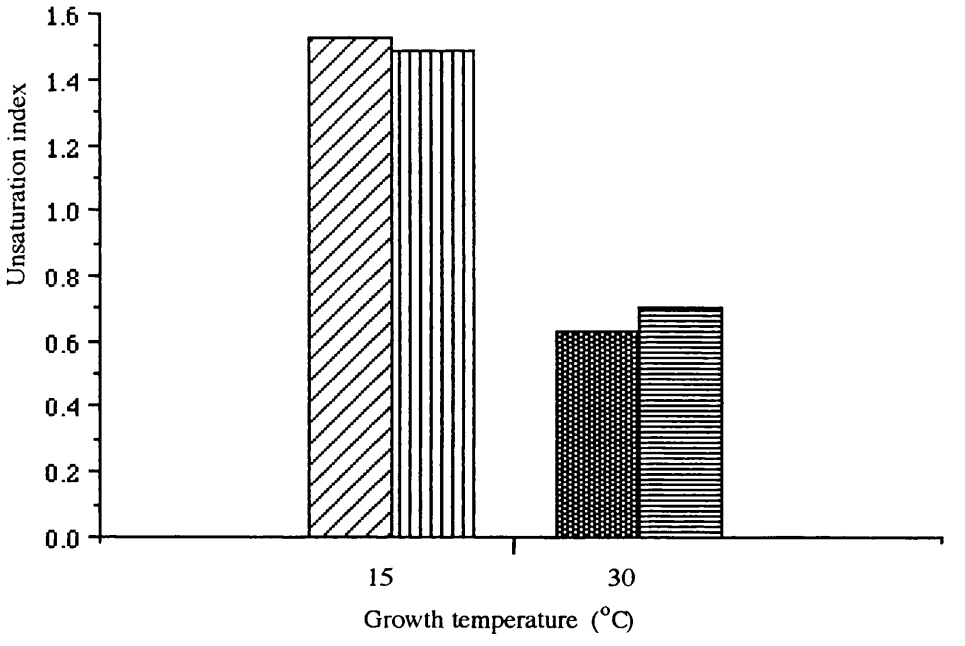
Comparison of the chain length and unsaturation indices of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$

Acinetobacter sp. NCIB 8250 was grown at 15 °C and 30 °C in continuous culture under oxygen limitation medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.5.3) with steady states achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested at each of the steady states achieved (Section 2.7), the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9) and then analysed by glc (Section 2.10.2). Each sample was analysed in triplicate and each wax ester component in that sample was then calculated as a percentage of the total.

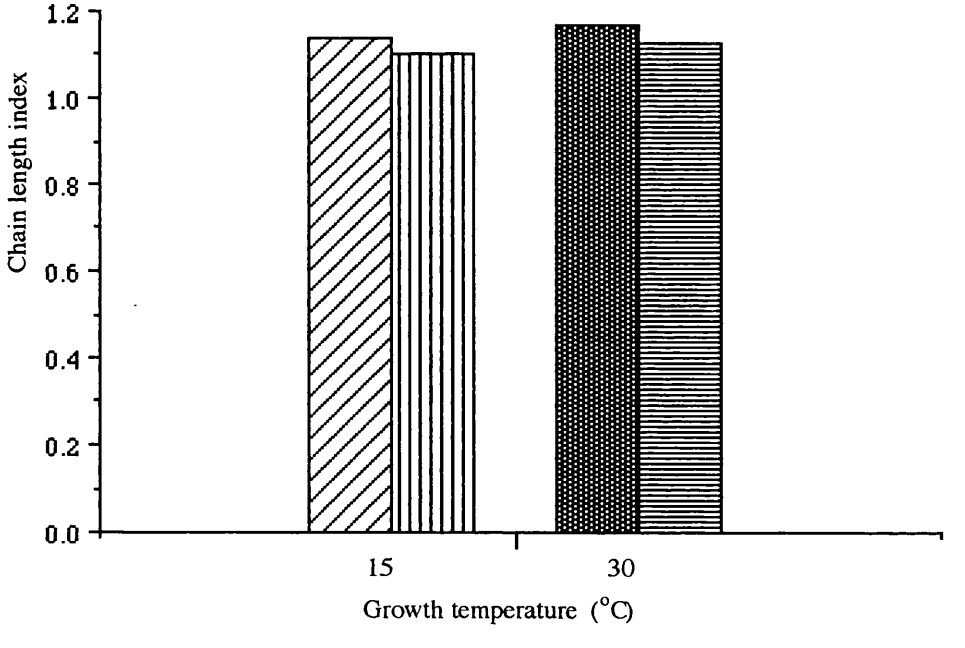
Figure 4.8a compares the values of the unsaturation index (ui) for each of the samples obtained under oxygen limitation and Figure 4.8b compares the values of the chain length index (cli) for each of the samples obtained under oxygen limitation.

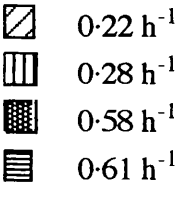
The data used in this Figure are taken from Table 4.11.

a) comparison of the unsaturation indices for the oxygen-limited samples



b) comparison of the chain length indices for the oxygen-limited samples





4.8a), although the overall chain length indices were similar regardless of the growth temperature (Table 4.10 and Figure 4.8b). The specific growth rate also had a slight but significant effect on the wax ester composition when *Acinetobacter* sp. NCIB 8250 was grown under oxygen limitation. Thus the effects of the growth temperature and specific growth rate were broadly the same as those observed when *Acinetobacter* sp. NCIB 8250 was grown in continuous culture under ammonium limitation (Tables 4.9 & 4.10).

4.3 Discussion

4.3.1 Growth rates and growth yields of *Acinetobacter* sp. NCIB 8250 grown in batch and continuous culture

The effect of growth temperature on the mean generation time of *Acinetobacter* sp. NCIB 8250 grown in batch culture has been studied previously by Fewson (1967), who showed that the growth rate was optimal between 29 - 33 °C. The results obtained in this present investigation are in agreement with this earlier report in that the optimum growth temperature in both batch and continuous culture was 30 °C. However, a specific growth rate of 0·69 h^{-1} (i.e. the mean generation time was 60 min) was determined in this present work for *Acinetobacter* sp. NCIB 8250 grown in batch culture at 30 °C, which is lower than that reported by Fewson (1967). Fewson reported a mean generation time of 45 min ($\mu = 0{\cdot}92\ h^{-1}$) when *Acinetobacter* sp. NCIB 8250 was grown in a minimal salts medium with 5 mM succinate as the sole source of carbon and energy.

Hardy (1982) also determined the specific growth rate of this bacterium grown in batch culture at 30 °C and reported values of 0·78 h^{-1} and 0·71 h^{-1} when grown on two minimal media containing 50 mM and 80 mM succinate respectively. Hardy (1982) accounted for this discrepancy from the results reported by Fewson (1967) on the basis that there was a linear relationship between the concentrations of the carbon source used and the specific growth rate obtained, i.e. the lower the succinate concentration then the higher the specific growth rate (Hardy, 1982). This relationship, however, was probably pure coincidence. A more probable explanation is that in Fewson's work the inocula were prepared by subculturing *Acinetobacter* sp. NCIB 8250 into nutrient broth before inoculating the growth flasks containing the minimal media (Fewson, 1967), whereas Hardy prepared the inocula by subculturing on succinate/agar plates and then in a liquid succinate-basal medium before inoculating the growth flask. Thus it would seem that the high value for the specific growth rate obtained by Fewson, compared with those obtained by Hardy

(1982) and in this present work (which are in agreement with each other), was probably an artifact of the inocula preparation.

In this present work the μ_{max} for *Acinetobacter* sp. NCIB 8250 grown in continuous culture at 30 °C was determined, by the method described by Tempest (1970), to be 1·11 h^{-1}. This value was much higher than the specific growth rate observed for this strain in exponential growth in batch culture (Hardy, 1982; this Thesis). A possible explanation for this would be that the inclusion of the trace metal supplement in the media used in the continuous culture experiments had a beneficial influence on the growth rate as the trace metal supplement was not included in any of the batch culture experiments (Fewson, 1967; Hardy, 1982; this thesis). Unfortunately, Hardy (1982), who included the trace metal supplement in the media used in the continuous culture experiments, did not determine the μ_{max} of *Acinetobacter* sp. NCIB 8250 in continuous culture by the method described by Tempest (1970) hence the value obtained in this investigation would appear to be the only determination.

A value of 42·9 g dry weight (mol succinate)$^{-1}$ was obtained in this work for the true molar growth yield of *Acinetobacter* sp. NCIB 8250 grown in continuous culture under succinate limitation. This value was in good agreement with the values of 42·09 - 42·56 g dry weight (mol succinate)$^{-1}$ and 41·1 g dry weight (mol succinate)$^{-1}$ reported by Hardy & Dawes (1985) and Fewson (1985) respectively. However, the maintenance coefficients, derived from the same data, do not agree. Hardy & Dawes (1985) and Fewson (1985) reported values of 0·53 - 0·56 mmol succinate (g dry weight)$^{-1}$ h^{-1} and 0·67 mmol succinate (g dry weight)$^{-1}$ h^{-1}, respectively and are three to four times lower then the value of 2·18 mmol succinate (g dry weight)$^{-1}$ h^{-1} obtained here. Hardy & Dawes (1985) also reported values of 42·66 g dry weight (mol succinate)$^{-1}$ and 1·50 mmol succinate (g dry weight)$^{-1}$ h^{-1} for the true molar growth yield and maintenance coefficient, respectively, for *Acinetobacter* sp. NCIB 8250 grown under oxygen limitation. Thus it would seem that the values obtained in this study of *Acinetobacter* sp. NCIB 8250 grown in continuous culture under succinate limitation agree better with those values for an oxygen-limited culture even though it was shown that the culture supernatants of the succinate-limited continuous culture contained insignificant amounts of organic carbon (see Section 3.2.3).

4.3.2 Wax ester content of *Acinetobacter* sp. NCIB 8250 grown in batch and continuous culture

The wax ester content *Acinetobacter* sp. NCIB 8250 has been studied previously in both batch (Fixter & Fewson, 1974; Fixter & McCormack, 1976; Nagi, 1981) and continuous culture (Hardy, 1982; Fixter *et al.*, 1986) and has been shown to

accumulate in only very low amounts under succinate limitation but in significant amounts under ammonium limitation. In succinate-limited batch culture the stationary phase bacteria contained only 1 - 2 mg wax esters (g dry weight)$^{-1}$ (Fixter & Fewson, 1974) but under ammonium limitation the wax ester content was as high as 40 - 60 mg (g dry weight)$^{-1}$ (Fixter & Sherwani, 1991). Fixter *et al.* (1986) also reported that during exponential phase the wax ester content was 5·82 mg (g dry weight)$^{-1}$. In this present work the amounts of wax esters accumulated during exponential phase were lower that those previously reported although under succinate limitation in batch culture there was agreement with these earlier reports. However, the amount accumulated under ammonium limitation, 10·4 - 15·4 mg (g dry weight)$^{-1}$, was lower than that reported by Fixter & Fewson (1974) and Fixter *et al.* (1986). Fixter *et al.* (1986) showed that the greatest rate of wax ester accumulation in batch culture occurs during the transition from exponential phase to stationary phase and that wax esters continue to accumulate during stationary phase (Fixter *et al.*, 1986) but that the amounts accumulated are dependant on the length of time the culture was in stationary phase before harvesting and on the amount of carbon source available during that time. This may account for the differences observed between this work and that of Fixter *et al.* (1986). In this work the medium supplement was added to the culture 12 - 13 h after inoculation (stationary phase being reached 7 - 9 h after inoculation) and harvested 2 h later, whereas previously the supplement was added 19 h after inoculation (Nagi, 1981; Fixter *et al.*, 1986) and harvested 2 h later.

Acinetobacter sp. NCIB 8250 has been grown in continuous culture under succinate, ammonium and oxygen limitation at a specific growth rate of 0·1 h^{-1} (Hardy, 1982). In that study the wax ester content was determined in each of those steady states as being 0·09 mg (g dry weight)$^{-1}$, 24·1 - 26·2 mg (g dry weight)$^{-1}$ and 11·9 - 19·0 mg (g dry weight)$^{-1}$, respectively. Fixter *et al.* (1986) have also studied *Acinetobacter* sp. NCIB 8250 in continuous culture and have shown that under ammonium limitation the wax ester content at specific growth rates approaching 0·1 h^{-1} was approximately 35 mg (g dry weight)$^{-1}$ and that as the specific growth rate was increased to 0·7 h^{-1} the wax ester content decreased to the levels observed during exponential phase (μ approximately 0·7 h^{-1}) in batch culture. Fixter *et al.* (1986) also grew *Acinetobacter* sp. NCIB 8250 under succinate limitation in continuous culture and showed that at high specific growth rates the wax ester content was similar to that found in cultures growing exponentially or in continuous culture at high specific growth rates under ammonium limitation. The results obtained in this present work are in very good agreement with those of Fixter *et al.* (1986) although both the results reported in this work and by Fixter *et al.* (1986) are in slight disagreement

with the values reported by Hardy (1982). This may be due to the fact that Hardy (1982) used a much higher succinate concentration in the continuous culture media and that this resulted in the inhibition of fatty acid, fatty alcohol or wax ester synthesis by a catabolite repression mechanism. However, this seems unlikely. Alternatively, the difference in the amount of wax esters accumulated in these studies may have been due to the different methods by which the wax esters were extracted. Hardy (1982), after washing the harvested cells in distilled water, lyophilised a cell suspension and then extracted the wax esters from a known weight of lyophilised cells; whereas in this work and in that of Fixter *et al.* (1986) the wax esters were extracted directly from the washed cells. The dry weight of cells used in the extraction being estimated from an optical density/dry weight calibration curve. Therefore, errors in the estimation of the dry weight of cells used may be the cause of the discrepancy between the results.

Under oxygen limitation, in this present work, the wax ester content was determined to be 2·4 - 4·7 mg (g dry weight)$^{-1}$ (Section 4.2.2), much lower than reported by Hardy (1982) for the same organism. However, Hardy (1982) determined the wax ester content of *Acinetobacter* sp. NCIB 8250 at a specific growth rate of 0·1 h^{-1} whereas in this work the specific growth rate was 0·58 - 0·61 h^{-1}. Therefore the observed difference would be expected if the relationship between wax ester content and specific growth rate are the same under oxygen limitation as under ammonium limitation, as it has been shown that the wax ester content decreased as the specific growth rate increased, under ammonium limitation.

There have been only a few reports where *Acinetobacter* species have been grown at different temperatures and the wax ester content and composition examined (e.g. Gallagher, 1971; Bryn *et al.*, 1977). Gallagher (1971) grew three strains of *Acinetobacter*, a mesophile and two psychrophiles, at growth temperatures ranging from 1 °C to 27 °C on a nutrient broth based medium and harvested at the end of the exponential phase of growth. Under these conditions the strains contained 0·8 - 7·9 % of the cell dry weight as wax esters [equivalent to 1 - 8 mg (g dry weight)$^{-1}$]. Bryn *et al.* (1977) grew a number of *Acinetobacter* strains at 33 °C but only one at 22 °C. All of these strains were grown in a nutrient broth/yeast extract medium and harvested 20 h after inoculation. The strain grown at 22 °C (MJT/F4/5,7) accumulated 4·7 mg wax esters (g dry weight)$^{-1}$, the highest of all the strains examined. In this present study *Acinetobacter* sp. NCIB 8250 was also grown in batch culture at different growth temperatures and when the cells were harvested in exponential phase during growth at 15 °C the wax ester content was 3·08 - 3·25 mg (g dry weight)$^{-1}$ whereas at 30 °C the wax ester content was lower at only 0·42 - 0·92 mg (g dry weight)$^{-1}$. However, this result can probably be explained in terms of

the growth rate of the culture. From continuous culture it was clear that the specific growth rate effects the accumulation of wax esters. Therefore at 15 °C where the cultures were growing at approximately 0·18 h^{-1} it could be expected that the wax ester content would be higher then when growing exponentially at 30 °C where the growth rate was approximately 0·7 h^{-1}. This explanation may also be applicable to the results of Bryn *et al.* (1977) where the strain MJT/F4/5,7 accumulated more wax esters than those strains grown at 33 °C. Alternatively it has been shown that some strains accumulate more wax esters than others, even under the same growth conditions (Fixter & McCormack, 1976) and strain MJT/F4/5,7 may be a naturally high producing strain.

In continuous culture where growth conditions are more rigorously controlled the effect of growth temperature on the wax ester content can be investigated independently of other parameters, such as growth rate. Using the data in Table 4.5a it is possible to assess if the growth temperature had an effect on the wax ester content of the cells. Comparing the wax ester content at similar specific growth rates from different growth temperatures e.g. at 0·28 h^{-1} the wax ester content at 15 °C and 30 °C were 3·29 mg (g dry weight)$^{-1}$ and 27·1 mg (g dry weight)$^{-1}$ respectively and at 20 °C and at 0·27 h^{-1} the wax ester content was 7·9 mg (g dry weight)$^{-1}$, it would appear that a decrease in the growth temperature had a negative effect on the wax ester content.

However, when considering data from different growth temperature it may be more pertinent to examine the data using the relative specific growth rate instead of the specific growth rate, that is dividing the specific growth rate by the maximum specific growth rate at that temperature. By treating the data in this way it would appear that the growth temperature in some instances had a positive effect. For instance, at 15 °C a specific growth rate of 0·22 h^{-1} equates to a relative specific growth rate of 0·60. A similar relative specific growth rate was achieved at 30 °C and under this criterion twice as much wax esters were accumulated at 15 °C than at 30 °C. This was also true for a relative specific growth rate of 0·22 - 0·25 at 25 °C and 30 °C where the wax ester contents were 43·4 mg (g dry weight)$^{-1}$ and 27·1 mg (g dry weight)$^{-1}$ respectively. However, at a relative specific growth rate of 0·42 - 0·45 the wax ester contents at 20, 30 and 33 °C were 7·9, 12·3 and 6·6 mg (g dry weight)$^{-1}$ respectively. Thus, there is still some doubt as to a direct effect of the growth temperature on the wax ester content, although, at each growth temperature under ammonium limitation (and probably under oxygen limitation), the actual specific growth rate had a very significant effect on the amount of wax esters accumulated.

4.3.3 Wax ester composition of *Acinetobacter* sp. NCIB 8250 grown in batch and continuous culture

The wax ester composition is perhaps the most studied aspect of wax ester metabolism in micro-organisms and in particularly, in *Acinetobacter*. Through this work several major factors have been shown to influence the wax ester composition: the nutrient composition of the medium; the growth temperature and in this present study, using continuous culture, the specific growth rate at which the culture was grown.

4.3.3.1 Effect of nutrient composition on the wax ester composition

It has been shown that the nutrient composition of the medium effects quite markedly the extent to which wax esters are accumulated. This was perhaps most evident in continuous culture at specific growth rates less than 0·2 h^{-1} where cultures growing at 30 °C under succinate limitation accumulate less than 1 mg (g dry weight)$^{-1}$ whereas under ammonium limitation they have been shown to accumulate to more than 40 mg (g dry weight)$^{-1}$. Several reports however, have also shown that the wax ester composition can be effected by the nutrient composition of the medium (Bryn *et al.*, 1977; de Witt *et al.*, 1982; Geigert *et al.*, 1984; Fixter *et al.*, 1986). These workers have grown a number of *Acinetobacter* strains on defined medium with hydrocarbons as the carbon and energy source (de Witt *et al.*, 1982; Geigert *et al.*, 1984), on defined medium with succinate as the carbon and energy source (Fixter *et al.*, 1986) and on a nutrient broth based medium (Bryn *et al.* , 1977). When hydrocarbons were used as the carbon and energy source assimilation of the hydrocarbons produced the corresponding fatty acids, via the terminal oxidation of the *n*-alkane first to the alcohol and then the aldehyde. The synthesis of the fatty acids from hydrocarbons results in the suppression of *de novo* fatty acid biosynthesis (Sampson & Finnerty, 1974). Geigert and co-workers using *Acinetobacter* sp. HO1-N grown on hydrocarbons as the carbon and energy source have shown that the acyl chains of the fatty acid and fatty alcohol moities in the wax esters reflected the carbon number of the growth substance used (de Witt *et al.*, 1982; Geigert *et al.*, 1984). For instance, if the strain was grown on hexadecane then the wax esters were exclusively C_{32} with the fatty acid and fatty alcohol moities both being C_{16}. Saturated and mono-unsaturated fatty acid and fatty alcohols giving saturated, mono- and di-unsaturated wax esters were observed. When growth on *n*-alkanes greater than C_{16} were used, e.g. *n*-C_{20}, there was greater variation in the chain length with C_{36}, C_{38} and C_{40} saturated, mono- and di-unsaturated wax esters being detected (de Witt *et al.*, 1982). Also, with the use of the longer chain length hydrocarbons as the carbon source there was an increase in the degree of

unsaturation with a predominance of the di-unsaturated wax esters. Presumably this increase in the degree of unsaturation was to counter the increased melting point caused by the longer chain length wax esters, thereby maintaining the fluidity of these lipids.

When acinetobacters are grown in a defined medium where the carbon source is not a long chain hydrocarbon, fatty alcohol, aldehyde or fatty acid then the fatty acids required for growth are synthesised *de novo*. Fixter *et al.* (1986) have reported that with *Acinetobacter* sp. NCIB 8250 growing on succinate as the carbon and energy source, that in ammonium-limited batch cultures the stationary phase cells accumulate predominantly the saturated C_{32} wax ester with smaller but significant amounts of the saturated C_{34} wax ester. This result has been confirmed in this investigation and it has also been shown that the wax ester composition of these cells was different to those found in succinate-limited batch culture stationary phase cells.

In the report by Bryn *et al.* (1977) where several strains of *Acinetobacter* where grown on a nutrient broth/yeast extract medium and harvested after 20 h the wax ester compositions were very different to those reported by Fixter *et al.* (1986) or Geigert and co-workers (de Witt *et al.*, 1982; Geigert *et al.*, 1984). The wax ester compositions reported by Bryn *et al.* (1977) consisted predominantly of di-unsaturated wax esters, even in those strains grown at 33 °C. In the strain MJT/F4/5,7 grown at 22 °C, the wax esters were still di-unsaturated but were predominantly C_{34} whereas the strains grown at 33 °C contained predominantly C_{36} wax esters.

4.3.3.2 Effect of growth temperature on the wax ester composition

It is well documented that the growth temperature has a profound effect on the fatty acid composition of the phospholipids. It has also been shown that the growth temperature effects the wax ester composition of *Micrococcus cryophilus* ATCC15174 (Russell & Volkman, 1980). This psychrophilic bacterium has a growth temperature range of minus 4 °C to 25 °C and the wax esters in this organism have been studied in cells grown at 1 °C and 20 °C. As the growth temperature was decreased there was a significant increase in the degree of unsaturation (ui = 1·07 at 20 °C to ui = 1·81 at 1 °C) resulting in approximately 98 % of the wax esters being unsaturated (83·3 % di-unsaturated and 14·8 % mono-unsaturated) at 1 °C (Russell & Volkman, 1980). These workers proposed that because there was very little change in the fatty acid composition of the phospholipids found in this bacterium throughout its growth temperature range, with only small changes in the chain length of the fatty acids occurring as the growth temperature was decreased, that the wax esters might

be used as membrane modifiers, adjusting the fluidity of the membranes as the growth temperature altered. However McGibbon *et al.* (1985) have shown using differential scanning calorimetry and fluorescence polarisation that the wax esters present in the membranes of *M. cryophilus* ATCC 15174 (Lloyd & Russell, 1984) are not involved in maintaining membrane fluidity and that the function of the wax esters in this bacterium is still unknown.

The changes in the wax ester composition observed in *Acinetobacter* sp. NCIB 8250 during this work were similar to those found by Russell & Volkman (1980) in that as the growth temperature was decreased from 33 °C to 15 °C the degree of unsaturation increased (ui = 0·62 - 0·67 at 33 °C and ui = 1·45 - 1·53 at 15 °C). Interestingly, the increase in the degree of unsaturation observed in the wax esters as the growth temperature was decreased cannot be entirely accounted for by the observed increase in the degree of unsaturation in the fatty acids (Sections 5.2.1.5 & 5.3.2) in the wax esters. Thus there was a biased increase in the degree of unsaturation in the fatty alcohols, i.e. there was a much greater increase in the degree of unsaturation in the fatty alcohols than in the fatty acids, as the growth temperature was decreased. At the higher growth temperatures of 30 °C and 33 °C the degree of unsaturation in both the fatty acid and fatty alcohols were very similar. This could mean that the fatty alcohol dehydrogenase involved producing the fatty alcohols used in the biosynthesis of wax esters has a greater preference for mono-unsaturated acyl chains than it does for saturated ones.

4.3.3.3 Effect of specific growth rate on the wax ester composition

A direct analysis of the specific growth rate on the wax ester composition has not been undertaken before, although Fixter *et al.* (1986) had used continuous culture to examine the effect of specific growth rate on total wax ester content. Russell and co-workers have also grown *Micrococcus cryophilus* ATCC 15174 at different specific growth rates, but in batch culture, by altering the growth temperature (Russell, 1971; 1974; 1978b; Russell & Volkman, 1980) and therefore it is not possible to separate any effect of the specific growth rate on the wax ester composition from an affect of the growth temperature. Indeed, unless continuous culture is used to examine both the growth temperature and the specific growth rate independently it is not possible to say absolutely that the effects observed by Russell and co-workers are a result of the growth temperature and not a result of the alteration in the specific growth rate.

In the present investigation continuous culture was used to study the effect of the specific growth rate and growth temperature independently of each other. The affect of the growth temperature (Section 4.3.3.2) was to increase the degree of

unsaturation in the wax esters as the growth temperature was decreased from 33 °C to 15 °C. The effect of decreasing the specific growth rate, at any one temperature, was to decrease the degree of unsaturation and increase the overall chain length of the wax esters. In terms of the biochemistry of wax ester synthesis this may give an indication of the route by which fatty acids are synthesised and modified in this bacterium. The implications being that the saturated C_{16} fatty acid is the end product of the fatty acid synthase. This could also be concluded from the work of Fixter *et al.* (1986) where the major product of wax ester accumulation during stationary phase under ammonium limitation was the $C_{32:0}$ wax ester. The C_{16} fatty acid, probably the end product of the fatty acid synthase and present as the ACP derivative, is then the substrate for an elongase or a desaturase. It would seem that the preferred route for the $C_{16:0}$ would be elongation as opposed to desaturation whereas the desaturase is more active at the higher specific growth rates. This in turn can probably be explained as the fatty acid composition of the phospholipids is known, and have been shown to have a requirement for unsaturated fatty acids, therefore, at higher specific growth rates the desaturase would be expected to be more active than at lower specific growth rates.

Chapter Five

Effect of Specific Growth Rate, Growth Temperature and Nutrient Limitation on the Fatty Acid Composition of the Wax Esters and Phospholipids in *Acinetobacter* sp. NCIB 8250

5.1 Introduction

The affect of growth temperature on the fatty acid composition of the phospholipids in micro-organisms has been studied extensively. As it had been shown that the growth temperature had a significant affect on the wax ester compositions found in *Acinetobacter* sp. NCIB 8250 it was of interest to see what affect the growth temperature, as well as other factors, such as nutrient limitation and specific growth rate had on the fatty acid composition of the phospholipids in this bacterium. Also, as fatty acids are components of both wax esters and phospholipids it was of interest to see if these different factors affected the fatty acid compositions of the wax esters and phospholipids in *Acinetobacter* sp. NCIB 8250 in the same way or in different ways.

The results of batch and continuous culture experiments on the effect of growth temperature, specific growth rate and nutrient limitation on the fatty acid compositions in the wax esters and phospholipids are presented in this chapter.

5.2 Results

5.2.1 Wax ester fatty acid compositions

The fatty acids observed in the wax esters and phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in batch and continuous culture on either the medium with a low concentration of succinic acid or the medium with a low concentration of $(NH_4)_2SO_4$ were $C_{14:0}$, $C_{16:1}$, $C_{16:0}$, $C_{18:1}$ and $C_{18:0}$ (Figure 5.1). These fatty acids were present, in varying proportions, under all growth conditions studied.

5.2.1.1 Fatty acid composition of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 15 °C and harvested during exponential phase

The fatty acid composition of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 15 °C on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase were all significantly different ($P < 0{\cdot}05$) from each other (Table 5.1a). However, the overall patterns in the fatty acid compositions of the wax esters were the same [Figure 5.2 (▤ , ▨)]. In these compositions the $C_{18:1}$ fatty acid was approximately 50 % of the total with the $C_{16:1}$, $C_{16:0}$ and $C_{18:0}$ fatty acids all being

Figure 5.1

Gas chromatogram of the fatty acids from the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture and harvested during the exponential phase of growth

Acinetobacter sp. NCIB 8250 was grown at 30 °C in batch culture in a 2 l flask on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The culture was harvested (Section 2.7) in exponential phase (Section 2.5.2) and the pellet washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0. The lipids were extracted (Section 2.8) and the phospholipids and wax esters separated by tlc (Section 2.9). The fatty acids in the phospholipids were then converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2).

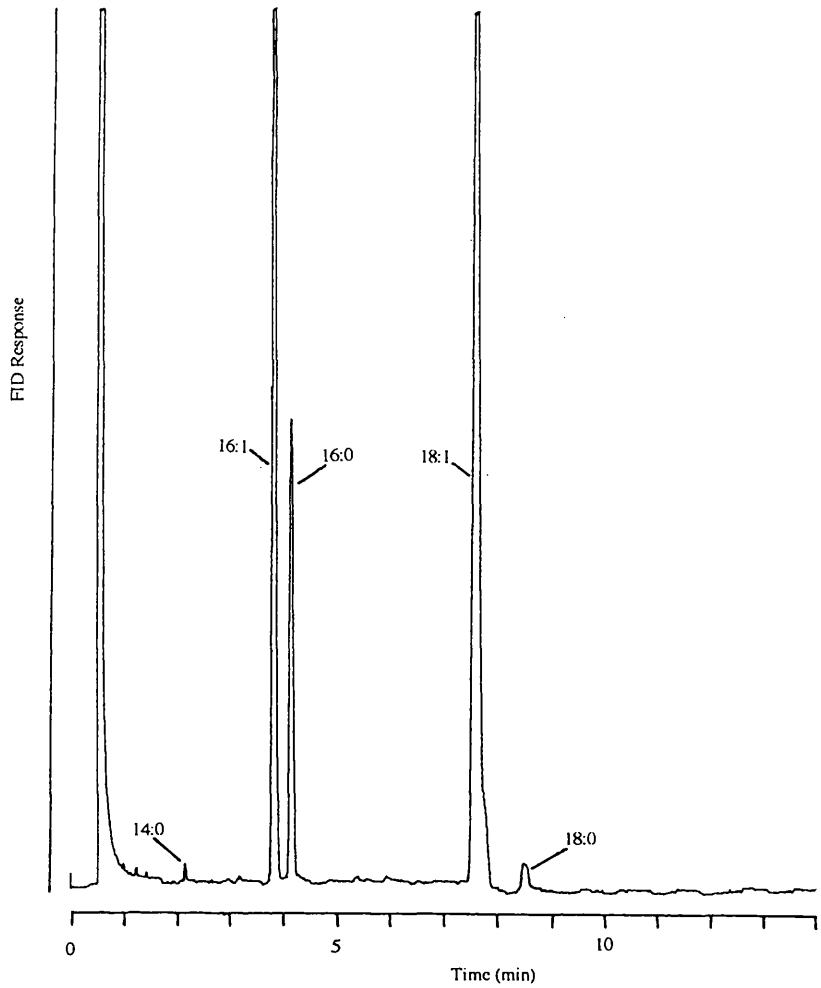

FID Response
16:1
16:0
18:1
14:0
18:0
0
5
10
Time (min)

Table 5.1

Fatty acid compositions of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 15 °C and 30 °C on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase of growth and in stationary phase

Acinetobacter sp. NCIB 8250 was grown at 15 °C and 30 °C in batch culture in 2 l flasks on the medium with a low concentration of succinic acid (Section 2.3.2.2) or on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in either the exponential phase of growth or in stationary phase (Section 2.5.2), the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9), the fatty acids in the wax esters converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in the sample was then calculated as a percentage of the total. The results are expressed as the mean ± standard deviation together with the chain length (cli) and unsaturation indices (ui).

Table 5.1a shows the fatty acid composition of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 15 °C and harvested during exponential phase, Table 5.1b shows the fatty acid composition of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C and harvested during the exponential phase of growth and Table 5.1c shows the fatty acid composition of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C and harvested during stationary phase.

‡There was insufficient sample available for the analysis of the fatty acids in the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown on the medium with a low concentration of succinic acid because of the low wax ester content of such cells (see Section 4.2.2).

#Average of only two analyses.

*Denotes that $P < 0{\cdot}05$ for a comparison of the fatty acid values between the media. None of the others were significantly different, i.e. $P > 0{\cdot}05$.

a) grown at 15 °C and havested during exponential phase

	Percentage of the total	
Fatty acid	Low succinate	Low ammonium
*14:0	1·7 ± 0·75	3·4 ± 0·34
*16:1	14·0 ± 0·22	18·7 ± 0·98
*16:0	15·9 ± 0·72	18·7 ± 0·26
*18:1	52·0 ± 0·39	45·4 ± 0·70
*18:0	16·3 ± 0·64	13·9 ± 0·47
cli	1·67	1·56
ui	0·66	0·64

b) grown at 30 °C and havested during exponential phase

	Percentage of the total	
Fatty acid	Low succinate‡	Low ammonium
14:0		13·2 ± 1·05
16:1		12·5 ± 0·88
16:0		25·8 ± 0·06
18:1		17·5 ± 2·84
18:0		31·0 ± 1·34
cli		1·35
ui		0·30

c) grown at 30 °C and havested during stationary phase

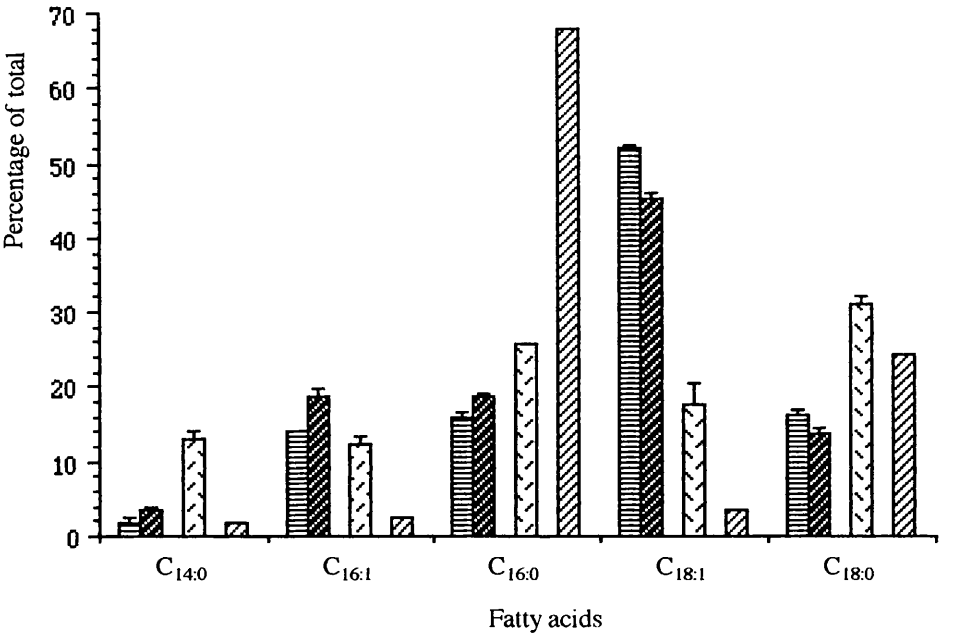
	Percentage of the total	
Fatty acid	Low succinate‡	Low ammonium#
14:0		1·7
16:1		2·4
16:0		67·9
18:1		3·6
18:0		24·4
cli		1·26
ui		0·06

Figure 5.2

Comparison of the fatty acid compositions of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 15 °C and 30 °C on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested either during the exponential phase of growth or in stationary phase

Acinetobacter sp. NCIB 8250 was grown at 15 °C and 30 °C in batch culture in 2 l flasks on the medium with a low concentration of succinic acid (Section 2.3.2.2) and on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2). The cultures were harvested (Section 2.7) in the exponential phase of growth or in stationary phase (Section 2.5.2), the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9), the fatty acids in the wax esters converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in the sample was then calculated as a percentage of the total.

The data used in this Figure are taken from Table 5.1.



Succinate-limited - exponential phase - 15 °C
Ammonium-limited - exponential phase - 15 °C
Ammonium-limited - exponential phase - 30 °C
Ammonium-limited - stationary phase - 30 °C

of a similar proportion to each other, i.e. 13·9 - 18·7 %.

5.2.1.2 Fatty acid composition of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 30 °C and harvested during exponential phase

The fatty acids of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C and harvested during exponential phase from the medium with a low concentration of $(NH_4)_2SO_4$ (Table 5.1b) showed that the saturated fatty acids, $C_{16:0}$ and $C_{18:0}$, were predominant, being 25·8 % and 31·0 % of the total, respectively. Each of the remaining fatty acids, $C_{14:0}$, $C_{16:1}$ and $C_{18:1}$, were approximately 15 % of the total (Table 5.1b). There was a significant difference ($P < 0·05$) in the fatty acid compositions of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown under these conditions with those extracted from *Acinetobacter* sp. NCIB 8250 grown at 15 °C (Tables 5.1a & b). These differences were because of changes in the degree of unsaturation, the fatty acids of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C being more saturated (ui = 0·30) than those extracted from *Acinetobacter* sp. NCIB 8250 grown at 15 °C (ui = 0·64 - 0·66) (Tables 5.1a & b and Figure 5.2).

5.2.1.3 Fatty acid composition of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 30 °C and harvested during stationary phase

The fatty acids in the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C and harvested during stationary phase from ammonium-limited cultures (Table 5.1c) consisted primarily of $C_{16:0}$ (67·9 %) and $C_{18:0}$ (24·4 %), whereas the fatty acid composition in the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C but harvested during exponential phase showed a more even distribution (Table 5.1b and Figure 5.2). Statistical analysis using the values for the fatty acid of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C and harvested from stationary phase was not possible because only two analyses of this sample were obtained (Table 5.1c). However, it was clear from the fatty acid compositions and the chain length and unsaturation indices that there were differences between the growth conditions (Tables 5.1b & c and Figure 5.2).

5.2.1.4 Fatty acid composition of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under succinate limitation

The major fatty acids observed in the wax esters extracted from *Acinetobacter* sp.

NCIB 8250 grown in continuous culture at 30 °C under succinate limitation were the $C_{16:0}$, $C_{18:1}$ and $C_{18:0}$ fatty acids (Table 5.2). Together these fatty acids comprised 80 - 85 % of the total. There was, apparently, a significant effect of the specific growth rate on the relative proportion of the $C_{16:0}$ and $C_{18:0}$ fatty acids, however, only two specific growth rates were examined.

5.2.1.5 Fatty acid composition of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under ammonium limitation

The predominant fatty acids present in the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under ammonium limitation were the $C_{16:0}$, $C_{18:1}$ and $C_{18:0}$ fatty acids (Table 5.3). The growth temperature had a significant effect ($P < 0.05$) on the fatty acid composition, however, the specific growth rate did not. The main effect of decreasing the growth temperature was to increase slightly the degree of unsaturation (ui = 0·29 - 0·39 at 30 °C and ui = 0·41 - 0·47 at 15 °C) with no real change in the overall chain length (cli = 1·54 - 1·67 at 30 °C and cli = 1·58 - 1·64 at 15 °C) (Table 5.3 and Figure 5.3). At 30 °C the fatty acid composition of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown under ammonium limitation (cli = 1·54 - 1·67 and ui = 0·29 - 0·39) were similar to those extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under succinate limitation (cli = 1·39 - 1·59 and ui = 0·28 - 0·30) (Tables 5.2 & 5.3) but showed some difference in the overall chain length to the fatty acid composition of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C and harvested from exponential phase (cli = 1·35) (Tables 5.1b & 5.3).

5.2.1.6 Fatty acid composition of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under oxygen limitation

At 30 °C under oxygen limitation the major fatty acids in the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture on the medium with a low concentration of $(NH_4)_2SO_4$ were the $C_{16:0}$, $C_{18:1}$ and $C_{18:0}$ fatty acids (Table 5.4), the same as those observed when *Acinetobacter* sp. NCIB 8250 was grown under succinate or ammonium limitation. When *Acinetobacter* sp. NCIB 8250 was grown at 15 °C under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$ the degree of unsaturation in the fatty acids of the wax esters increased from 0·37 - 0·40 at 30 °C to 0·58 - 0·60 at 15 °C. This increase in the unsaturation index at 15 °C was due to the increase in the proportion of the $C_{16:1}$ and $C_{18:1}$ fatty acids with the concomitant decrease of the $C_{18:0}$ fatty acid when compared with the

Table 5.2

Fatty acid compositions of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under succinate limitation

Acinetobacter sp. NCIB 8250 was grown at 30 °C in continuous culture under succinate limitation (Section 2.5.3) and steady states were achieved at 0·26 h^{-1} and 0·46 h^{-1}. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9), the fatty acids in the wax esters converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in the sample was then calculated as a percentage of the total. The results are expressed as the mean ± standard deviation together with the chain length (cli) and unsaturation indices (ui).

*Denotes that the specific growth rate had a significant effect ($P < 0·05$) on the composition of those fatty acids. None of the others were significantly different, i.e. $P > 0·05$.

Specific growth rate	0·26 h^{-1}	0·46 h^{-1}
Fatty acid		
14:0	5·6 ± 1·27	8·0 ± 2·17
16:1	8·4 ± 0·75	12·1 ± 4·79
*16:0	28·5 ± 0·81	31·2 ± 1·46
18:1	21·9 ± 0·81	16·1 ± 6·31
*18:0	35·8 ± 0·59	31·7 ± 1·77
cli	1·52	1·39
ui	0·30	0·28

Table 5.3

Fatty acid compositions of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under ammonium limitation

Acinetobacter sp. NCIB 8250 was grown in continuous culture at 15 20, 25 and 30 °C under ammonium limitation (Section 2.5.3) and steady states were achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9), the fatty acids in the wax esters converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in the sample was then calculated as a percentage of the total. The results are expressed as the mean ± standard deviation together with the chain length (cli) and unsaturation indices (ui).

*Denotes that the growth temperature and the specific growth rate had a significant effect ($P < 0·05$) on the compositions of those fatty acids. None of the others were significantly different, i.e. $P > 0·05$.

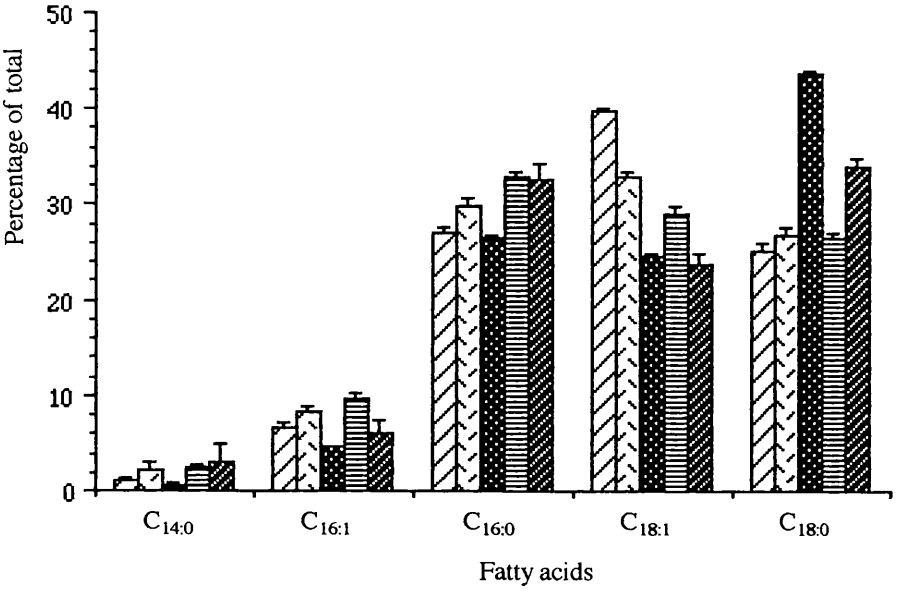
Growth temperature	15 °C		20 °C	25 °C		30 °C	
Specific growth rate	0·22 h^{-1}	0·28 h^{-1}	0·27 h^{-1}	0·18 h^{-1}	0·14 h^{-1}	0·62 h^{-1}	0·77 h^{-1}
Fatty acid							
*14:0	1·1 ± 0·37	2·1 ± 0·84	0·9 ± 0·30	4·2 ± 0·65	0·5 ± 0·40	2·4 ± 0·29	3·1 ± 1·90
*16:1	6·7 ± 0·40	8·3 ± 0·47	7·5 ± 0·44	5·3 ± 0·12	4·7 ± 0·05	9·6 ± 0·58	6·2 ± 1·25
*16:0	27·0 ± 0·67	29·8 ± 0·94	17·6 ± 0·09	17·3 ± 0·31	26·6 ± 0·27	32·8 ± 0·68	32·5 ± 1·88
*18:1	39·9 ± 0·24	32·8 ± 0·70	49·0 ± 0·45	28·5 ± 0·90	24·5 ± 0·25	29·0 ± 0·77	23·7 ± 1·05
*18:0	25·1 ± 0·77	26·9 ± 0·60	25·0 ± 0·36	44·6 ± 0·13	43·6 ± 0·33	26·5 ± 0·70	33·9 ± 1·01
cli	1·64	1·58	1·73	1·69	1·67	1·54	1·54
ui	0·47	0·41	0·57	0·34	0·29	0·39	0·30

Figure 5.3

Comparison of the fatty acid compositions in the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture at 15 °C and 30 °C under ammonium limitation

Acinetobacter sp. NCIB 8250 was grown at 15 °C and 30 °C in continuous culture under ammonium limitation (Section 2.5.3) and steady states were achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states achieved, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9), converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in that sample was then calculated as a percentage of the total.

The data used in this Figure are taken from Table 5.3.



15 °C - 0·22 h^{-1}

15 °C - 0·28 h^{-1}

30 °C - 0·14 h^{-1}

30 °C - 0·62 h^{-1}

30 °C - 0·77 h^{-1}

Table 5.4

Fatty acid compositions of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$

Acinetobacter sp. NCIB 8250 was grown at 15 °C and 30 °C in continuous culture under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.5.3). A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states achieved, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9), the fatty acids in the wax esters converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in the sample was then calculated as a percentage of the total. The results are expressed as the mean ± standard deviation together with the chain length (cli) and unsaturation indices (ui).

*Denotes that the growth temperature and the specific growth rate had a significant effect ($P < 0{\cdot}05$) on the composition of those fatty acids. None of the others were significantly different, i.e. $P > 0{\cdot}05$

Growth temperature	15 °C		30 °C	
Specific growth rate	0·22 h^{-1}	0·28 h^{-1}	0·58 h^{-1}	0·61 h^{-1}
Fatty acid				
*14:0	4·1 ± 0·37	3·0 ± 0·24	3·7 ± 1·97	2·5 ± 0·09
*16:1	20·7 ± 0·33	16·6 ± 1·56	11·5 ± 2·79	10·9 ± 0·39
*16:0	21·3 ± 0·23	20·1 ± 0·23	27·9 ± 1·12	30·2 ± 0·59
*18:1	39·6 ± 0·35	41·8 ± 0·75	25·1 ± 1·58	29·1 ± 0·85
*18:0	14·4 ± 0·62	18·5 ± 0·58	31·9 ± 2·74	27·3 ± 0·41
cli	1·50	1·57	1·53	1·54
ui	0·60	0·58	0·37	0·40

proportion of the corresponding fatty acids at 30 °C (Table 5.4 and Figure 5.4).

At 15 °C the fatty acid composition in the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$ were more unsaturated (ui = 0·58 - 0·60) than the fatty acids in the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown at 15 °C under ammonium limitation (ui = 0·41 - 0·47) (Tables 5.3 & 5.4). The increase in the degree of unsaturation under oxygen limitation was because of the increase in the proportion of the $C_{16:1}$ fatty acid and the decrease of the proportion of the $C_{18:0}$ fatty acid (Tables 5.3 & 5.4).

5.2.2 Phospholipid fatty acid compositions

5.2.2.1 Fatty acid composition of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 15 °C and harvested during exponential phase

At 15 °C the fatty acid composition of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown on the medium with a low concentration of succinic acid and harvested during the exponential phase was similar to the fatty acid composition of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase (Table 5.5a). The chain length (cli = 1·55 - 1·57) and unsaturation (ui = 0·82 - 0·83) indices showed that the overall patterns in these two conditions were nearly identical (Figure 5.5) with the $C_{16:1}$, $C_{16:0}$ and $C_{18:1}$ fatty acids being predominant. The $C_{18:1}$ fatty acid was greater than 50 % of the total and was approximately twice that of the $C_{16:1}$ fatty acid and four times that of the $C_{16:0}$ fatty acid (Table 5.5a & Figure 5.5). There was a small but significant difference ($P < 0·05$), however, between the values for the $C_{16:1}$ and $C_{18:1}$ fatty acids of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown on the medium with a low concentration of succinic acid with the values for the $C_{16:1}$ and $C_{18:1}$ fatty acids of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown on the medium with a low concentration of $(NH_4)_2SO_4$ (Table 5.5a).

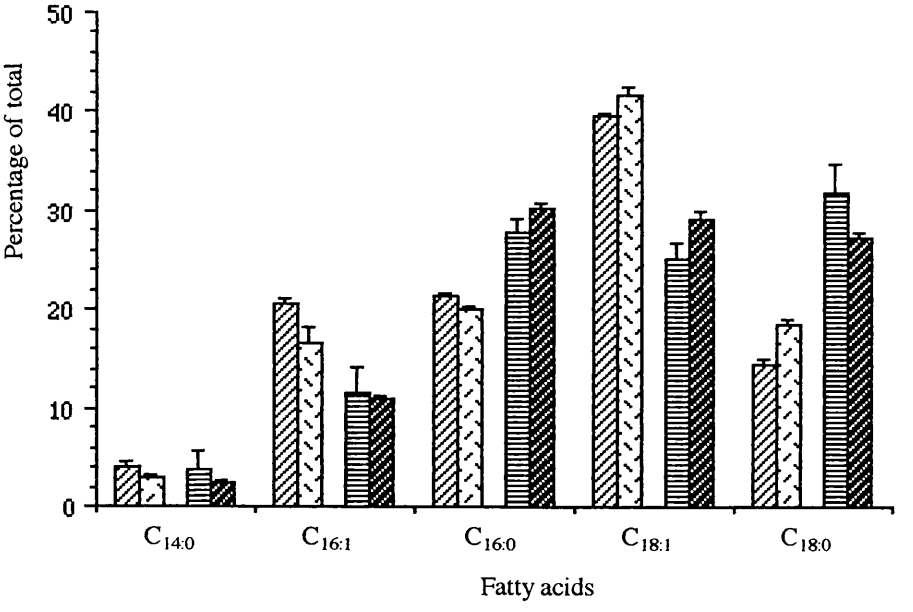
Comparison of the fatty acid compositions of the wax esters and phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown at 15 °C and harvested during the exponential phase of growth from the medium with a low concentration of succinic acid showed a significant difference ($P < 0·05$) in all but the $C_{14:0}$ fatty acid (Tables 5.1a & 5.5a and Figure 5.6a). In a similar comparison but with the fatty acid compositions in the wax esters and phospholipids extracted from *Acinetobacter* sp. NCIB 8250 harvested from the medium with a low concentration of $(NH_4)_2SO_4$

Figure 5.4

Comparison of the fatty acid compositions of the wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under oxygen limitation

Acinetobacter sp. NCIB 8250 was grown at 15 °C and 30 °C in continuous culture under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.5.3). A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states achieved, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The wax esters were separated from the other lipid components in the sample by tlc (Section 2.9) and the fatty acids in the wax esters were then converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in that sample was then calculated as a percentage of the total.

The data used in this Figure are taken from Table 5.4.



15 °C - 0·22 h^{-1}

15 °C - 0·28 h^{-1}

30 °C - 0·58 h^{-1}

30 °C - 0·61 h^{-1}

Table 5.5

Fatty acid compositions of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase of growth and in stationary phase

Acinetobacter sp. NCIB 8250 was grown at 15 °C and 30 °C in batch culture in 2 l flasks on the medium with a low concentration of succinic acid (Section 2.3.2.2) and on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in either the exponential phase of growth or in stationary phase (Section 2.5.2), the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9), the fatty acids in the phospholipids converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in the sample was then calculated as a percentage of the total. The results are expressed as the mean ± standard deviation together with the chain length (cli) and unsaturation indices (ui).

Table 5.5a shows the fatty acid compositions of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown at 15 °C and harvested during exponential phase, Table 5.5b shows the fatty acid compositions of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C and harvested during exponential phase and Table 5.5c shows the fatty acid compositions of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C and harvested during stationary phase.

*Denotes that $P < 0{\cdot}05$ for a comparison of the fatty acid values between the two media. None of the others were significantly different, i.e. $P > 0{\cdot}05$.

a) grown at 15 °C and harvested during exponential phase

	Percentage of the total	
Fatty acid	Low succinate	Low ammonium
14:0	1·9 ± 0·17	1·1 ± 0·52
*16:1	28·6 ± 0·09	27·4 ± 0·53
16:0	13·1 ± 0·27	13·2 ± 0·45
*18:1	53·6 ± 0·33	55·8 ± 0·97
18:0	2·8 ± 0·24	2·4 ± 0·36
cli	1·55	1·57
ui	0·82	0·83

b) grown at 30 °C and harvested during exponential phase

	Percentage of the total	
Fatty acid	Low succinate	Low ammonium
14:0	6·0 ± 1·83	7·4 ± 0·66
*16:1	22·7 ± 1·81	18·4 ± 1·63
16:0	23·1 ± 2·09	20·2 ± 2·33
*18:1	43·0 ± 2·06	49·8 ± 1·82
*18:0	5·1 ± 0·83	7·8 ± 1·18
cli	1·42	1·54
ui	0·66	0·68

c) grown at 30 °C and harvested during stationary phase

	Percentage of the total	
Fatty acid	Low succinate	Low ammonium
14:0	3·1 ± 0.68	2·3 ± 0·45
16:1	16·2 ± 1.25	15·1 ± 0·51
*16:0	29·7 ± 1.98	35·0 ± 0·50
18:1	48·4 ± 2.32	44·8 ± 0·61
18:0	2·6 ± 1.26	2·8 ± 0·50
cli	1·48	1·45
ui	0·65	0·60

Figure 5.5

Comparison of the fatty acid compositions of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 15 °C on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase of growth

Acinetobacter sp. NCIB 8250 was grown at 15 °C in batch culture in 2 l flasks on the medium with a low concentration of succinic acid (Section 2.3.2.2) and on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in exponential phase (Section 2.5.2), the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9), the fatty acids in the phospholipids converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in that sample was then calculated as a percentage of the total.

The data used in this Figure are taken from Table 5.5.

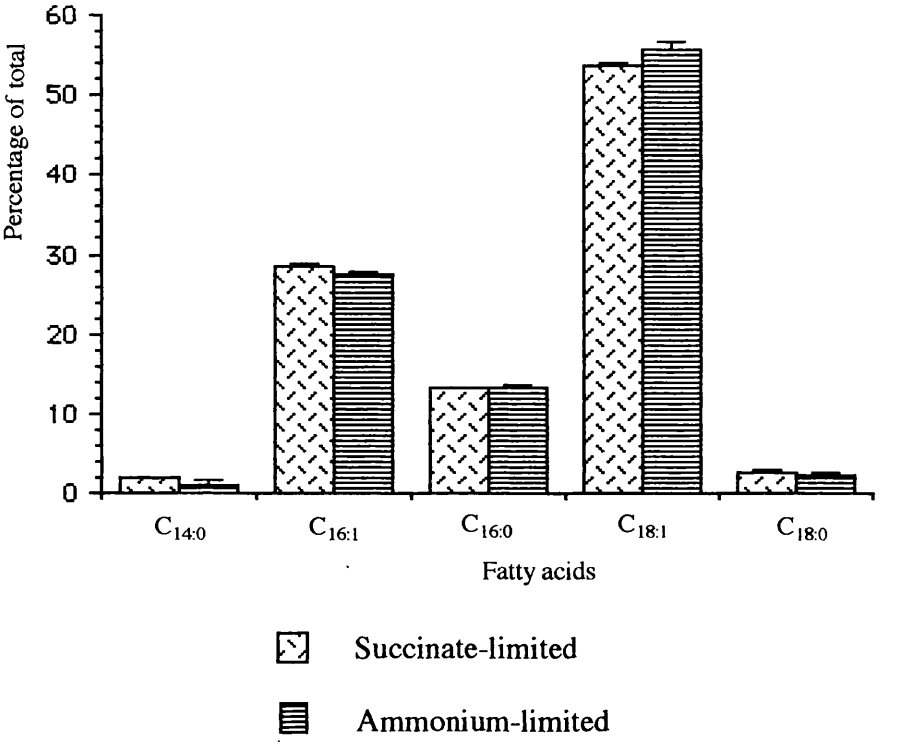

Percentage of total
60
50
40
30
20
10
0
C14:0
C16:1
C16:0
C18:1
C18:0
Fatty acids
Succinate-limited
Ammonium-limited

Figure 5.6

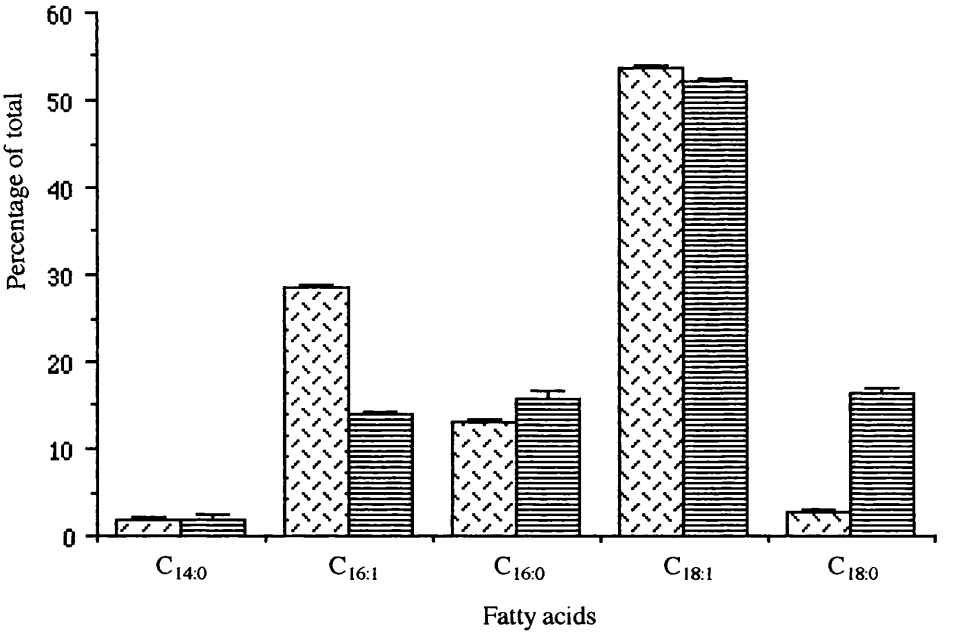
Comparison between the fatty acid compositions of the phospholipids and wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 15 °C and harvested during the exponential phase of growth

Acinetobacter sp. NCIB 8250 was grown at 15 °C in batch culture in 2 l flasks on the medium with a low concentration of succinic acid (Section 2.3.2.2) and on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in exponential phase (Section 2.5.2), the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9) and the fatty acids in the phospholipids and wax esters were then converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in that sample was then calculated as a percentage of the total.

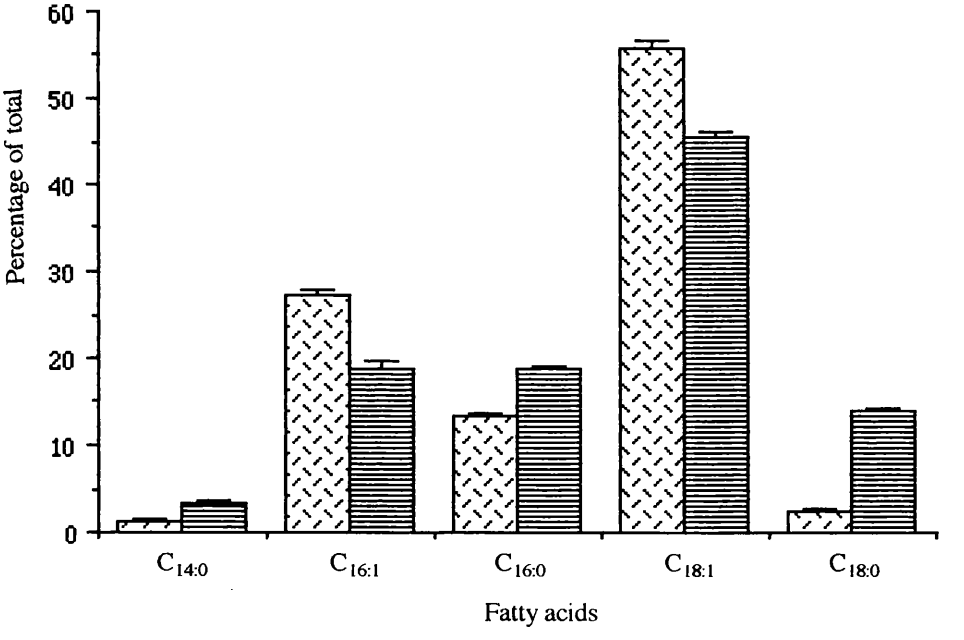
Figure 5.6a compares the fatty acid compositions in the phospholipids and wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown on the medium with a low concentration of succinic acid and Figure 5.6b compares the fatty acid compositions in the phospholipids and wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown on the medium with a low concentration of $(NH_4)_2SO_4$.

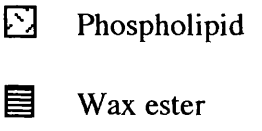
The data used in this Figure are taken from Tables 5.1 & 5.5.

a) growth on the medium with a low concentration of succinic acid



b) growth on the medium with a low concentration of $(NH_4)_2SO_4$





there was a significant difference ($P < 0{\cdot}05$) between the fatty acids of the wax esters and the fatty acids of the phospholipids (Tables 5.1a & 5.5a and Figure 5.6b). A major difference between the fatty acid compositions in the wax esters and phospholipids was in the degree of unsaturation, the fatty acids in the phospholipids being more unsaturated (ui = 0·82 - 0·83) than those in the wax esters (ui = 0·64 - 0·66). This was due largely to a greater proportion of the $C_{16:1}$ fatty acid in the phospholipids than in the wax esters (Tables 5.1a & 5.5a & Figure 5.6). Another difference in the compositions between the fatty acids in the wax esters and phospholipids was that in the wax esters the proportion of the $C_{18:0}$ fatty acid was greatly increased, to approximately 15 % of the total, the same proportion as the $C_{16:1}$ and $C_{16:0}$ fatty acids (both approximately 16 %), whereas in the phospholipids the $C_{18:0}$ fatty acid was a minor component being only 2 - 3 % (Tables 5.1a & 5.5a and Figures 5.5 & 5.6).

5.2.2.2 Fatty acid composition of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 30 °C and harvested during exponential phase

The fatty acid composition of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown on the medium with a low concentration of succinic acid and harvested during the exponential phase was similar to that extracted from *Acinetobacter* sp. NCIB 8250 grown on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase (Table 5.5b). Although the patterns in the fatty acid compositions of these phospholipids were the same [Figure 5.7 (▤, ▨)] there was a significant difference ($P < 0{\cdot}05$) between the values for the $C_{16:1}$, $C_{18:1}$ and $C_{18:0}$ fatty acids.

The predominant fatty acids observed in the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown on either media at 30 °C were the same as those observed at 15 °C (Tables 5.5a & b and Figure 5.7), but, the $C_{18:1}$ fatty acid was present in a smaller proportion at 30 °C (43 - 50 %) than at 15 °C (54 - 56 %). This was also true for the $C_{16:1}$ fatty acid which was 18 - 23 % of the total at 30 °C and 27 - 29 % of the total at 15 °C. This reduction in the proportion of the unsaturated fatty acids was reflected in a decrease in the unsaturation index at 30 °C (ui = 0·66 - 0·68) compared to that at 15 °C (ui = 0·82 - 0·83) (Tables 5.5a & b). Also, there was a significant difference ($P < 0{\cdot}05$) between the values for the fatty acids in the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C and for those extracted from *Acinetobacter* sp. NCIB 8250 grown at 15 °C.

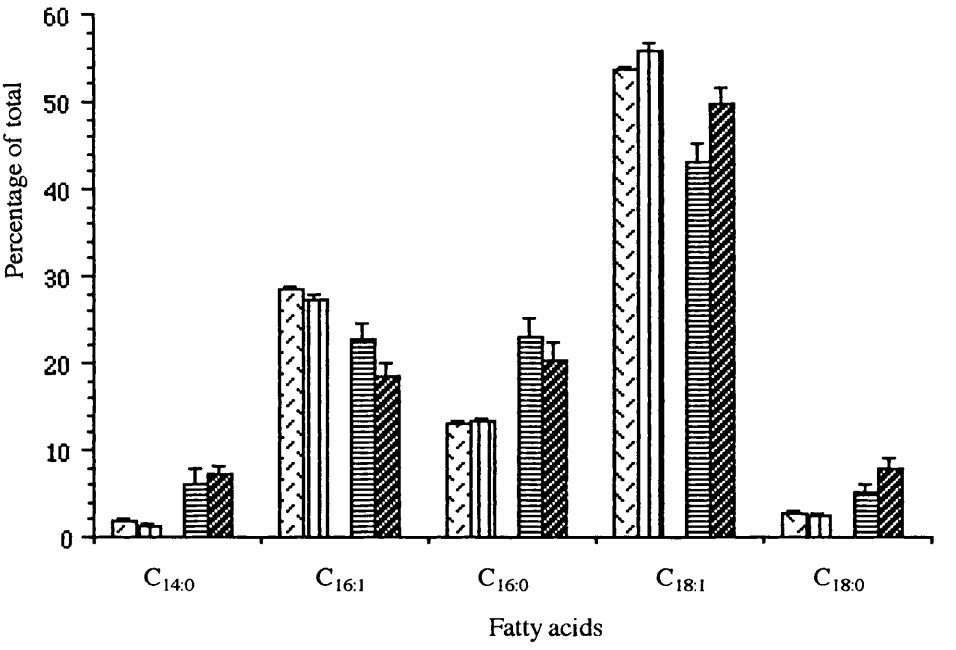
The fatty acid composition of the wax esters extracted from *Acinetobacter* sp. NCIB

Figure 5.7

Comparison of the fatty acid compositions of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 15 °C and 30 °C on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase of growth

Acinetobacter sp. NCIB 8250 was grown at 15 °C and 30 °C in batch culture in 2 l flasks on the medium with a low concentration of succinic acid (Section 2.3.2.2) and on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in exponential phase (Section 2.5.2), the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9) and the fatty acids in the phospholipids converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in that sample was then calculated as a percentage of the total.

The data used in this Figure are taken from Table 5.5.



Succinate-limited - 15 °C

Ammonium-limited - 15 °C

Succinate-limited - 30 °C

Ammonium-limited - 30 °C

8250 grown at 30 °C on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during exponential phase was significantly different ($P < 0{\cdot}05$) to the fatty acid composition of the phospholipids extracted from the same sample (Tables 5.1b & 5.5b and Figure 5.8). The greater proportion of the fatty acids of the wax esters were saturated ($C_{18:0}$, 31 %; $C_{16:0}$, 26 % and $C_{14:0}$, 13 %), whereas in the phospholipids the greater proportion of the fatty acids were unsaturated ($C_{18:1}$, 50 % and $C_{16:1}$, 18 %). Also, at 15 °C the fatty acid compositions of the wax esters were more saturated than the fatty acid compositions observed in the phospholipids from the same sample (Tables 5.1 & 5.5).

5.2.2.3 Fatty acid composition of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 30 °C and harvested during stationary phase

When *Acinetobacter* sp. NCIB 8250 was grown at 30 °C in batch culture and harvested from succinate- or ammonium-limited stationary phase cultures the fatty acid composition of the phospholipids were similar to each other with only the values of the $C_{16:0}$ fatty acid being significantly different ($P < 0{\cdot}05$) between the two limitations (Table 5.5c). The major fatty acid found in these compositions was $C_{18:1}$ which was of a similar proportion to the $C_{18:1}$ fatty acid found in the fatty acid compositions of the phospholipids extracted from *A. calcoaceticus* grown at 30 °C but harvested from exponential phase (Table 5.5b). The only major differences between the fatty acid compositions of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 when grown at 30 °C and harvested from the exponential phase or stationary phase were the $C_{16:1}$ and $C_{16:0}$ fatty acids. With these fatty acids there was an increase in the proportion of the $C_{16:0}$, with a similar decrease in the $C_{16:1}$, as the culture conditions changed from exponential growth to stationary phase (Tables 5.5b & c).

The fatty acid compositions of the wax esters and phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown to stationary phase on the medium with a low concentration of $(NH_4)_2SO_4$ were very different. The fatty acids in the wax esters were of a shorter chain length (cli = 1·26) and very much more saturated (ui = 0·06) than the fatty acids in the phospholipids (cli = 1·45 - 1·48 and ui = 0·60 - 0·65) (Tables 5.1c & 5.5c and Figure 5.9).

5.2.2.4 Fatty acid composition of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under succinate limitation

Acinetobacter sp. NCIB 8250 was grown in continuous culture at 30 °C and steady

Figure 5.8

Comparison of the fatty acid compositions of the phospholipids and wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 30 °C on the medium containing a low concentration of $(NH_4)_2SO_4$ and harvested during the exponential phase of growth

Acinetobacter sp. NCIB 8250 was grown at 30 °C in batch culture in 2 l flasks on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in exponential phase (Section 2.5.2), the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9) and the fatty acids in the phospholipids and wax esters were then converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in that sample was then calculated as a percentage of the total.

The data used in this Figure are taken from Tables 5.1 & 5.5.

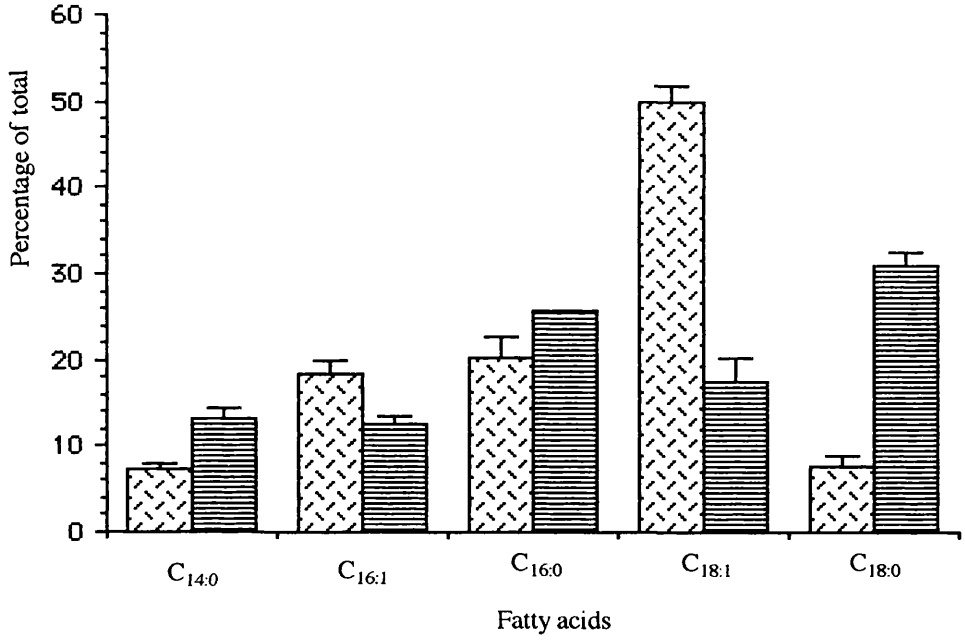
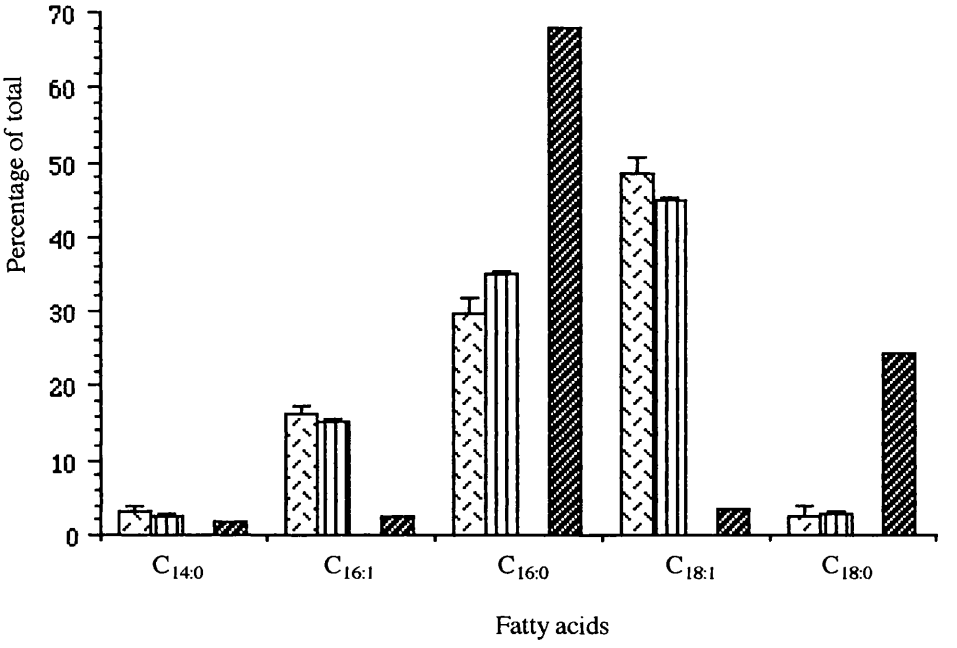

60
50
40
30
20
10
0
Percentage of total
C14:0
C16:1
C16:0
C18:1
C18:0
Fatty acids
Phospholipid
Wax ester

Figure 5.9

Comparison of the fatty acid compositions of the phospholipids and wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 30 °C on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested during the stationary phase

Acinetobacter sp. NCIB 8250 was grown at 30 °C in batch culture in 2 l flasks on the medium with a low concentration of succinic acid (Section 2.3.2.2) and on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in the stationary phase (Section 2.5.2), the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9) and the fatty acids in the phospholipids and wax esters converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in that sample was then calculated as a percentage of the total.

The data used in this Figure are taken from Tables 5.1 & 5.5.



Succinate-limited - phospholipid

Ammonium-limited - phospholipid

Ammonium-limited - wax ester

states were achieved under succinate limitation over the specific growth rate range of 0·14 h^{-1} to 0·80 h^{-1} (Table 5.6). Over this range the pattern of the fatty acid compositions of the phospholipids were the same (Figure 5.10) with only the values for the $C_{14:0}$ and $C_{18:1}$ fatty acids being significantly affected ($P < 0·05$) by the specific growth rate (Table 5.6). The major fatty acid, $C_{18:1}$, accounted for nearly 50 % of the total. The $C_{16:1}$ and $C_{16:0}$ fatty acids together represented 38 - 44 % and the remaining 10 - 12 % of the total were represented by the $C_{14:0}$ and $C_{18:0}$ fatty acids (Table 5.6 and Figure 5.10). The fatty acid compositions of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under succinate limitation (cli = 1·42 - 1·57 and ui = 0·62 - 0·68) were similar to the fatty acid compositions of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 30 °C on either media and harvested during exponential phase (cli = 1·42 - 1·54 and ui = 0·66 - 0·68) or in stationary phase (cli = 1·45 - 1·48 and ui = 0·60 - 0·65) (Tables 5.5 & 5.6).

There were, however, differences in the fatty acid compositions of the phospholipids and the wax esters when *Acinetobacter* sp. NCIB 8250 was grown at 30 °C in continuous culture under succinate limitation. These differences were largely in the degree of unsaturation, the fatty acids in the wax esters being more saturated (ui = 0·28 - 0·30) than those in the phospholipids (ui = 0·62 - 0·68) (Tables 5.2 & 5.6). These differences between the fatty acid compositions of the wax esters and phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown under succinate limitation were in the relative proportions of the $C_{16:1}/C_{16:0}$ and $C_{18:1}/C_{18:0}$ (Figure 5.11). For instance, the $C_{18:1}$ fatty acid in the wax esters was a smaller proportion of the total than in the phospholipids, however, the $C_{18:0}$ fatty acid in the wax esters was a much greater proportion of the total compared with the $C_{18:0}$ fatty acid in the phospholipids (Figure 5.11).

5.2.2.5 Fatty acid composition of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under ammonium limitation

Acinetobacter sp. NCIB 8250 was grown over a range of specific growth rates at 25, 30 and 33 °C in continuous culture under ammonium limitation, the phospholipids extracted and the fatty acid compositions determined. Under these conditions the values for the $C_{16:1}$ and $C_{16:0}$ fatty acids were significantly effected ($P < 0·05$) by both the growth temperature and the specific growth rate (Table 5.7). Throughout the range of growth temperatures and specific growth rates studied the $C_{18:1}$ fatty acid was predominant, comprising 40 - 48 % of the total, with the $C_{18:0}$ and $C_{14:0}$ being minor components, except in the 0·48 h^{-1} steady state sample at 30 °C where the

Table 5.6

Fatty acid compositions of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under succinate limitation

Acinetobacter sp. NCIB 8250 was grown at 30 °C in continuous culture under succinate limitation (Section 2.5.3) and steady states were achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9), the fatty acids in the phospholipids converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in the sample was then calculated as a percentage of the total. The results are expressed as the mean ± standard deviation together with the chain length (cli) and unsaturation indices (ui).

*Denotes that the specific growth rate had a significant effect ($P < 0{\cdot}05$) on the composition of those fatty acids. None of the others were significantly different, i.e. $P > 0{\cdot}05$.

Specific growth rate	0·14 h^{-1}	0·31 h^{-1}	0·49 h^{-1}	0·56 h^{-1}	0·80 h^{-1}
Fatty acid					
*14:0	3·2 ± 0·45	3·1 ± 0·13	3·3 ± 0·28	2·3 ± 0·15	5·3 ± 0·45
16:1	15·6 ± 0·30	16·8 ± 0·26	23·0 ± 0·77	19·6 ± 0·57	24·8 ± 0·31
16:0	25·1 ± 0·65	22·6 ± 0·47	21·3 ± 1·14	19·5 ± 1·01	22·4 ± 0·27
*18:1	46·9 ± 0·25	49·2 ± 0·29	45·4 ± 2·03	48·6 ± 1·70	38·5 ± 0·55
18:0	9·5 ± 0·76	8·3 ± 0·53	7·0 ± 0·18	10·0 ± 1·41	8·8 ± 0·68
cli	1·54	1·54	1·49	1·57	1·42
ui	0·62	0·66	0·68	0·68	0·63

Figure 5.10

Comparison of the fatty acid compositions of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture at 30 °C under succinate limitation

Acinetobacter sp. NCIB 8250 was grown at 30 °C in continuous culture under succinate limitation (Section 2.5.3) and steady states were achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9) and the fatty acids in the phospholipids were then converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in that sample was then calculated as a percentage of the total.

The data used in this Figure are taken from Table 5.6.

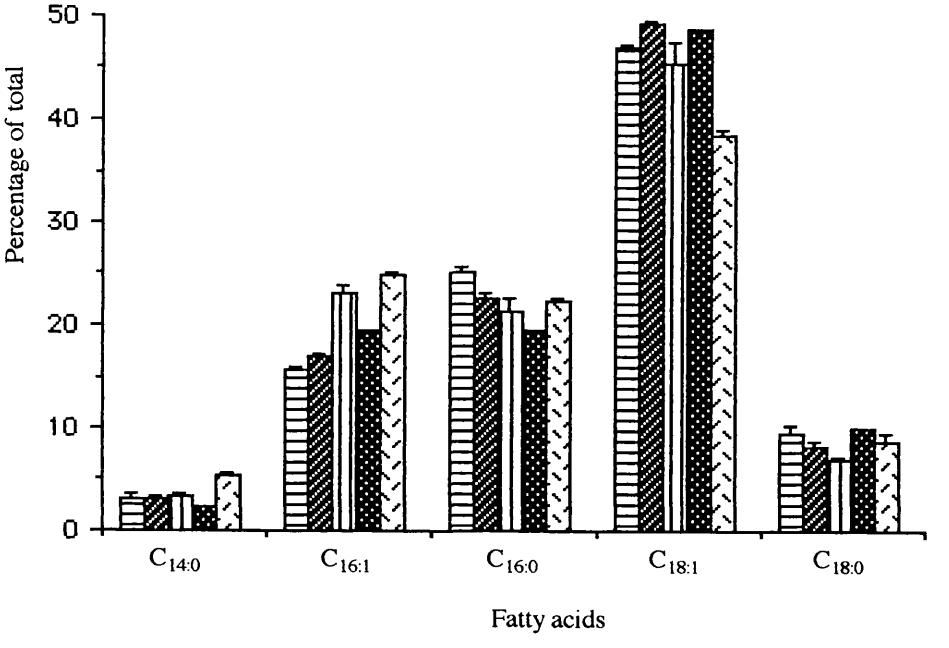
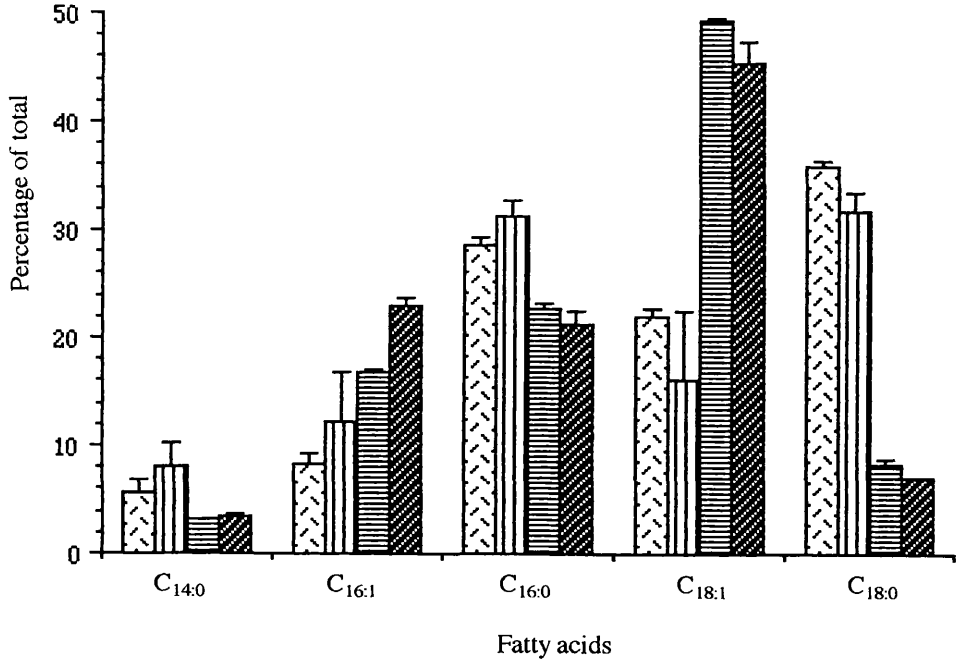

Percentage of total
50
40
30
20
10
0
C14:0
C16:1
C16:0
C18:1
C18:0
Fatty acids
0·14 h-1
0·31 h-1
0·49 h-1
0·56 h-1
0·80 h-1

Figure 5.11

Comparison of the fatty acid compositions of the phospholipids and wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture at 30 °C under succinate limitation

Acinetobacter sp. NCIB 8250 was grown at 30 °C in continuous culture under succinate limitation (Section 2.5.3) and steady states were achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states achieved, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9), the fatty acids in the phospholipids and wax esters converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in that sample was then calculated as a percentage of the total.

The data used in this Figure are taken from Tables 5.2 & 5.6.



Wax ester - 0·26 h^{-1}

Wax ester - 0·46 h^{-1}

Phospholipid - 0·31 h^{-1}

Phospholipid - 0·49 h^{-1}

Table 5.7

Fatty acid compositions of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under ammonium limitation

Acinetobacter sp. NCIB 8250 was grown in continuous culture at 25, 30 and 33 °C under ammonium limitation (Section 5.3) and steady states were achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9), the fatty acids in the phospholipids converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in the sample was then calculated as a percentage of the total. The results are expressed as the mean ± standard deviation together with the chain length (cli) and unsaturation indices (ui).

*Denotes that the growth temperature and the specific growth rate had a significant effect ($P < 0{\cdot}05$) on the composition of those fatty acids. None of the others were significantly different, i.e. $P > 0{\cdot}05$.

Growth temperature	25 °C	30 °C		33 °C	
Specific growth rate	0·18 h^{-1}	0·28 h^{-1}	0·48 h^{-1}	0·44 h^{-1}	0·66 h^{-1}
Fatty acid					
14:0	2·9 ± 0·42	2·7 ± 0·18	3·2 ± 1·18	4·4 ± 1·06	3·7 ± 2·11
*16:1	20·2 ± 0·82	19·8 ± 0·36	19·5 ± 1·24	20·1 ± 1·12	19·1 ± 0·49
*16:0	22·5 ± 0·65	24·5 ± 0·49	17·9 ± 1·03	27·1 ± 0·84	28·6 ± 0·27
18:1	48·0 ± 1·58	47·4 ± 0·81	40·5 ± 1·11	44·2 ± 2·34	44·6 ± 2·29
18:0	6·5 ± 0·22	5·5 ± 0·25	18·9 ± 2·17	4·2 ± 0·39	3·9 ± 0·28
cli	1·52	1·50	1·56	1·44	1·45
ui	0·68	0·67	0·60	0·64	0·64

$C_{18:0}$ fatty acid was an unusually large proportion of the total (Table 5.7 and Figure 5.12). Similar fatty acid compositions of the phospholipids were observed whether *Acinetobacter* sp. NCIB 8250 was grown at 30 °C in continuous culture under succinate limitation (cli = 1·42 - 1·57 and ui = 0·62 - 0·68), ammonium limitation (cli = 1·50 - 1·56 and ui = 0·60 - 0·67) or at 30 °C in batch culture on either media and harvested during exponential phase (cli = 1·42 - 1·54 and ui = 0·66 - 0·68) (Tables 5.5b, 5.6 & 5.7).

Differences were observed in the fatty acid compositions of the wax esters and phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under succinate limitation (Tables 5.2 & 5.6) and there were also differences in these fatty acid compositions when *Acinetobacter* sp. NCIB 8250 was grown in continuous culture under ammonium limitation. These differences were a smaller proportion of the $C_{16:1}$ and $C_{18:1}$ fatty acids in the wax esters compared to the phospholipids and a greater proportion of the $C_{16:0}$ and $C_{18:0}$ fatty acids in the wax esters compared to the phospholipids. The $C_{14:0}$ fatty acid, however, remained approximately the same percentage of the total in all the samples (Tables 5.3 & 5.7 and Figure 5.13).

5.2.2.6 Fatty acid composition of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under oxygen limitation

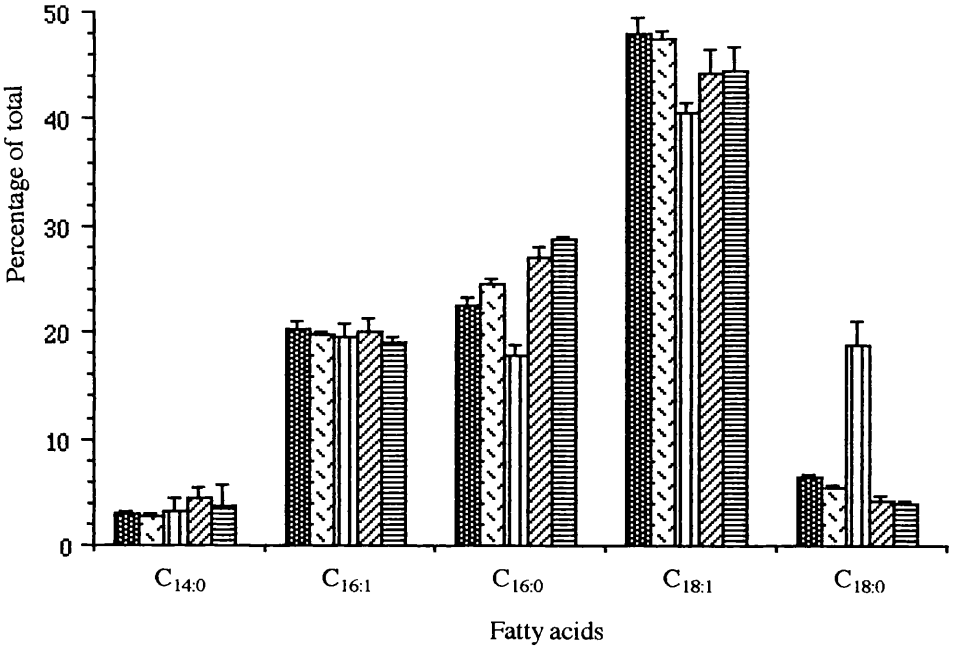
Acinetobacter sp. NCIB 8250 was grown in continuous culture at 15 °C and 30 °C under oxygen limitation using the medium with a low concentration of $(NH_4)_2SO_4$ (Table 5.8). Under these conditions there was a change in the ratios of the $C_{16:1}/C_{16:0}$ fatty acids; at 15 °C the proportion of the $C_{16:1}$ fatty acid was greater than that of the $C_{16:0}$, whereas at 30 °C the proportions of these fatty acids were nearly equal. The other fatty acids remained at approximately the same percentage at both 15 °C and 30 °C (Table 5.8 and Figure 5.14). The fatty acid compositions of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C in continuous culture under oxygen limitation were different to those extracted from *Acinetobacter* sp. NCIB 8250 grown under succinate or ammonium limitation. Under oxygen limitation the $C_{18:1}$ fatty acid was a higher proportion of the total (54·8 - 57·6 %) than under succinate limitation (38·5 - 49·2 %) or ammonium limitation (40·5 - 47·4 %) (Tables 5.6 to 5.8 and Figure 5.15). Thus under oxygen limitation the fatty acid composition of the phospholipids was more unsaturated (ui = 0·75) than under succinate (ui = 0·62 - 0·68) or under ammonium limitation (ui = 0·60 - 0·67). Under oxygen limitation the fatty acid composition of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown at 15 °C were

Figure 5.12

Comparison of the fatty acid compositions of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture at 25, 30 and 33 °C under ammonium limitation

Acinetobacter sp. NCIB 8250 was grown at 25, 30 and 33 °C in continuous culture under ammonium limitation (Section 2.5.3) and steady states were achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states achieved, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9) and the fatty acids in the phospholipids converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in that sample was then calculated as a percentage of the total.

The data used in this Figure are taken from Table 5.7.



25 °C - 0·18 h^{-1}

30 °C - 0·28 h^{-1}

30 °C - 0·48 h^{-1}

33 °C - 0·44 h^{-1}

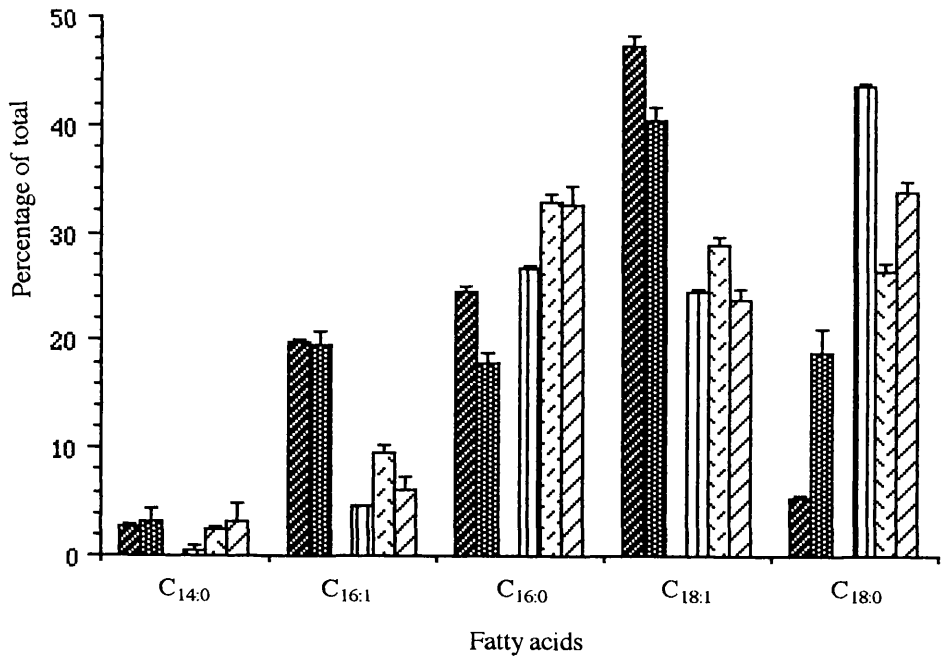
33 °C - 0·66 h^{-1}

Figure 5.13

Comparison of the fatty acid compositions of the phospholipids and wax esters extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture at 30 °C under ammonium limitation

Acinetobacter sp. NCIB 8250 was grown at 30 °C in continuous culture under ammonium limitation (Section 2.5.3) and steady states were achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states achieved, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9), the fatty acids in the phospholipids and wax esters converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in that sample was then calculated as a percentage of the total.

The data used in this Figure are taken from Tables 5.3 & 5.7.



Phospholipid - 0·28 h^{-1}

Phospholipid - 0·48 h^{-1}

Wax ester - 0·14 h^{-1}

Wax ester - 0·62 h^{-1}

Wax ester - 0·77 h^{-1}

Table 5.8

Fatty acid compositions of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$

Acinetobacter sp. NCIB 8250 was grown at 15 °C and 30 °C in continuous culture under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.5.3). A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states achieved, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9), the fatty acids in the phospholipids converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in the sample was then calculated as a percentage of the total. The results are expressed as the mean ± standard deviation together with the chain length (cli) and unsaturation indices (ui).

*Denotes that the growth temperature had a significant effect ($P < 0{\cdot}05$) on the composition of those fatty acids. None of the others were significantly different, i.e. $P > 0{\cdot}05$.

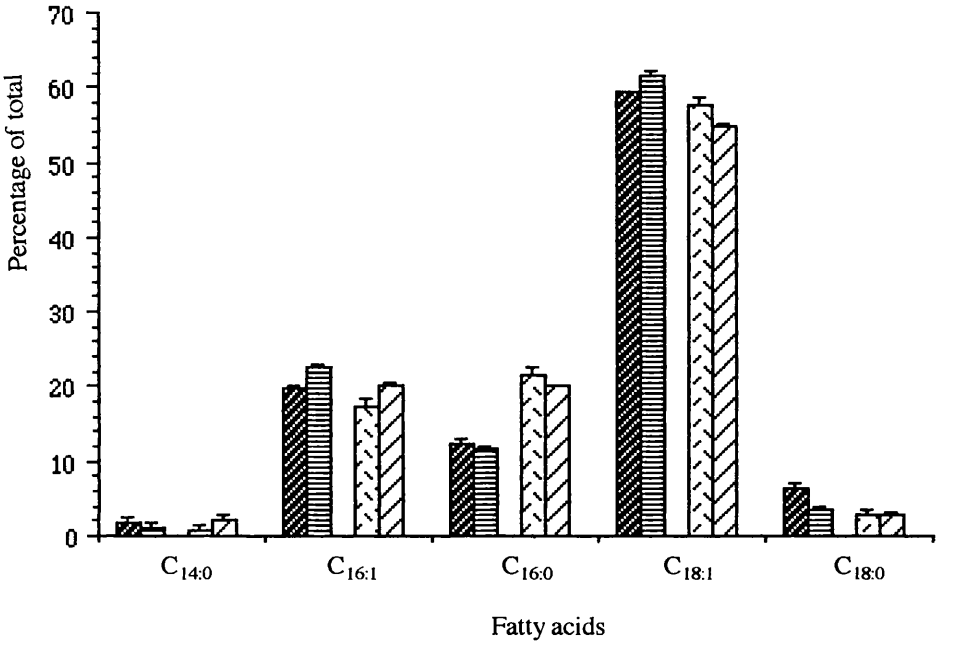
Growth temperature	15 °C		30 °C	
Specific growth rate	0·22 h^{-1}	0·28 h^{-1}	0·58 h^{-1}	0·61 h^{-1}
Fatty acid				
14:0	1·8 ± 0·69	0·9 ± 0·83	0·8 ± 0·44	2·2 ± 0·51
16:1	19·7 ± 0·43	22·6 ± 0·26	17·4 ± 1·07	20·3 ± 0·19
16:0	12·4 ± 0·58	11·5 ± 0·46	21·4 ± 1·15	20·0 ± 0·12
18:1	59·5 ± 0·06	61·4 ± 0·82	57·6 ± 1·08	54·8 ± 0·27
18:0	6·2 ± 0·82	3·6 ± 0·29	2·7 ± 0·81	2·7 ± 0·61
cli	1·63	1·64	1·59	1·55
ui	0·79	0·84	0·75	0·75

Figure 5.14

Effect of growth temperature and specific growth rate on the fatty acid composition of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in continuous culture under oxygen limitation

Acinetobacter sp. NCIB 8250 was grown at 15 °C and 30 °C in continuous culture under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.5.3) and steady states were achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states achieved, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9) and the fatty acids in the phospholipids converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each sample was analysed in triplicate and each fatty acid component in that sample was then calculated as a percentage of the total.

The data used in this Figure are taken from Table 5.8.



15 °C - 0·22 h^{-1}

15 °C - 0·28 h^{-1}

30 °C - 0·58 h^{-1}

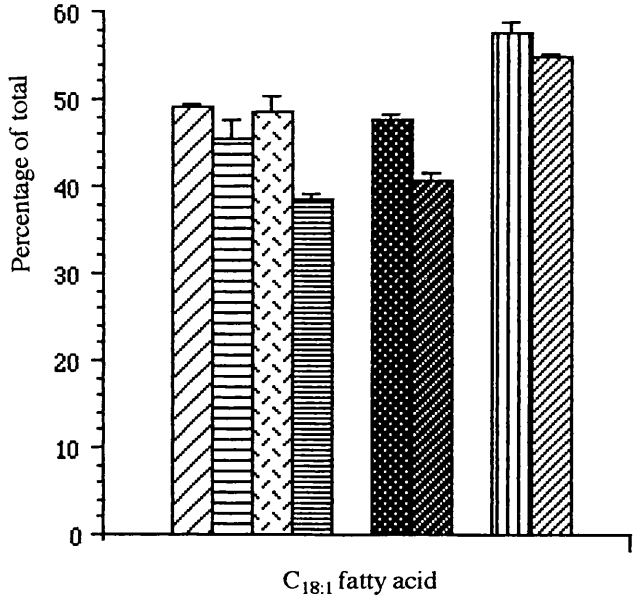
30 °C - 0·61 h^{-1}

Figure 5.15

Comparison of the values for the $C_{18:1}$ fatty acids of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown at 30 °C in continuous culture under succinate, ammonium and oxygen limitation

Acinetobacter sp. NCIB 8250 was grown at 30 °C in continuous culture under succinate, ammonium and oxygen limitation (Section 2.5.3) and steady states were achieved over a wide range of specific growth rates. A 30 ml sample of culture was harvested (Section 2.7) at each of the steady states achieved, the pellets washed with chilled 10 mM KH_2PO_4 buffer, pH 7·0 and the lipids extracted (Section 2.8). The phospholipids and wax esters were separated by tlc (Section 2.9) and the fatty acids in the phospholipids converted to their methyl ester derivatives (Section 2.10.3.1) and analysed by glc (Section 2.10.3.2). Each fatty acid component was then calculated as a percentage of the total for that sample but only the values for the $C_{18:1}$ fatty acids are used in the Figure.

The data used in this Figure are taken from Tables 5.6, 5.7 & 5.8.



Succinate-limited - 0·31 h^{-1}

Succinate-limited - 0·49 h^{-1}

Succinate-limited - 0·56 h^{-1}

Succinate-limited - 0·80 h^{-1}

Ammonium-limited - 0·28 h^{-1}

Ammonium-limited - 0·48 h^{-1}

Oxygen-limited - 0·58 h^{-1}

Oxygen-limited - 0·61 h^{-1}

similar to the fatty acid composition of the phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown in batch culture at 15 °C, although the $C_{18:1}$ fatty acid was a slightly greater proportion and the $C_{16:1}$ fatty acid a slightly smaller proportion of the total under oxygen limitation than in batch culture (Tables 5.5a & 5.8).

The differences in the fatty acid compositions of the wax esters and phospholipids extracted from *Acinetobacter* sp. NCIB 8250 grown under oxygen-limitation were that the fatty acids in the wax esters had a smaller proportion of $C_{18:1}$ and a greater proportion of $C_{18:0}$ when compared with the fatty acid compositions in the phospholipids (Tables 5.4 & 5.8). These were the same differences as those found in the fatty acid compositions of the wax esters and phospholipids when *Acinetobacter* sp. NCIB 8250 was grown under succinate or ammonium limitation (Tables 5.2, 5.3, 5.6 & 5.7).

5.3 Discussion

5.3.1 Effect of the growth medium on the fatty acid and fatty alcohol composition of the wax esters

There have been several reports on the fatty acid and/or fatty alcohol compositions of the wax esters extracted from *Acinetobacter* strains grown in batch culture on complex media (Gallagher, 1971; Makula *et al.*, 1975; Bryn *et al.*, 1977) and on defined media with hydrocarbons (Makula *et al.*, 1975) or on non-hydrocarbons (Fixter *et al.*, 1986) as the sole source of carbon and energy. However, the results reported in this present work are the first time that the fatty acid composition of the wax esters have been determined in continuous culture grown cells.

Gallagher (1971) grew three strains of *Acinetobacter* on nutrient broth which contained 0·5 % ($^{v}/_{v}$) Tween 80, presumably as an antifoam agent. The fatty alcohol composition of the wax esters in these strains contained a substantial proportion of short chain alcohols, i.e. C_3 to C_5, although longer chain length alcohols, C_8 to C_{20}, were also observed. Gallagher (1971) unfortunately made no comment on the chain length or degree of unsaturation of the fatty acids or of the wax esters, thus this work is of limited use for comparing these compositions with those obtained by other investigators. Also, there is some doubt about the work by Gallagher (1971) as no other investigators, even those using a similar complex medium, have reported the occurrence of such short chain alcohols in the wax esters. Of course, these other investigators may have missed the short chain alcohols as they would have eluted

with or near to the solvent peak. Alternatively, the short chain alcohols observed by Gallagher (1971) may be an artifact due to the inclusion of Tween 80 in the growth medium, or the strains may not have been acinetobacters but members of another closely related genus, one which does produce short chain alcohols.

Bryn *et al.* (1977) also grew a number of acinetobacters on a complex medium (Muller-Hinton broth/yeast extract) and report the occurrence of only C_{13} to C_{24} fatty alcohols with the C_{16} to C_{18} chain lengths accounting for nearly 90 % of the total (cli = 1·35 - 1·59) in most of the strains examined. In the strains used there were differences in the fatty alcohol compositions, possibly a reflection that different species within the *Acinetobacter* genus had been used although at the time of the work only one species, *calcoaceticus*, was recognized. Of the five *Acinetobacter* strains that produced wax esters, four were grown at 33 °C and one at 22 °C. Three of the strains, 8, BD4 and MJT/F4/5,7, had similar fatty alcohol compositions characterised by a large proportion of mono-unsaturates (ui = 0·79 - 0·90) whereas the other two strains had a much lower degree of unsaturation in the fatty alcohol composition (ui = 0·47 for strain ATCC 15149 and ui = 0·37 for strain ATCC 17959). Although no comment was made on the fatty acid composition of the wax esters extracted, other than "no di-unsaturated fatty acids were observed", the wax ester composition was reported (cli = 0·87 - 1·36 and ui = 1·32 - 1·87 for strains 8, BD4 and MJT/F4/5,7) and therefore it is possible to make some comment on the fatty acid composition in these strains. Thus, using the data from Bryn *et al.* (1977) the fatty acids in the wax esters are as unsaturated as the corresponding fatty alcohol composition but they are probably of a much shorter overall chain length, i.e the fatty acid composition would be predicted as being predominantly $C_{16:1}$ followed by $C_{16:0}$ with a smaller proportion of $C_{18:1}$ and trace amounts of $C_{18:0}$ and $C_{14:0}$.

Makula *et al.* (1975) grew *Acinetobacter* sp. HO1-N on both a nutrient broth/yeast extract (NBYE) medium and on a defined medium with hexadecane as the sole source of carbon and energy. When this strain was grown on the NBYE medium the fatty acid composition of the wax esters present in the cells, as a percentage of the total, were determined as being 23 % $C_{16:0}$, 30 % $C_{16:1}$ and 47 % $C_{18:1}$ with trace amounts of $C_{14:0}$ and $C_{18:0}$ (cli = 1·47 and ui = 0·77). The corresponding fatty alcohol composition was somewhat different; 5 % $C_{16:0}$, 45 % $C_{16:1}$ and 50 % $C_{18:1}$ with trace amounts of $C_{14:0}$ and $C_{18:0}$ (cli = 1·50 and ui = 0·95). This fatty alcohol composition was very similar to that obtained by Bryn *et al.* (1977) for strains 8, BD4 and MJT/F4/5,7 which were also grown on a NBYE based medium. When the defined medium with hexadecane as the carbon source was used, the fatty acid and fatty alcohol compositions were much simpler, being 100 % $C_{16:0}$. Thus, the media in which this strain of *Acinetobacter* was grown had a definite affect on the fatty acid

and fatty alcohol compositions and therefore an affect on the wax ester composition.

Fixter *et al.* (1986) grew *Acinetobacter* sp. NCIB 8250 on a defined medium and harvested cultures during stationary phase. Both the fatty acid and fatty alcohol compositions of the wax esters were determined and although the same chain lengths were present as observed by Makula *et al.* (1975) and Bryn *et al.* (1977), i.e. C_{14} to C_{18}, the degree of unsaturation was very different. The major species in both the fatty acid and fatty alcohol compositions reported by Fixter *et al.* (1986) was the $C_{16:0}$, being 55·7 % and 58·2 % of the totals respectively. The $C_{18:0}$ was the next most abundant species, which differs with both Makula *et al.* (1975) and Bryn *et al.* (1977) as in the strains used in these investigations the $C_{18:0}$ was a trace component. Thus, with the $C_{16:0}$ and $C_{18:0}$ representing over 70 % of the total in both the fatty acid and fatty alcohol compositions reported by Fixter *et al.* (1986) these compositions, and therefore the wax ester composition as well, were more saturated (ui = 0·26 for fatty acid and 0·16 for fatty alcohol) than the compositions reported by Makula *et al.* (1975) and Bryn *et al.* (1977).

This pattern of a greater abundance of $C_{16:0}$ and $C_{18:0}$ fatty acids in the wax esters was also observed in this present work in cultures harvested from exponential phase and in ammonium-limited stationary phase. However, differences were observed. The fatty acid composition of the wax esters in the ammonium-limited stationary phase cultures in this present work were almost exclusively $C_{16:0}$ and $C_{18:0}$ (92·3 % of the total) whereas in the compositions reported by Fixter *et al.* (1986) the $C_{16:1}$ and $C_{18:1}$ fatty acids account for approximately 25 % of the total. Indeed the fatty acid composition obtained from mid-exponential phase cultures in this present work resembled more closely that of the fatty acid composition of Fixter *et al.* (1986) than did the ammonium-limited stationary phase culture.

Perhaps this was a reflection of the fact that the ammonium-limited stationary phase cultures of Fixter *et al.* (1986) were in stationary phase for longer than those analysed in this present work. If at the end of exponential growth, in nitrogen-limited/carbon excess cultures, the fatty acid synthase continues to function as Fixter *et al.* (1986) have shown by the continued accumulation of wax esters, then the fatty acid synthase would continue to produce $C_{16:0}$ fatty acids. Also, if the preferred substrate of the acyl desaturase in *Acinetobacter* species were acyl chains incorporated into phospholipid molecules (Foot *et al.*, 1983b) and the acyl chains of the wax esters were less preferred then it could be envisaged that harvesting the culture shortly after stationary phase had been entered would create a predominance of saturated fatty acids and hence saturated wax esters. Only if the culture had been left in stationary phase for a longer period of time would the acyl chains in the wax

esters become desaturated.

Several reasons could account for the differences observed between the fatty acid and fatty alcohol compositions obtained by Fixter *et al.* (1986) and those workers who used NBYE based media. The most dominant factor is probably the media itself although the growth phase from which the cultures were harvested could also have had an affect. Complex media, such as those used by Makula *et al.* (1975) and Bryn *et al.* (1977) may contain significant amounts of preformed lipids, such as fatty acids and as has been demonstrated with *Acinetobacter* sp. HO1-N, a growing cell gives preference to ready made fatty acids rather than relying on *de novo* synthesis (Sampson & Finnerty, 1974; Makula *et al.*, 1975). Therefore, it is possible that the fatty acid and fatty alcohol compositions of the wax esters found in the strains used by Makula *et al.* (1975) and Bryn *et al.* (1977) may be more a reflection of the fatty acid content of the media than the fatty acids composition obtained from *de novo* synthesis. Fixter *et al.* (1986) grew *Acinetobacter* sp. NCIB 8250 in a defined medium and therefore this strain would have synthesised fatty acids *de novo.* Therefore, the fatty acid and fatty alcohol compositions in this strain would be a reflection of those fatty acids that *Acinetobacter* sp. NCIB 8250 had synthesised.

It is difficult to comment on exactly how much influence the phase of growth from which the cultures were harvested had on the fatty acid and fatty alcohol compositions because only Fixter *et al.* (1986) state clearly how the cultures were grown and when they were harvested. However, the fatty acid and fatty alcohol compositions obtained by Fixter *et al.* (1986) are definitely a feature of both the growth phase from which the cultures were harvested and of the medium composition. It has been shown in this present work, using the same organism and media, that the fatty acid composition of the wax esters in cultures harvested during stationary phase, which were very similar to the fatty acid composition obtained by Fixter *et al.* (1986), were different from the fatty acid composition obtained when the cultures were harvested from exponential phase. The cultures grown by Makula *et al.* (1975) were probably harvested in late exponential phase (Makula & Finnerty, 1968; 1970) and Bryn *et al.* (1977) state that their cultures were grown for 20 h, therefore they were probably harvested from stationary phase. The similarities in the compositions obtained by Makula *et al.* (1975) and Bryn *et al.* (1977) imply that the media composition, unlike the defined medium used by Fixter *et al.* (1986) and in this present work, had such a dominant effect on the fatty acid and fatty alcohol compositions that the growth phase from which the cultures were harvested probably had little effect.

Finally, each of the studies used a different growth temperature and it is known,

indeed it has been shown in this present work, that the growth temperature can have an effect on the fatty acid composition of the lipids. Gallagher (1971) used three growth temperatures; 27 °C, 13 °C and 1 °C. Makula *et al.* (1975) grew *Acinetobacter* sp. HO1-N at 25 °C, Bryn *et al.* (1977) grew all but one strain at 33 °C (MJT/F4/5,7 was grown at 22 °C) and Fixter *et al.* (1986) grew *Acinetobacter* sp. NCIB 8250 at 30 °C. The effect of growth temperature on the fatty acid and fatty alcohol compositions are discussed below.

5.3.2 Effect of the growth temperature on the fatty acid and fatty alcohol composition of the wax esters

In the work reported by Gallagher (1971) there was a clear increase in the proportion of the unsaturated fatty alcohols in the strain G37 as the growth temperature was decreased from 27 °C to 13 °C. The other strains examined by Gallagher (1971) did not increase the degree of unsaturation in the fatty alcohols as the growth temperature was lowered, but, in all three strains examined the proportion of the shorter chain alcohols (C_3 - C_5) increased. Both these responses, an increase in the degree of unsaturation and a decrease in the chain length, would help to maintain the wax esters in a fluid state as the growth temperature was decreased. However, as was stated earlier (Section 5.3.1), Gallagher (1971) made no comment on the fatty acid composition of the wax esters so it is not possible to say what affect, if any, the growth temperature had on the degree of unsaturation in these fatty acids.

Bryn *et al.* (1977) studied five wax ester producing strains of *Acinetobacter* and reported both the wax ester and fatty alcohol compositions. Unfortunately, each of the strains were grown at only one temperature; four strains were grown at 33 °C and one at 22 °C. It is evident from comparisons of the fatty alcohol compositions from the four strains grown at 33 °C that the differences observed must be as a result of strain specific variations, as the growth temperature and medium were the same in all four cases. Two strains, 8 and BD4, had a similar composition to each other but had a much higher degree of unsaturation than the strains ATCC 15149 and ATCC 17959. These differences in the fatty alcohol compositions indicate that Bryn *et al.* (1977) were probably dealing with at least two species within the *Acinetobacter* genus (Hansen & Yourassowski, 1991). However, the degree of unsaturation in the strain MJT/F4/5,7 (approximately 95 % unsaturated when grown at 22 °C) was even greater that that observed in strains 8 and BD4 (approximately 81 - 85 % unsaturated). This difference may still only be strain related but it is probably due in part at least to the lower growth temperature.

Russell & Volkman (Russell, 1978b; Russell & Volkman, 1980) have also reported

on the fatty acid and fatty alcohol compositions of the wax esters from a related bacterium, *Micrococcus cryophilus* ATCC 15174. In their work it was shown that decreasing the growth temperature from 20 °C to 1 °C had very little affect on the fatty acid and fatty alcohol chain length indices (1·75 - 1·77 for the fatty acids and 1·70 - 1·74 for the fatty alcohols) whereas there is a significant affect on the degree of unsaturation in both the fatty acid and fatty alcohol moities. At 20 °C the unsaturation indices were 0·67 and 0·50 for the fatty acid and fatty alcohol respectively and at 1 °C these indices were 0·92 and 0·89 (Russell & Volkman, 1980). These changes in the fatty acid and fatty alcohol moities in the wax esters were in stark contrast to the affect of temperature on the fatty acid moities in the phospholipids in this organism where a decrease in the growth temperature only had the affect of decreasing the chain length of the fatty acids in the phospholipids (see Section 5.3.4).

In this present work, using *Acinetobacter* sp. NCIB 8250, there was also an increase in the unsaturation index as the growth temperature was decreased. There was also a slight but noticeable increase in the overall chain length of the fatty acids of the wax esters. The amount by which the unsaturation index was altered was less in *Acinetobacter* sp. NCIB 8250 than in the bacterium used by Russell and Volkman (Russell, 1978b; Russell & Volkman, 1980) and the increase in the chain length index probably reflect that fact that the two organisms have different growth temperature optima; *Micrococcus cryophilus* ATCC 15174 being a psychrophile and *Acinetobacter* sp. NCIB 8250 being a mesophile.

5.3.3 Effect of the growth medium on the fatty acid composition of the phospholipids

Makula & Finnerty (1968), Nishimura *et al.* (1979) and Hansen & Yourassowsky (1991) have examined the cellular fatty acid composition of several strains of *Acinetobacter*. In all three studies complex media were used, although Makula & Finnerty (1968) also used defined media. It is unlikely that when grown on the complex media, any of the strains would have accumulated significant amounts of wax esters as complex media are more likely to result in carbon-limited cultures than in nitrogen-limited cultures. Therefore, in these studies the cellular fatty acid compositions would approximate to that of the fatty acid composition of the phospholipids had the phospholipids first been isolated from any other lipids present. In this present work the phospholipids were separated from the other lipid components and then the fatty acids analysed.

In the study by Makula & Finnerty (1968), who were interested in the effect of

hydrocarbons on the growth of *Acinetobacter* sp. HO1-N, the cellular fatty acid compositions were analysed after growth of this strain at 25 °C on nutrient broth and on a defined medium with either acetate or a hydrocarbon (ranging from C_{10} - C_{18}) as the carbon source. With nutrient broth as the growth medium the fatty acid composition was very similar to that obtained in this present work (Table 5.5c), although Makula & Finnerty (1968) also found a $C_{7:0}$ fatty acid, which was present as an appreciable proportion of the total. When the defined medium was used with acetate (or succinate) as the carbon source the $C_{7:0}$ fatty acid was not observed, although the proportion of the $C_{18:0}$ fatty acid increased (from 3 % obtained in growth on nutrient broth) to nearly 20 % of the total. The proportion of the $C_{16:0}$ fatty acid also increased and the proportion of the $C_{18:1}$ fatty acid decreased by 10 %. Although the overall pattern in the fatty acid composition of *Acinetobacter* sp. HO1-N grown on acetate (or succinate) was similar to that obtained in this present work, there is a marked discrepancy in the proportion of the $C_{18:0}$ fatty acid, which was only a minor component in this present work (Table 5.5).

It is not clear why there should be such a large discrepancy. One explanation might have been that the cultures grown by Makula & Finnerty (1968) were not carbon-limited but nitrogen- or oxygen-limited as this present work has shown, that under these limitations wax esters accumulate and that the proportion of the $C_{18:0}$ fatty acid of the wax esters is much higher than in the phospholipids. Therefore, in a total cellular fatty acid analysis inclusion of the fatty acids in the wax esters would mean that the results would not be a reflection solely of the fatty acids in the phospholipids. However, Hardy (1982) grew *Acinetobacter* sp. NCIB 8250 in continuous culture under carbon limitation using a similar defined medium that had the same $(NH_4)_2SO_4$ concentration as that used by Makula & Finnerty (1968) but had an even higher succinate concentration. Therefore, it seems unlikely that the cultures grown by Makula & Finnerty (1968) would have been nitrogen-limited. However, these authors do not state when the cultures of *Acinetobacter* sp. HO1-N were harvested and if it was during stationary phase then the cultures could have been oxygen-limited and this would probably account for the observed differences.

When these workers used a hydrocarbon as the carbon and energy source the fatty acid composition was influenced quite strongly by the length of the hydrocarbon used (Makula & Finnerty, 1968). With hydrocarbons of $C_{14:0}$ and above the major fatty acid observed was the same length as the hydrocarbon substrate but was never more than 50 % of the total. When the $C_{10:0}$ hydrocarbon was used the major fatty acid was $C_{16:0}$ and with the $C_{12:0}$ hydrocarbon it was the $C_{18:1}$ fatty acid. Indeed, the fatty acid composition obtained when dodecane was used as the carbon source was almost identical to that obtained with the nutrient broth medium (Makula &

Finnerty, 1968).

Nishimura *et al.* (1979) investigated eight *Acinetobacter* strains grown at 30 °C and all had the same fatty acid compositional patterns. The $C_{18:1}$ was the predominant fatty acid and was at least 30 % of the total, with an average of over 40 %. The C_{16} fatty acids were the next most abundant with $C_{16:0}$ being approximately 23 % - 31 % and $C_{16:1}$ being approximately 16 % - 22 %, with one strain containing only 8·6 % $C_{16:1}$. The $C_{18:0}$ and $C_{14:0}$ fatty acids were present in trace quantities. Overall the cellular fatty acid compositions obtained by Nishimura *et al.* (1979) and the fatty acid compositions of the phospholipids in *Acinetobacter* sp. NCIB 8250 grown in batch culture and harvested during stationary phase in this work are in very good agreement. Nishimura *et al.* (1979), however, also report the presence of significant amounts of $C_{17:0}$, $C_{17:1}$ and $C_{18:2}$ fatty acids as well as 1 - 5 % unidentified fatty acids. These fatty acids are probably an artifact of the medium used which was a heart infusion liquid containing 10 % calf serum.

In the study by Hansen & Yourassowsky (1991) an older nomenclature system for the genus *Acinetobacter* was used. In this nomenclature there was one species, *calcoaceticus*, which was divided into four biotypes; namely *anitratus*, *lwoffi*, *haemolyticus* and *alcaligenes*. These workers examined several strains from each of the four biotypes. The strains were grown at 36 °C on a complex medium of heart infusion agar supplemented with 5 % ($^{v}/_{v}$) defibrinated horse blood and the total fatty acid compositions determined. The results show that strains within the same biotype had a similar fatty acid composition and that differences in the fatty acid composition between the four biotypes could be discerned. *Acinetobacter* sp. NCIB 8250, under the classification system used by Hansen & Yourassowsky (1991) falls into the biotype *anitratus*. With the exception of the presence of odd chain length and hydroxylated fatty acids, the fatty acid compositions of the phospholipids obtained by Hansen & Yourassowsky (1991) for biotype *anitratus* and in this present work are very similar.

Therefore, from the four studies it would appear that there was very little effect of the medium on the fatty acid composition of the phospholipids from strains of *Acinetobacter*. It is, however, possible to alter the fatty acid composition by suppling primary hydrocarbons, alcohols and fatty acids exogenously. It is also possible to alter the fatty acid composition of the phospholipids by altering the growth temperature. The affect of growth temperature on the fatty acid composition of the phospholipids is discussed below.

5.3.4 Effect of growth temperature on the fatty acid composition of the phospholipids

The effect of temperature on the fatty acid composition in the phospholipids has not been studied in *Acinetobacter* species before. However, it has been studied in considerable detail in both Gram-negative and Gram-positive micro-organisms, especially *Escherichia coli* (e.g. de Mendoza & Cronan, 1983) and in Bacilli (e.g. Fulco, 1984). Russell and co-workers (e.g. Sandercock & Russell, 1980) have studied in some detail the effect of temperature on the fatty acid composition in the phospholipids of a bacterium classified as *Micrococcus cryophilus* but which is more like the acinetobacters than the micrococci., (Russell, 1974)

The classical response of a Gram-negative bacterium to a decrease in the growth temperature is to increase the degree of unsaturation of the fatty acids in the membrane phospholipids (Russell, 1984; Neidleman, 1987). This is achieved by *E. coli* through the temperature sensitive β-ketoacyl ACP synthase II which has a faster rate of catalysis at lower temperatures, i.e. 17 °C, than at higher temperatures, e.g. 37 °C. This enzyme produces relatively more *cis* -Δ^{11} $C_{18:1}$ fatty acids at lower temperatures than at higher temperatures (de Mendoza & Cronan, 1983). In *E. coli* the *sn*-2 position of the phospholipids is occupied almost exclusively by the *cis* -Δ^{9} $C_{16:1}$ fatty acid. The $C_{16:0}$ and *cis* -Δ^{11} $C_{18:1}$ fatty acids compete for the *sn*-1 position. Thus, at the lower temperatures because of the increased activity of the β-ketoacyl ACP synthase II enzyme there is more $C_{18:1}$ *cis* -Δ^{11} available and therefore more phospholipids are synthesised with two unsaturated fatty acids. The result of this mechanism, which relies on the *de novo* biosynthesis of phospholipids and not the modification of the acyl chains in existing phospholipid molecules, is to lower the phase transition temperature of the membrane of the cell, the whole culture becoming adapted to the new growth temperature after several cell divisions.

In the bacilli, where the predominant fatty acids are iso and anteiso C_{15} and C_{17}, the response to a decrease in the growth temperature is a general decrease in the overall chain length, an increase in the anteiso:iso ratio and a large increase in the degree of unsaturation in the *n*-C_{16} and branched C_{17} fatty acids. This increase in the degree of unsaturation in the fatty acids is achieved in *B. megaterium* ATCC 14581 by the *de novo* synthesis of a Δ^5 desaturase at temperatures below approximately 28 °C (Fulco, 1984). This oxygen dependent desaturase acts on the acyl chains already incorporated into the phospholipids and acts predominantly on the cytosolic mono-layer of the membrane phospholipids. Thus, the mechanism of lowering the phase transition temperature of the membrane is very different to that observed in *E. coli*

and other Gram negative bacteria.

In *M. cryophilus* ATCC 15174 the mechanism of altering the phase transition of the membrane is solely by decreasing the chain length of the fatty acids in the phospholipids (Russell, 1971; Sandercock & Russell, 1980) and is effected through a temperature sensitive elongase. This enzyme, *in vivo*, elongates $C_{16:0}$ to $C_{18:0}$ but the activity of this enzyme is greatly decreased by a decrease in the growth temperature and therefore at lower temperatures the proportion of the C_{18} fatty acids is decreased. The degree of unsaturation in the fatty acids, however, is unaffected by the growth temperature and remains relatively constant at greater than 95 % of the total at all growth temperatures (Russell, 1971). The desaturase probably acts directly on the acyl chains of the phospholipids and not on acyl-CoA or ACP derivatives (Foot *et al.*, 1983b) and at least in this respect it is similar to the desaturase found in Bacilli species. Also, *M. cryophilus* ATCC 15174 has a preferred fatty acid configuration in the phospholipids of *sn*-1-long and *sn*-2-short (McGibbon & Russell, 1983). This configuration is beneficial to an organism that grows at lower temperatures as it disrupts the packing of the acyl chains in the membranes more than the *sn*-1-short and *sn*-2-long configuration.

Bacteria, therefore, have developed numerous ways of adapting to a change in their environmental temperature, maintaining membrane fluidity so that physiological functions can continue. The affect of decreasing the growth temperature on the fatty acid composition in the phospholipids of *Acinetobacter* sp. NCIB 8250 was to increase the degree of unsaturation (Table 5.5), the overall chain length index remained constant. In *E. coli* a decrease in growth temperature increased both the overall chain length and degree of unsaturation of those fatty acids. This change in the phospholipid fatty acid composition was sufficient to allow the physiological functions of *E. coli* to continue. The pertinent questions with respect to *Acinetobacter* sp. NCIB 8250 are: is the change in the fatty acid composition of the phospholipids sufficient to maintain the membrane fluidity? Or are the wax esters, whose degree of unsaturation increases much more than the fatty acids of the phospholipids, also required? And, if so, do they perform an analogous role to cholesterol in the membranes of eukaryotes?

The answers are most probably, yes, the changes in the fatty acid composition of the phospholipids, observed in *Acinetobacter* sp. NCIB 8250, are sufficient to maintain membrane fluidity. It has been shown, in *M. cryophilus* ATCC 15174 for instance, that although the fatty acid composition of the phospholipids changed as environmental factors, such as growth temperature, were altered, the affect that this change had on the gel - liquid crystalline transition temperature was small, i.e. the

gel - liquid crystalline transition temperature was almost unaltered over the growth temperature range investigated (Foot *et al.*, 1983a; McGibbon *et al.*, 1985). The reason for this is probably that the combined affect of changes in the fatty acid composition and the polar head group composition of the phospholipids, for any given phospholipid content have a wide spectrum over which the there is sufficient fluidity to maintain the metabolic functions of the micro-organism (Baldassare *et al.*, 1976; Goldfine, 1984). Therefore, the wax esters are probably not required as membrane modifiers. Indeed this question has already been asked and answered by Russell and co-workers, for the bacterium *M. cryophilus* ATCC 15174. McGibbon *et al.* (1985) report that in *M. cryophilus* ATCC 15174 the wax esters did not alter the gel - liquid crystalline temperature of the phospholipids and they concluded that in *M. cryophilus* ATCC 15174 that the wax esters were not used as membrane modifiers.

Chapter Six

Some Studies on the NADP-dependent Alcohol Dehydrogenase of *Acinetobacter* sp. NCIB 8250

6.1 Introduction

Fixter & Nagi (1984) suggested that an NADP-dependent alcohol dehydrogenase, that was known to occur in *Acinetobacter* sp. NCIB 8250, might be involved with wax ester biosynthesis and/or degradation in this bacterium. This enzyme has now been purified to homogeneity and characterized (Wales, 1992). As part of that work samples were taken from the continuous culture experiments described earlier in this thesis to study the affects of the different growth parameters on the activity of the NADP-dependent alcohol dehydrogenase. An attempt was then made to correlate these results with those obtained for the wax ester content and composition under the same growth conditions to see if any conclusions could be drawn about whether or not the enzyme was involved in wax ester biosynthesis and/or degradation.

6.2 Results

6.2.1 Storage, disruption and assaying of NADP-dependent alcohol dehydrogenase activity in *Acinetobacter* sp. NCIB 8250 harvested from continuous culture

As part of the investigation into the *in vivo* role of the enzyme NADP-dependent alcohol dehydrogenase (Wales, 1992), it was decided to measure the enzyme's activity in samples from cultures grown under very different physiological conditions. These samples were obtained from continuous culture experiments from which only small samples, in terms of cell dry weight, were collected. However, during the development of a purification protocol for the enzyme, cell suspensions were routinely broken using a French pressure cell (Wales, 1992) but because of the small volume of the cell suspensions (approximately 1 ml) obtained from the continuous culture experiments the samples could not be disrupted by this method; whereas this volume could easily be handled by sonication.

Therefore, an experiment was carried out to verify that the NADP-dependent alcohol dehydrogenase activity in samples of *Acinetobacter* sp. NCIB 8250 harvested from continuous culture could be assayed after storage of the cell pellets at minus 80 °C and after disruption of the cell suspension by sonication. To test this method of storage and cell disruption, *Acinetobacter* sp. NCIB 8250 was grown in batch culture in medium containing a low concentration of succinic acid and in medium containing a low concentration of $(NH_4)_2SO_4$. The cultures were harvested during stationary phase and a sample of the cells from each nutrient limitation, equivalent to

the amount expected in the samples harvested from continuous culture, were broken by sonication and the resulting cell free extract assayed for NADP-dependent alcohol dehydrogenase activity (Table 6.1a). The remainder of the harvested cells were stored at minus 80 °C. After all the samples had been collected from the continuous culture the control samples were thawed, sonicated and assayed for NADP-dependent alcohol dehydrogenase activity (Table 6.1b). Comparison of the results showed that the average enzyme activity of the control samples assayed before and after storage and sonication varied by approximately 5 %. These results (Table 6.1) indicated that the method of storage and cell disruption of the samples harvested from the batch cultures were not deleterious to the activity of the NADP-dependent alcohol dehydrogenase and on the basis of these results it was decided that the procedure of harvesting the continuous culture samples, storing them at minus 80 °C and disruption of the cell suspensions by sonication was acceptable. Therefore, this protocol was followed for the samples harvested from the continuous culture experiments.

6.2.2 NADP-dependent alcohol dehydrogenase activity

The activity of the enzyme NADP-dependent alcohol dehydrogenase was measured in cell free homogenates of *Acinetobacter* sp. NCIB 8250 harvested from continuous culture grown under succinate, ammonium and oxygen limitation. Under ammonium limitation the specific activity of the enzyme at 15 °C was lower than at 30 °C regardless of the specific growth rate (Figure 6.1a). This was also true for samples obtained from oxygen-limited cultures [grown on the medium with a low concentration of $(NH_4)_2SO_4$] where the activities at 15 °C and 20 °C were lower than at 30 °C (Figure 6.1b). However, because of the limited amount of data obtained under any of the nutrient limitations in order to have sufficient data for significant statistical analysis the data from slightly different nutrient limitations were grouped together. Thus all the samples of *Acinetobacter* sp. NCIB 8250 grown at 30 °C on the medium with a low concentration of succinic acid (succinate and oxygen-limited) were grouped together as were the samples of *Acinetobacter* sp. NCIB 8250 grown at 30 °C on the medium with a low concentration of $(NH_4)_2SO_4$ (NH_3 and oxygen-limited). Using these two groups of data there was a decrease in enzyme activity as the specific growth rate was increased (Figure 6.2). The decrease in enzyme activity being greater in the samples of *Acinetobacter* sp. NCIB 8250 grown on the medium with a low concentration of $(NH_4)_2SO_4$ than in the samples of *Acinetobacter* sp. NCIB 8250 grown on the medium with a low concentration of succinic acid.

Correlation of enzyme activity with total wax ester content (Figure 6.3), using three

Table 6.1

Effect of storage and method of cell disruption on the specific activity of the enzyme NADP-dependent alcohol dehydrogenase in *Acinetobacter* sp. NCIB 8250 grown in batch culture on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$

Acinetobacter sp. NCIB 8250 was grown in batch culture at 30 °C in a 2 l flask on the medium with a low concentration of succinic acid (Section 2.3.2.2) and on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.3.2.3). The cultures were harvested (Section 2.7) in stationary phase (Section 2.5.2) and a sample of the cells from each nutrient limitation, equivalent to the amount expected in the samples harvested from continuous culture, were broken by sonication (Section 2.13.1) and the resulting cell free extract assayed in duplicate for NADP-dependent alcohol dehydrogenase activity (Section 2.13.2). The remainder of the harvested cells were stored at minus 80 °C and after a period of time were thawed, sonicated (Section 2.13.1) and assayed four times for NADP-dependent alcohol dehydrogenase activity (Section 2.13.2).

Table 6.1a shows the average specific activity obtained for NADP-dependent alcohol dehydrogenase in the samples assayed before storage at minus 80 °C, the numbers in parentheses are the actual determinants. Table 6.1b shows the average specific activity obtained for NADP-dependent alcohol dehydrogenase in the samples assayed after storage at minus 80 °C.

a) before storage

Nutrient limitation	Specific activity of NADP-dependent alcohol dehydrogenase [nmol min^{-1} (mg protein)$^{-1}$]	
Succinic acid	145	(141, 148)
$(NH_4)_2SO_4$	209	(207, 211)

b) after storage

Nutrient limitation	Specific activity of NADP-dependent alcohol dehydrogenase [nmol min^{-1} (mg protein)$^{-1}$]	
Succinic acid	138 ± 4	(n = 4)
$(NH_4)_2SO_4$	198 ± 12	(n = 4)

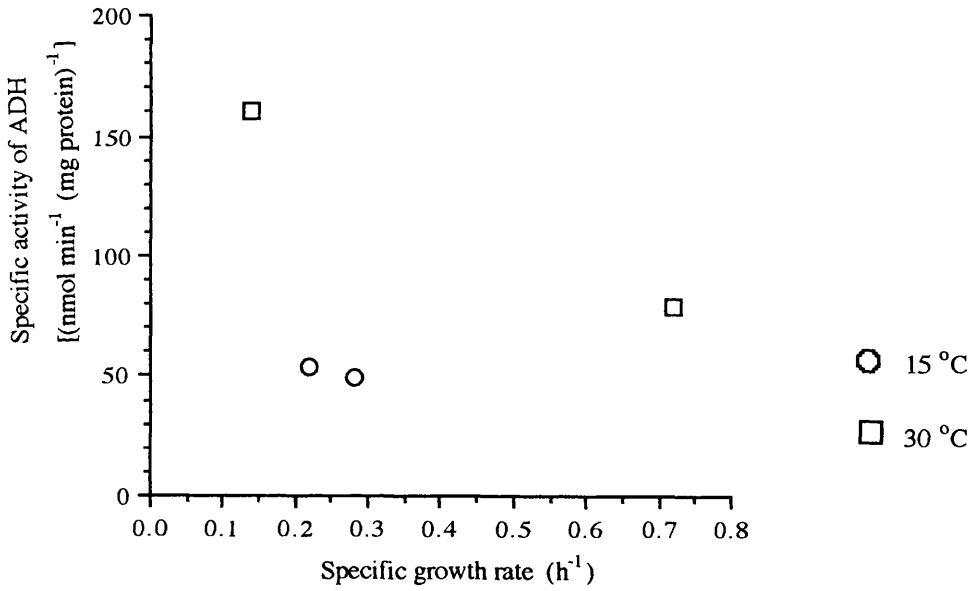
Figure 6.1

Effect of specific growth rate and growth temperature on the activity of NADP-dependent alcohol dehydrogenase when assayed in extracts of *Acinetobacter* sp. NCIB 8250 grown in continuous culture under ammonium and oxygen limitation

Acinetobacter sp. NCIB 8250 was grown in continuous culture at 15 °C and 30 °C under ammonium limitation and at 15, 20 and 30 °C under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.5.3). The samples were collected over ice from the effluent line, harvested (Section 2.13.1) and assayed for NADP-dependent alcohol dehydrogenase activity (Section 2.13.2).

Figure 6.1a shows the specific activities obtained from the samples of *Acinetobacter* sp. NCIB 8250 grown at 15 °C and 30 °C under ammonium limitation and Figure 6.1b shows the specific activities obtained from the samples of *Acinetobacter* sp. NCIB 8250 grown at 15, 20 and 30 °C under oxygen limitation on the medium with a low concentration of $(NH_4)_2SO_4$.

a) at 15 °C and 30 °C under ammonium limitation



b) at 15 °C, 20 °C and 30 °C under oxygen limitation

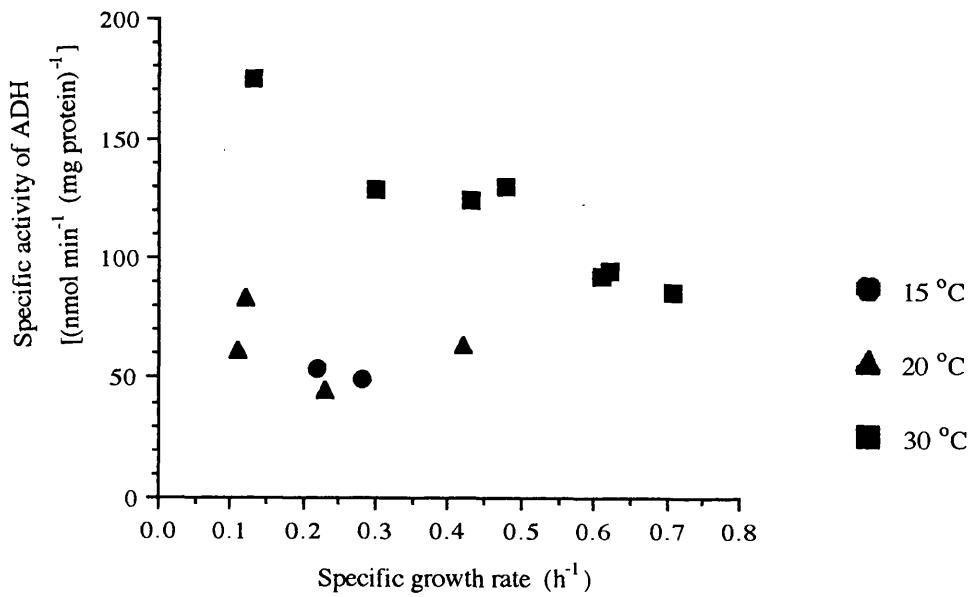
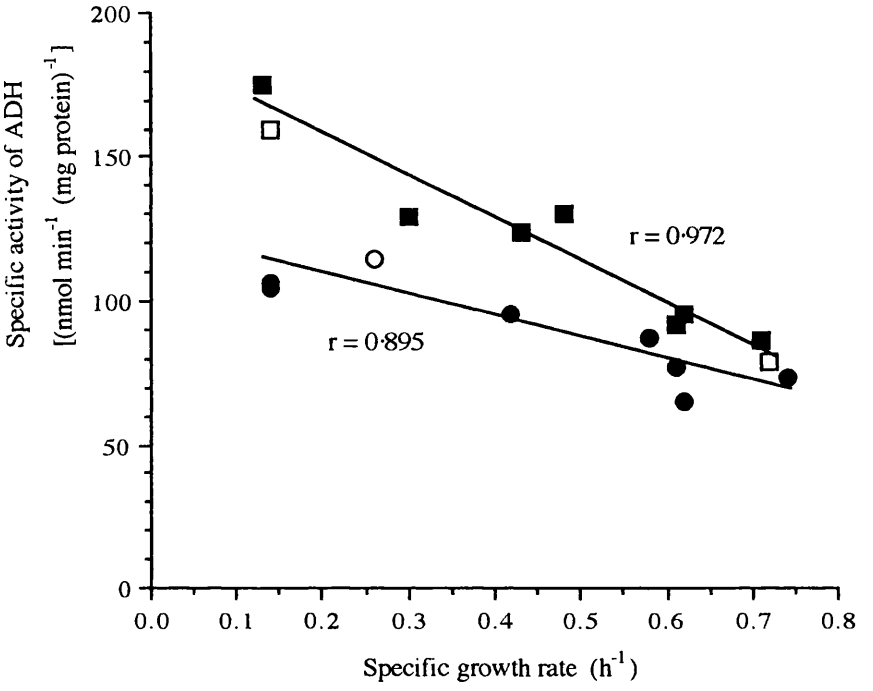


Figure 6.2

Correlation of the NADP-dependent alcohol dehydrogenase activity assayed in extracts of *Acinetobacter* sp. NCIB 8250 grown in continuous culture under succinate, ammonium and oxygen limitation on the medium with a low concentration of succinic acid and on the medium with a low concentration of $(NH_4)_2SO_4$ with the specific growth rate

Acinetobacter sp. NCIB 8250 was grown in continuous culture at 30 °C under succinate and ammonium limitation and under oxygen limitation using both the medium with a low concentration of succinic acid and the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.5.3). The samples were collected over ice from the effluent line, harvested (Section 2.13.1) and assayed for NADP-dependent alcohol dehydrogenase activity (Section 2.13.2).

The Figure shows the two groups of data obtained from cells (i) grown on the medium with a low concentration of succinic acid and harvested under succinate or oxygen limitation and (ii) grown on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested under ammonium or oxygen limitation (see also Section 6.2.2).



○ Succinate-limited - medium with a low concentration of succinic acid

● Oxygen-limited - medium with a low concentration of succinic acid

□ Ammonium-limited - medium with a low concentration of $(NH_4)_2SO_4$

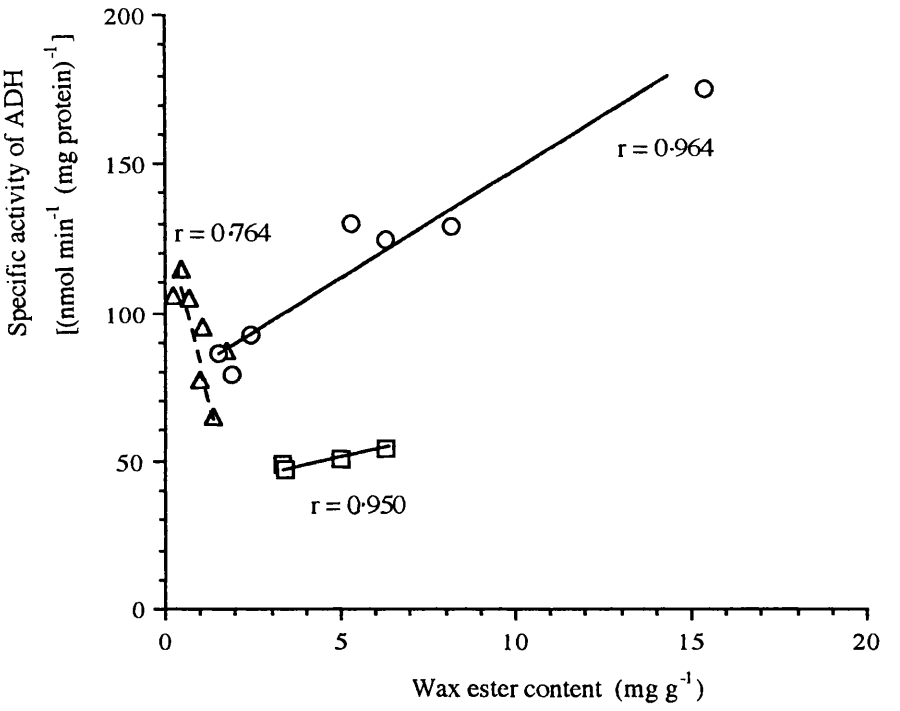
■ Oxygen-limited - medium with a low concentration of $(NH_4)_2SO_4$

Figure 6.3

Correlation of the NADP-dependent alcohol dehydrogenase activity assayed in extracts of *Acinetobacter* sp. NCIB 8250 grown in continuous culture under succinate, ammonium and oxygen limitation with the total amount of wax esters accumulated

Acinetobacter sp. NCIB 8250 was grown in continuous culture at 30 °C under succinate limitation and under oxygen limitation using the medium with a low concentration of succinic acid and at 15 °C and 30 °C under ammonium limitation and under oxygen limitation using the medium with a low concentration of $(NH_4)_2SO_4$ (Section 2.5.3). The samples were collected over ice from the effluent line, harvested (Section 2.13.1) and assayed for NADP-dependent alcohol dehydrogenase activity (Section 2.13.2).

The Figure shows the three groups of data obtained for cells (i) grown at 30 °C on the medium with a low concentration of succinic acid and harvested under succinate or oxygen limitation (ii) grown at 30 °C on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested under ammonium or oxygen limitation and (iii) grown at 15 °C on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested under ammonium or oxygen limitation (see also Section 6.2.2).



△ medium with a low concentration of succinic acid - 30 °C

○ medium with a low concentration of $(NH_4)_2SO_4$ - 30 °C

□ medium with a low concentration of $(NH_4)_2SO_4$ - 15 °C

groups of data: (i) *Acinetobacter* sp. NCIB 8250 grown at 30 °C on the medium with a low concentration of succinic acid and harvested under succinate or oxygen limitation: (ii) *Acinetobacter* sp. NCIB 8250 grown at 30 °C on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested under ammonium or oxygen limitation and (iii) *Acinetobacter* sp. NCIB 8250 grown at 15 °C on the medium with a low concentration of $(NH_4)_2SO_4$ and harvested under ammonium or oxygen limitation, was more difficult. For group (i) [△] there was no significant relationship between enzyme activity and total wax ester content, as judged by the statistical analysis used, although it should be noted that the total wax ester content for this group are much smaller than for the other groups. However, when the Figure (6.3; [△]) was examined by eye there did appear to be an apparent correlation, indicating a decrease in enzyme activity as the total wax ester content increased but the correlation coefficient for these data was only 0·764. For group (ii) [○] the sample with the highest wax content [41·1 mg wax esters (g dry weight)$^{-1}$] did not appear to conform to the pattern of the rest of the data and had a disproportionate amount of influence on any models fitted to these data. If this sample was ignored there was a strongly linear relationship (r = 0·964) between enzyme activity and total wax ester content, enzyme activity increasing with increasing total wax ester content. However, there were only two samples of *Acinetobacter* sp. NCIB 8250 obtained under ammonium limitation in this set of data and it could be argued that if more samples had been available that a different correlation between samples of *Acinetobacter* sp. NCIB 8250 harvested from ammonium and oxygen limitation (grown on the medium with a low concentration of $(NH_4)_2SO_4$) would have been revealed and therefore excluding the sample with the highest wax content may not be justified. For group (iii) [□] there was also a significant linear relationship between enzyme activity and total wax ester content (r = 0·950), enzyme activity increasing with total wax ester content but not as steeply as seen in group (ii).

6.3 Discussion

It has been shown that *Mycobacterium tuberculosis* has an NADP-dependent alcohol dehydrogenase that has a wide specificity for alkan-1-ols and that this enzyme was involved in wax ester synthesis in this bacterium (Wang *et al.*, 1972; de Bruyn *et al.*, 1981). Fixter & Nagi (1984) reported the occurrence of an NADP-dependent alcohol dehydrogenase in *Acinetobacter* sp. NCIB 8250 that also had a broad specificity for alkan-1-ols. As acinetobacters can synthesise wax esters it was postulated that this enzyme could be involved in the synthesis and/or degradation of the wax esters found in *Acinetobacter* sp. NCIB 8250.

When *Acinetobacter* sp. NCIB 8250 was grown at 30 °C under ammonium limitation there was good correlation between the enzyme's activity and the wax ester content of the cells, i.e. the activity of the enzyme was high when the wax ester content was high and low when the wax ester content was low. This correlation was also true when *Acinetobacter* sp. NCIB 8250 was grown at 15 °C under ammonium limitation. There was however, poor correlation of the enzyme's activity with the wax ester content when *Acinetobacter* sp. NCIB 8250 was grown under succinate limitation but this was probably because of the low amounts of wax ester present in *Acinetobacter* sp. NCIB 8250 grown under these conditions.

These correlations are in agreement with the enzyme being involved in the synthesis of wax esters. Indeed Wales (1992) reported that the NADP-dependent alcohol dehydrogenase showed a preference for reducing the aldehyde to the corresponding alcohol, a requirement for an enzyme involved in the synthesis of wax esters. Mutants of *Acinetobacter* sp. HO1-N reported to accumulate more wax esters than the parent strain (Geigert *et al.*, 1984) because of reduced wax ester breakdown were investigated by Wales who showed that there was no change in the activity of the NADP-dependent alcohol dehydrogenase in the mutants compared with the parent (Wales, 1992). This is perhaps further evidence that if the enzyme is involved in the metabolism of wax esters that its role is in the biosynthesis of wax esters

It has also been suggested that the net accumulation of wax esters by *Acinetobacter* sp. NCIB 8250 is regulated by the rate of their degradation (Nagi & Fixter, 1981). Evidence that the NADP-dependent alcohol dehydrogenase is constitutive has been provided by Wales (1992) who grew *Acinetobacter* sp. NCIB 8250 in batch culture on a variety of different carbon sources. These experiments showed that the NADP-dependent alcohol dehydrogenase activity was present regardless of the carbon source and varied less than two to three fold, being greatest in cells grown under ammonium limitation. This result is also in agreement with the enzyme being involved in the synthesis of the wax esters whose accumulation is regulated by their rate of degradation.

Wales (1992), however, concluded that the enzyme was not involved in the synthesis of fatty alcohols for the biosynthesis of wax esters because the enzyme is soluble and not associated with membranes, which might be expected to contain the substrate as it is lipophilic. Also, the enzyme did not oxidise primary alcohols greater that C_{14} whereas the majority of the alcohols in the wax esters are C_{16} and C_{18}. The conclusions drawn by Wales (1992) may be correct but it is possible that the enzyme is associated with the wax ester synthesis in processes which one would expect to be membrane associated. Moreover, the longer chain length alcohols are notoriously

chain length alcohols are notoriously insoluble in aqueous assay systems and this inability to detect oxidation of primary alcohols longer than C_{14} may have been a result of the substrates' insolubility and not because they are not a substrate for the enzyme.

Chapter Seven

Conclusions and Future Work

7.1 Conclusions

From these studies on *Acinetobacter* sp. NCIB 8250 it is clear that the optimum growth temperature range is within a few degrees of 30 °C and that the specific growth rate during the exponential phase of batch culture was lower than the maximum specific growth rate observed in continuous culture. It is not clear why there was such a difference. It has also been established, by three independent workers, that the true molar growth yield at 30 °C is 41·1 - 42·9 g dry weight (mol succinate)$^{-1}$, when *Acinetobacter* sp. NCIB 8250 is grown in a defined medium with succinic acid as the sole source of carbon and energy and $(NH_4)_2SO_4$ as the nitrogen source. However, the actual value for the maintenance coefficient is still in some doubt as a different value is obtained in this present work from the values obtained by the other workers.

The wax ester content found in *Acinetobacter* sp. NCIB 8250 is influenced quite dramatically by the environment in which the bacteria are grown. In continuous culture under conditions of excess carbon and energy, and limiting concentrations of nitrogen, wax esters are accumulated. The extent to which they accumulate being influenced by the specific growth rate of the culture. Thus at low specific growth rates significantly more wax esters accumulate than at specific growth rates approaching the maximum specific growth rate. In batch culture, under nitrogen limited stationary phase, the extent to which wax esters accumulate is also dependent on the length of time the culture is in stationary phase. Conversely in either batch or continuous culture under conditions of excess nitrogen and limiting concentrations of carbon, wax esters are not accumulated to any great extent. In batch culture it would appear that the growth temperature has an affect on the total amount of wax esters accumulated by exponentially growing cultures. However, it is probably the specific growth rate of the culture that is influencing the amount of wax esters accumulated as there is a direct relationship between the growth temperature and the specific growth rate of the culture, i.e. as the growth temperature is reduced so is the specific growth rate and it has been shown using continuous culture the accumulation of wax esters is very dependent on the specific growth rate of the culture.

The specific growth rate, however, does not have a significant affect on the fatty acid composition of the phospholipids found in *Acinetobacter* sp. NCIB 8250 grown in either batch or continuous culture. Thus at 30 °C the fatty acid composition of the phospholipids is essentially the same whether *Acinetobacter* sp. NCIB 8250 is grown on the medium with a low concentration of succinic acid or on the medium with a

low concentration of $(NH_4)_2SO_4$, in continuous or batch culture and harvested from exponential phase or stationary phase. However, there is evidence from batch culture experiments, where *Acinetobacter* sp. NCIB 8250 was grown at 15 °C and 30 °C, that the growth temperature affects the fatty acid composition of the phospholipids. This is likely to be a genuine effect of growth temperature, as it has been shown that the fatty acid composition of the phospholipids is not affected by changes in the specific growth rate of the culture. However, when *Acinetobacter* sp. NCIB 8250 was grown in continuous culture at different growth temperatures there was very little change in the fatty acid composition of the phospholipids, although, it should be noted that only an 8 °C range was used. As most of the range studied covered the optimum growth temperature range, it is probably of inadequate size to show if there is any affect of the growth temperature on the fatty acid composition of the phospholipids. Thus it would have been necessary to extend the growth temperature range studied down to 15 °C, perhaps, in order to demonstrate clearly if there was an affect of growth temperature on the fatty acid composition of the phospholipids when *Acinetobacter* sp. NCIB 8250 was grown in continuous culture.

The same affects of specific growth rate and growth temperature on the fatty acid composition of the wax esters were observed as was found for the fatty acid composition of the phospholipids. Thus, the specific growth rate had no affect but the growth temperature did. This result, however, applies only in continuous culture. In batch culture during stationary phase, where the specific growth rate is effectively zero, there was a significant change in fatty acid composition of the wax esters as the culture conditions changed from exponential growth to stationary phase. In stationary phase the majority of the fatty acids in the wax esters were $C_{16:0}$. This suggests that the end product of fatty acid synthesis is the $C_{16:0}$ fatty acid and that fatty acid synthase is not affected by the specific growth rate of the culture but that both the elongase and desaturase enzymes are affected. If this is the case, are these enzymes linked more closely with phospholipid biosynthesis, which presumably ceases during stationary phase, than they are with fatty acid synthesis?

Unlike the fatty acid compositions of the wax esters and phospholipids the wax ester composition is affected by both the specific growth rate and the growth temperature. As the fatty acid composition of the wax esters is not affected by the specific growth rate it must be concluded that the affect of the specific growth rate on the wax ester composition is exerted through a specific growth rate affect on the fatty alcohol composition. As indicated the growth temperature has a major influence on the wax ester compositions. Although, probably more accurately would be that the growth temperature influenced the fatty acid compositions through the enzymes of the fatty acid synthesis and therefore it only has an indirect affect on the wax ester

compositions. Therefore, to understand more fully the effects of environmental factors, such as growth temperature, on the wax ester composition a complete understanding of the synthesis of fatty acids and the modifications made to them by elongase and desaturase enzymes is required.

7.2 Future work

Due to the paucity in our knowledge with respect to wax ester metabolism there is an enormous amount of work still left to be done before we have a complete understanding of the control of wax ester synthesis and degradation. Indeed it is probably a necessity that we also elucidate the pathways of fatty acid and phospholipid biosynthesis. Our knowledge of the requirements of the enzymes involved in these pathways is very limited, as the majority of the work done to date has concentrated on what affects changes in the growth conditions have on the wax ester content and composition (Fixter *et al.*, 1986; this Thesis) and to a lesser extent on the fatty acid compositions of the wax esters and phospholipids (this Thesis). There has, however, been some preliminary work carried out on the overall synthesis of wax esters using both whole cells and homogenates supplied with radio-labelled [1-^{14}C] hexadecanoic acid (Sampson & Finnerty, 1974) and on the enzymes involved in the degradation pathway (Sherwani & Fixter, 1989) but virtually no detailed work on individual enzymes has been undertaken.

This then must be the next line of investigation.

It is imperative that we study the enzymes of fatty acid synthesis [including the elongase and the acyl desaturase(s)] and those involved in the synthesis of phosphatidic acid (assuming that the pathway is similar to that found in *E. coli*) as well as the enzyme(s) responsible for the conversion of the fatty acid derivatives to the corresponding fatty alcohol (via the aldehyde). It would be hoped that the study of these enzymes (assays for some of which would have to be developed), which would probably be initially undertaken in crude or partially purified homogenates, would identify the true substrates for the enzymes. For instance, do the enzymes of phosphatidic acid synthesis use the acyl-ACP derivatives while the enzyme(s) responsible for the synthesis of the fatty alcohol uses the acyl-CoA derivative and therefore is there an acyl-ACP-CoA transacylase at the regulatory point in the synthesis of the wax esters? Or do both sets of enzymes use the same acyl derivatives and if so how then is wax ester synthesis regulated? Or is it that only the degradation of the wax esters is regulated? Also, does the enzyme responsible for the desaturation of the acyl chains function similar to the desaturase of *M. cryophilus*

ATCC 15174, which probably acts directly on the acyl chains of the phospholipids or by some other mechanism? Perhaps *Acinetobacter* sp. NCIB 8250 has different desaturases, one that uses the acyl chains of the phospholipids as a substrate and another with a preference for the acyl chains of the wax esters. It would also be of interest to study the enzyme responsible for the condensation of the fatty alcohol and fatty acid derivative, i.e. the wax ester synthase. Does this enzyme use an acyl-ACP or CoA derivative as the source of the fatty acid? This might be one way of determining if an acyl-ACP-CoA transacylase was involved in the control of wax ester biosynthesis.

There has also been a lack of work on the degradation of wax esters and fatty acids in *Acinetobacter* species, although Sherwani & Fixter (1989) have investigated some of the properties of the esterase and lipases that they found in several *Acinetobacter* species.. It is assumed that the esterase (or lipase) responsible for the hydrolysis of the wax esters produces a free fatty acid and fatty alcohol and that the fatty alcohol is then reduced to a free fatty acid. It is thought that the fatty acids would then be activated to the CoA derivatives which would then be used in the fatty acid degradation pathway to produce acetyl-CoA for energy production. However, there is no biochemical evidence from work carried out in *Acinetobacter* species to support these assumptions. Therefore, similar studies to those conducted on the biosynthetic enzymes and on the equivalent enzymes in *E. coli* would need to be carried out.

Therefore, it is only by the identification of the enzymes, the true substrates for the enzymes and by identification of the co-factors and regulatory molecules that evidence for a control mechanism for the biosynthesis and degradation of the wax esters can be obtained.

An alternative route, perhaps even complementary to that of the enzymology route suggested above, would be one of molecular biology. By the production of a gene library and mutants lacking certain enzymes it would be possible to not only clone the gene for the enzyme of interest but to overexpress it. This would facilitate the purification of these enzymes which due to their lipophilic nature, is difficult. However, it must be noted that although producing mutants is not usually a problem it would be very difficult to easily identify isolates with the desired mutations, although strategies similar to those used to isolate mutants of the glycogen biosynthesis pathway in *Saccharomyces cerevisiae* might be employed (Chester, 1968). In those experiments mutants lacking the ability to synthesis glycogen were identified by flooding the agar plates with an iodine solution. The colonies still able to produce glycogen.turned brown as the iodine was bound by the glycogen whereas

the mutants remained white.

Therefore, with *Acinetobacter*, it might be possible to identify mutants using a lipophilic stain, such as Sudan Black. This particular method might not be satisfactory for a number of reasons, however, a strategy along the lines of mutation, growth on nitrogen-limited agar plates followed by a specific test for the presence/absence of wax esters would probably be successful. The crucial part is undoubtable the "specific test for the presence/absence of wax esters".

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