THE UTILISATION OF OIL DURING STREPTOMYCETE FERMENTATIONS

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for the degree of

MASTER OF PHILOSOPHY

by

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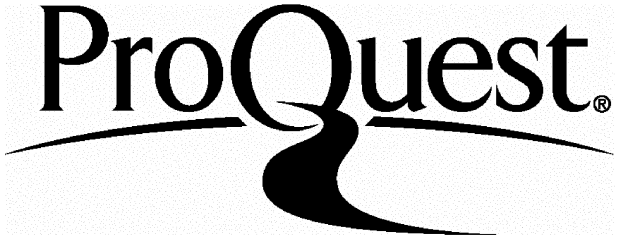

ABSTRACT

Many industrial antibiotic fermentations use mixed substrates such as complex sugars and oils as sources of carbon. Oils are included because they may result in higher antibiotic titres and can be cheaper than the alternative refined sugar carbon sources.

This study examines the effects of oil substrate utilisation in an antibiotic producing *Streptomyces clavuligerus* fermentation and illustrates the factors which influence the efficient utilisation of these oil substrates. Batch fermentations at 5l scale show both the utilisation of oil in the presence of other carbon sources and that the oil is degraded by the action of lipase. The use of a complex medium containing both oil and sugar appears to stimulate the rate of growth of the organism and also results in an increase in antibiotic titre.

The lipase produced appears to be cell-associated and its expression is dependent on medium composition as well as the environmental conditions. For example, variation in the carbon and nitrogen sources leads to changes in the time of lipase expression and the amount of lipase produced whilst maximum activity is exhibited at 30°C and pH 7.2. The level of lipase activity was affected by the dissolved oxygen concentration and the agitation rate and was inactivated during the later stages of the fermentation. It was proposed that this was due to either the effect of shear or denaturation of the enzyme by the gas/liquid interface.

At the end of the fermentation a residual amount of oil was found to be present. The composition of the residual oil was found to be similar to the oil added at the start of the fermentation. The study showed that metabolism of the oil ceased due to both the physical limitation of oil mass transfer in the fermenter environment as well as lipase inactivation. Increasing the agitation rate had a negligible effect with respect to oil utilisation as did increasing the dissolved oxygen concentration.

To my mother & father

for their continual encouragement & support

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NOMENCLATURE

Greek symbols

μ	=	viscosity (N/m^2s)
τ	=	shear stress (N/m^2)
γ	=	shear rate (s^{-1})
ρ	=	density (g/l)
τ_o	=	yield stress (N/m^2)
η	=	flow behaviour index
σ	=	surface tension (N/m)
υ	=	kinematic viscosity (m^2/s)

Letters

a	=	specific surface area (m^{-1})
Dc	=	bubble column diameter (m)
D_0	=	initial droplet size (μm)
Di	=	impeller diameter (m)
g	=	gravitational acceleration (m/s^2)
k	=	consistency index (N/m^2)
Kc	=	Casson viscosity $(N/m^2s)^{0.5}$
L_e	=	length of hyphae (μm)
L_{hgu}	=	length of the hyphal growth length (μm)
M_F	=	morphology factor
N	=	impeller tip speed (s^{-1})
og	=	interfacial tension between oil and gas phases (dynes/cm)
ow	=	interfacial tension between oil and water phases (dynes/cm)
P	=	power consumption (Watts)
Sp	=	spreading coefficient (dynes/cm)
T	=	tank diameter (m)
V	=	volume (l)
Vs	=	superficial gas velocity (m/s)
wg	=	interfacial tension between water and gas phases (dynes/cm)

Italics

a	=	radius of the droplet (μm)
d	=	droplet diameter (μm)
k	=	proportionality constant (6.3 for a Rushton turbine)

Subscripts

c	=	continuous phase
d	=	dispersed phase

Chapter 1 The Actinomycetes

Actinomycetes have been recognised as a distinct group known as *Actinomycetales* for more than a century. This group of organisms is sometimes regarded as "procaryotic fungi" due to their ability to adapt to growth on a solid substrate resulting in filamentous morphological characteristics similar to that of fungi. However, actinomycetes are more correctly defined as "procaryotic bacteria with elongated cells or filaments that usually show some degree of branching", (Goodfellow *et al.*, 1983), which gives them their characteristic appearance; a hyphal network of individual tubular cells. Actinomycetes are Gram positive sporulating bacteria which are saprophytic by nature, (Goodfellow *et al.*, 1988), and encompass a wide range of micro-organisms isolated from a variety of different sources such as soil, water, dust and other natural environments.

Interest in actinomycetes began in the late nineteenth century when it was found that the bacteria were the causal agents of a wide range of infections in man, animals and plants. Further studies discovered the ability of actinomycetes to produce antibiotics, bioactive compounds which appear to have no apparent function in the organisms producing them (Doull & Vining, 1991). The first of these new compounds was given the name actinomycin and was isolated by Waksman & Woodruff in 1940 from a bacterium they named *Streptomyces antibioticus*. This was followed by the discovery by Schatz, (1944) of streptomycin which proved to be a more effective antibiotic.

Further secondary metabolite producing actinomycetes have been isolated and commercially exploited. The industrial importance of actinomycetes and their respective secondary metabolites has been extensively reviewed by Bushell, (1983). However, their distinctive morphology presents several problems in relation to the production process. Their filamentous nature results in a viscous broth exhibiting pseudoplastic behaviour which causes problems with respect to mixing and mass transfer, such as oxygen limitation. Therefore, the organisms demonstrate low specific growth rates and low metabolite production. These variables may also affect microbial physiology and morphology. The morphological changes, which occur during the growth of this group of organisms, are associated with the physiological changes from primary metabolism, responsible for cell growth and maintenance, to secondary metabolism.

There is a complex relationship between mycelial morphology and broth rheology, (Chapter 7), and this can be further complicated by the growth conditions used. For example, oils have been used as carbon sources in fermentation media for both economic and production reasons since they are cheaper than refined sugars and have been found to stimulate antibiotic production. These compounds are highly viscous which complicates an already complex morphology/rheology interaction. Therefore, this study concerns the effect of fermentation conditions, both physical and chemical, on *Streptomyces clavuligerus* morphology and clavulanic acid production.

Chapter 1.1 The Streptomycetes

Streptomycetes are considered to be the most common aerobic Gram positive members of the actinomycetes. They belong to the category of sporoactinomycetes which are morphologically complex because they form spores at some point in their life cycle and are highly branched (Williams *et al.*, 1976). The Streptomycetes exhibit characteristic branching hyphae during vegetative growth and can also produce aerial mycelia depending on the composition of the medium, the incubation temperature and nutrient depletion. These aerial branches usually bear long chains of spores which are their reproductive units.

Streptomycetes have a typical procaryotic structure with the nuclear material consisting of fine fibrils which are usually situated in the central region of the cell (Hirsch & McCormick, 1985). This material may be dispersed or organised into more compact aggregates with the fibrils lying longitudinally along the hyphae. The hyphae contain cross walls which have been found to be associated with the age of the hyphae where the septa appear to form in the older areas of the hyphae and then spread to the hyphal apex. However, Kretschmer, (1982) showed that septation during the ageing process proceeds by placing new cross walls at points approximately intermediate between the existing cross walls. The presence of these cross walls is important when considering the morphology of the organism since their presence should reduce fragmentation and reduce loss of cytoplasmic cell contents during fermentation and hence maintain viability. Streptomycete mycelia rarely spontaneously fragment.

During sporulation, the Streptomycete spores are formed by the regular septation of a hypha enclosed within a fibrous sheath which persists on the spores and contributes to its surface and hydrophobic nature. This is indicated by their increased Gram positiveness (Kalakoutskii & Pouzharitskaja, 1973). The sporulation process is influenced by a number of factors such as medium composition and growth conditions, which results in variation in the morphology and physiology of the organism although sporulation is rarely observed in submerged culture.

However, it has been shown that exhaustion of certain medium components such as phosphate will induce sporulation thus reducing antibiotic titre (Kalakoutskii & Agre, 1976). This implies that the use of oil in the fermentation medium could affect this process such as repressing sporulation, thus resulting in an increased antibiotic titre. Sporulation may also result in a decrease in optical density and viscosity but an increased respiratory activity and therefore, can be used as an indicator of physiological and morphological state of the organism. This suggests that there is a direct link between production of antibiotics and the morphology of the antibiotic producer.

The Streptomycetes have proved to be the most successful source of antibiotics and other secondary metabolic products. The most lucrative genus of this family is the *Streptomyces.* They produce valuable bioactive substances for use in agriculture, industry, medicine and animal husbandry and account for 93% of actinomycete products. The most important secondary metabolites produced are antibiotics, the main groups of which are illustrated in table 1.1. From the studies carried out it becomes apparent that *Streptomyces* represent an important source of bioactive metabolic products with applications to the industrial sector.

TABLE 1.1 **Antibiotics Produced by *Streptomyces***

Organism	Antibiotic(s) product(s)	Antibiotic group
Streptomyces clavuligerus	Clavams	β-lactams
Streptomyces cattleya	Carbapenems	
Streptomyces griseus	Streptomycins	Aminoglycosides
Streptomyces fradiae	Neomycin	
Streptomyces kanamycetius	Kanamycin	
Streptomyces erythraeus	Erythromycin	Macrolides
Streptomyces ambofaciens	Spiramycin	
Streptomyces fradiae	Tylosin	
Streptomyces aureofaciens	Chlortetracycline	Tetracyclines

Chapter 1.2 *Streptomyces clavuligerus*

Streptomyces clavuligerus is a species from the genus termed *Streptomycete.* It was first observed by Waksman & Henrici in 1943 and produces a number of different structurally related antibiotics such as β-lactam compounds including clavulanic acid, cephalosporin and penicillin N.

When grown on solid media *Streptomyces clavuligerus* produces aerial mycelia which are composed of a network of branched aerial hyphae that segment into spores. Spores are oblong to short-cylindrical averaging from 0.64 to 1.53μm in size. Sporulation is indicated by dark greyish green aerial mycelia but the colour can range from white to grey depending on the medium (Higgens & Kastner, 1971). Neither fragmentation of hyphae nor formation of spores occurs in the substrate mycelia (Higgens & Kastner, 1971).

Streptomyces clavuligerus is unusual however, in its inability to assimilate a wide range of carbon sources. It has the ability to grow on glycerol, starch, maltose and other simple sugars but it is incapable of growing in glucose (Aharanowitz & Demain, 1978). Since maltose and starch support growth, this inability to utilise glucose, and perhaps other sugars as well, is thought to be due to inadequate mechanisms for the uptake of the monomeric substrate to a glycolytic intermediate (Vining *et al.,* 1987).

Chapter 2 *Streptomyces clavuligerus* and clavulanic acid

Streptomyces clavuligerus produces a variety of secondary metabolites. During this study the effect of oil on clavulanic acid production has been investigated and therefore, it is necessary to consider the biosynthesis of clavulanic acid. Subsequent to its isolation and identification as a producer of 7α-methoxycephalosporin derivatives, (cephamycins), *Streptomyces clavuligerus* has served as a source of alternative β-lactam antibiotics based on the 7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane or clavam ring system. Clavulanic acid is one of the newer antibiotics and a potent antibiotic inhibitor of ersine, (or Classes A, C and D), β-lactamases (Brown *et al.*, 1978 and Napier, 1981).

Clavulanic acid is used as its potassium salt in conjunction with amoxycillin, (Augmentin™), and with ticarcillin as Tigmentin™. The formulation of amoxycillin with the lactamase inhibitor clavulanic acid was first introduced into the UK in 1981. The rationale for the formulation was to counteract the growing prevalence of β-lactamase mediated resistance to β-lactam antibiotics. It is especially active against β-lactamases produced by the TEM group of plasmid mediated β-lactamases produced by *Enterobacteriaceae, Haemophilus influenzae* and *Neisseria gonorrhoeae* as well as the chromosomally mediated β-lactamases from *Klebsiella aerogenes and Proteus vulgaris.*

Clavulanic acid was shown to be a fused bicyclic β-lactam structurally different from the penicillins and the cephalosporins in having an oxygen atom in place of the sulphur normally found in the β-lactam ring structure (Howarth *et al.*, 1976). The structure of the clavulanic acid molecule is illustrated in figure 2.1. In total the organism produces five β-lactam antibiotics which are clavulanic acid, deacetoxycephalosporin, penicillin N, cephamycin C and the O-carbamoyl derivative of deacetylcephalosporin C (Hu *et al.*, 1984).

Clavulanic acid is a potent β-lactamase inhibitor and possesses weak broad spectrum antibacterial activity (Reading & Cole, 1977). The enzyme lactamase which brings about the inactivation of many penicillins, cephalosporins and related antibiotics was first recognised by Abraham and Chain, (1940) in a strain of *Escherichia coli.* In 1944 the enzyme was established as the mechanism of resistance in penicillin resistant strains

of *Staphylococcus aureus*. A wide range of pathogenic bacteria have evolved highly efficient β-lactamases with varying substrate specificities which inactivate penicillins and cephalosporins by hydrolysing their β-lactam rings to give penicilloic acid derivatives and analogous degradation products in the case of cephalosporins.

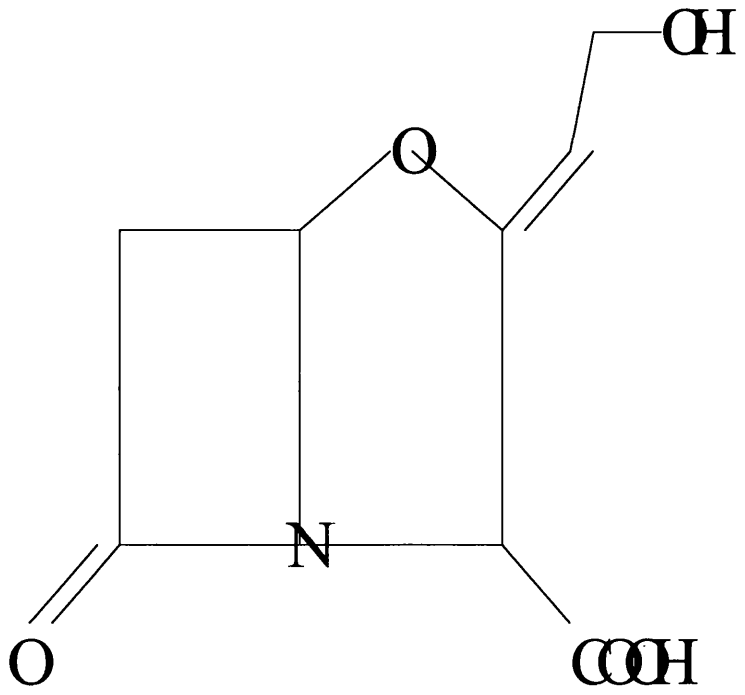


FIGURE 2.1 The structure of the clavulanic acid molecule
(z-(2R,5R)-3-(β-hydroxyethylidene)-7-oxo-4oxa-1 azabicyclo-
(3,2,0) heptane-2-carboxcylic acid)

In order to counter β-lactamase mediated resistance to β-lactam antibiotics two approaches were taken. The first was to discover or synthesise β-lactam antibiotics which were inherently stable to β-lactamase attack and the second was to employ an inhibitor of the enzyme. The first lactamase inhibitors were discovered in *Streptomyces olivaceus* which were later found to be members of the olivanic acid and carbapenem family of β-lactam antibiotics (Brown *et al.*, 1976). Further work lead to the discovery of clavulanic acid, MM, (or BRL), 14151, from *S. clavuligerus*.

Clavulanic acid inhibits the activity of β-lactamase enzymes, produced by many clinical pathogens, by irreversibly binding to the penicillin binding protein 2, (PBP2), thus inactivating the enzyme (Spratt *et al.*, 1977). This is an unusual mechanism since most lactamase resistant β-lactams are normally competitive inhibitors and as such do not physically bind to the enzymes. This last property has made clavulanic acid industrially important as it can be combined with a β-lactam antibiotic to enhance antibiotic activity and overcome bacterial resistance. For example, Brown *et al.,* (1981) demonstrated that the minimum inhibitory concentration, (MIC), of ampicillin could be reduced from 500μg/ml to less than 0.1μg/ml by supplementation with 5μg/ml of clavulanic acid. This clearly illustrated the potential of clavulanic acid to pharmaceutical and other industrial companies.

Chapter 2.1 Biosynthesis of clavulanic acid

It has become evident that *Streptomyces clavuligerus* is very important commercially but due to its unusual metabolic requirements, clavulanic acid biosynthesis is controlled by complex control mechanisms and is derived from mixed biosynthetic origin. Preliminary studies carried out by SmithKline Beecham Pharmaceuticals suggested that carbon atoms 2, 3, 8, 9 and 10, the C5 unit, were derived from 2-oxoglutarate and the three carbon skeleton of the β-lactam ring, the C3 unit, had originated from the tricarboxylic, (TCA), acid cycle.

Further studies by Elson and his co-workers, using radiolabelled precursors of clavulanic acid, deduced that the carbon atoms of the β-lactam ring were derived from gluconeogenesis products, possibly pyruvate (Elson *et al.,* 1978; Elson *et al.,* 1980 and Elson *et al.*, 1981). These findings are supported by Pitlik & Townsend, (1997). However, Romero *et al.*, (1988) proposed that the three carbon atoms of the lactam ring are derived from glycerol via a β-hydroxy-propionate intermediate whilst the other five carbons of the molecule are derived from orthinine (Townsend & Ho, 1985 and Romero *et al.,* 1986).

These studies demonstrated that arginine and ornithine stimulated clavulanic acid production and arginine was converted to ornithine suggesting that this C5 amino acid is a direct precursor of clavulanic acid. However, further work has demonstrated that ornithine has to be converted before it can be incorporated into clavulanic acid (Valentine *et al.,* 1993). This confirms the mixed biosynthetic origin.

Clavulanic acid biosynthesis is also regulated by metabolic activity which tends to take the form of carbon and phosphate regulation. Carbon regulation is not well understood in *S. clavuligerus* although it is known that nutritional conditions that support high specific growth rates are not suitable for antibiotic synthesis.

For example, the use of glycerol or maltose as a carbon source will limit both volumetric and specific antibiotic production with increasing carbon source concentration although both will support extensive biomass growth (Hu *et al.,* 1984 and Romero *et al.*, 1984). Whilst Lebrihi *et al.*, (1988) found that glycerol hinders cephamycin C production by the repression of the cephamycin C synthetase system. It is also known that the use of oils will enhance antibiotic synthesis (chapter 4). This could be due to either a lower specific growth rate and therefore, growth is not limited or the oil and/or fatty acids may work as a precursor or a cofactor for antibiotic synthesis.

Regulating the levels and sources of phosphate and nitrogen may also control clavulanic acid production. In the later stages of fermentation phosphate is required for maintenance of cellular metabolism and therefore, low phosphate concentration can result in reduced levels of biomass. This in turn will cause a reduction in antibiotic production. However, growth dissociated specific antibiotic production has been shown to increase under both phosphate and nitrogen limitation although clavulanic acid production ceased under carbon limitation (Ives & Bushell, 1997).

The effects of phosphate on secondary metabolite production have been extensively reviewed by Martin & Demain, (1980) and the effects include changes in carbohydrate metabolism, stimulation of primary metabolism and inhibition of antibiotic precursor formation. The negative effects of phosphate on β-lactam antibiotic production were first noted by Aharonowitz & Demain, (1977) and confirmed by Romero *et al.*, (1984);

Lubbe *et al.*, (1985); Lebrihi *et al.*, (1987) and Zhang *et al.*, (1989a). It is proposed that phosphate levels control clavulanic acid production by repression on the clavulanic acid synthetase enzymatic system although this has not been confirmed. However, there are reports of the enhancement of antibiotic production by phosphate. Omura *et al.*, (1980) reported that 1% magnesium phosphate increased leucomycin production by *Streptomyces kitasatoensis* several fold due to an inhibitory effect on nitrogen catabolite regulation.

Nitrogen has a key role in regulatory mechanisms and the form in which the nitrogen source is provided determines the pattern of formation of enzymes, intermediates and end products. Nitrogen nutrition and regulation, with respect to glutamate formation, was first reviewed by Magasanik, (1976) and later discussed by Aharonowitz & Demain, (1977) when investigating cephalosporin production in *Streptomyces clavuligerus*. Antibiotic production was closely linked with the presence or absence of ammonium ions which appeared to exhibit a negative inhibitory and/or repressive effect on cephalosporin production. However, amino nitrogen was reported to have stimulatory effects on antibiotic production. Similar effects were reported by Brana *et al.*, (1985) and Zhang *et al.*, (1989b). These negative regulatory effects can be removed by restricting the level of aeration (Fang & Demain, 1995). This is a rare example of regulation reversal by environmental modification.

It should also be noted that variations in clavulanic acid production can also arise from the use of different strains of the organism since this can result in differences in metabolite production. For example, Vining *et al.*, (1987) found differences in cephalosporin production by *S. clavuligerus* when compared with previous studies done by Aharanowitz & Demain, (1978).

Chapter 2.2 Cultivation & production of clavulanic acid

At present the commercial market for clavulanic acid is in coformulation with other antibiotics such as ampicillin or amoxycillin. SmithKline Beecham Pharmaceuticals are manufacturing one example of the commercial success of clavulanic acid under the

trade name "Augmentin" and sales now exceed £1 billion per annum. This is a combination of 250mg of amoxycillin as the trihydrate and 125mg of clavulanic acid as the potassium salt, potassium clavulanate (Rollinson & Watson, 1980).

The industrial process for the production of clavulanic acid has not been fully disclosed but one of the most important considerations is the fermentation nutrients. Clavulanic acid synthesis requires soybean protein; a high carbon sugar source and the addition of a lipid/oil as both an alternative carbon source and an antifoam. Lard or soybean oils produce the best clavulanic acid yields. Besides the economic reasons, this complex medium is used because it has been observed that the clavulanic acid producing strains of *S.clavuligerus* grow very poorly if at all when low carbon compounds such as glucose are provided as the sole carbon source.

From the limited information available, the fermentation process employed involves the use of a series of stirred, aerated fermentation vessels ending with the final large scale production vessel. The process is described in detail in British Patent No 1,571,888. The culture source is usually a mycelia or spore suspension, although the mycelial form is preferred, with culture conditions of a temperature range of 26-30^{o}C and pH range of between 6.0 and 7.5. The media consists of 0.1-1.0% complex organic nitrogen source or chemically defined sources of nitrogen, 0.1-5.0% carbon source, mineral salts and trace elements (Cole *et al.*, 1978). The actual yields of clavulanic acid attained in large scale production have not been disclosed but an average titre of 500µg/ml has been reported although titres as high as 1100µg/ml have been attained (Kitano *et al.*, 1984).

The fermentation process has been described at smaller scale. For example, Tarbuck *et al.*, (1985) investigated clavulanic acid production at 10 litre scale using a complex medium of glycerol, malt extract and bacteriological peptone. The stirrer speed and air flow rate were varied in order to maximise clavulanic acid production. The maximum titre observed was 36mg/l with a biomass of 3.6g dry weight/l at 375rpm and 0.25vvm. However, an increase in air flow rate at this stirrer speed to 1vvm resulted in a decrease in clavulanic acid production.

Chapter 3 Lipids and oils

Oils are of great importance during this study since rape seed oil is used as a carbon source during *Streptomyces clavuligerus* fermentation. The structure of the oil has been shown to be significant in determining the rate of oil uptake with respect to fatty acid composition and therefore, an understanding of the structure oils/lipids is essential for this study. This chapter discusses the structure and function of oils and their use in industrial processes.

No satisfactory or widely accepted definition exists for the term "lipid" although many have been proposed. Generally, lipids are described as a group of naturally occurring compounds which are water-insoluble but soluble in non-polar substances such as chloroform, hydrocarbons or alcohols. It is necessary to use a description based on the physical properties of these compounds since there may be no chemical relationship between the numerous different substances now classified as lipids.

The main classes of lipids of plant and animal origin consist of fatty acids linked by an ester bond to the trihydric alcohol glycerol, or to other alcohols such as cholesterol, or by amide bonds to long chain bases or to other amines. There is also a further subdivision into two broad classes. Simple, or neutral, lipids yield at most two types of primary product per mole on hydrolysis whilst complex, or polar, lipids yield three or more primary hydrolysis products per mole.

The simplest and most abundant lipids are the triacylglycerols, commonly termed triglycerides which consist of a glycerol moiety, each hydroxyl group of which is esterified to fatty acid. Structurally, a triglyceride is the reaction product of one molecule of glycerol and three molecules of fatty acid to yield three molecules of water and one molecule of triglyceride (figure 3.1). Nearly all the commercially important fats and oils of animal and plant origin consist almost exclusively of this simple lipid class.

The fatty acid component of lipids contributes 94-96 % of the total weight of the molecule. Fatty acids are a class of compounds containing a long hydrocarbon chain and a terminal carboxylate group. The systemic name for a fatty acid is derived from the name of its parent hydrocarbon by substitution of the "oic" for the final "e", for example,

a C18 saturated fatty acid is called octadecanoic acid because the parent hydrocarbon is octadecane. A C18 fatty acid with one double bond would be called octadecenoic acid, with two double bonds, octadecadienoic acid and so on. The symbol 18:0 denotes a C18 fatty acid with no double bonds whereas 18:2 signifies that there are two double bonds.

The position of the double bond is represented by a symbol Δ followed by a superscript number indicating between which carbon atoms the double bond is situated. For example, *cis*- Δ9 means that there is a *cis* double bond between carbon atoms 9 and 10 and *trans*- Δ2 means that there is a *trans* double bond between carbon atoms two and three. The cis- and trans- represent the geometric isomers possible with fatty acids. The most abundant fatty acids are shown in table 3.1.

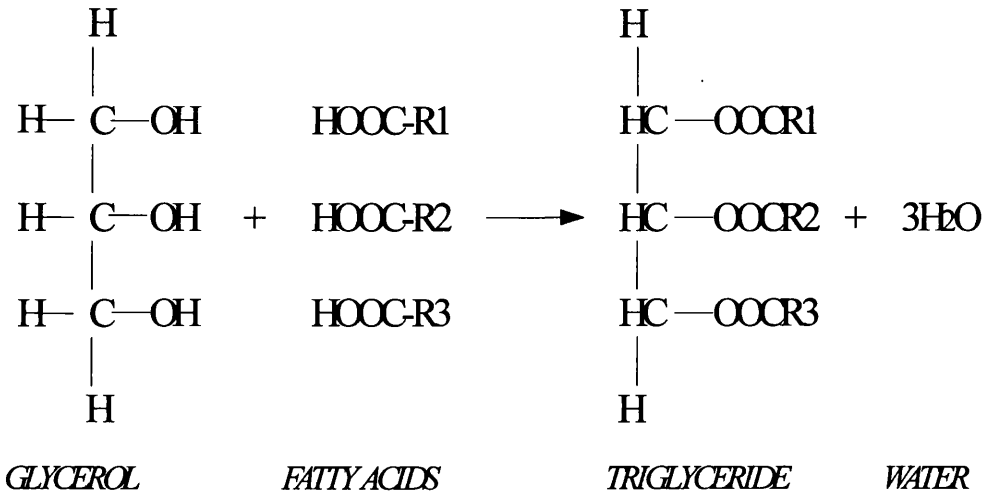


FIGURE 3.1 Triglyceride synthesis

The ability to utilise fatty acids and oils, which contain these fatty acids, is found widely amongst bacteria, yeasts and moulds. In order for the organism to utilise the oils and fats as an energy source, the ester linkage between the glycerol backbone and the fatty acids must be hydrolysed. This is achieved by the production of a lipase which may be intracellular or extracellular and yields free fatty acids (three moles) and glycerol (one mole). The glycerol is then utilised by the Embden-Meyerhof pathway where it is phosphorylated and oxidised to dihydroxyacetone. This in turn is isomerised to glyceraldehyde-3-phosphate. This is then used as a glycolytic intermediate later in the oxidation cycle.

Many organisms can utilise free fatty acids but it is not known whether the acids are taken into the cell or manufactured at the cell. However, it is known that these acids are extremely toxic to the cell due to their surfactant properties and hence the metabolism of fatty acids needs to be efficient and must be converted immediately into their coenzyme A thioesters. The sequence for fatty acid degradation, known as the β-oxidation cycle, continues until the substrate for the final reaction is the C2 compound, acetyl-CoA which is then converted to pyruvate and is used in the Tricarboxycylic Acid Cycle (TCA).

TABLE 3.1 Some common fatty acids

Systematic name	Trivial name	Designation
butanoic	butyric	4:0
dodecanoic	lauric	12:0
hexadecanoic	palmitic	16:0
octadecanoic	stearic	18:0
cis-9-octadecanoic	oleic	18:1(n-9)
9,12-octadecadienoic	linoleic	18:2(n-6)
9,12,15-octadecatrienoic	linolenic	18:3(n-3)

Chapter 3.1 The use of oils in industrial fermentation

Oils were first used in antibiotic processes as antifoam agents due to the fact that it was possible for the organism(s) to metabolise the oil which meant that the downstream processing of the product would not be hindered by the presence of residual and unnecessary chemicals. This would have an economic benefit since the overall cost of downstream processing could be reduced. Therefore, this is an important consideration when utilising oils. However, it soon became clear that oils could provide a readily available, consistent and economic source of energy for the growth and maintenance of the organism and were hence employed as co-substrates for use with other carbohydrates, such as sugars, in a wide variety of fermentations (Pan *et al.*, 1959 and Stowell, 1987).

However, oils are now considered to be essential components of industrial fermentation media. Due to their high viscosities and the hydrophilic nature of natural oils provided by three ester linkages, there is limited spreading ability which limit their efficiency as antifoam agents. Hence, they tend to be used where their antifoam characteristics are secondary to their value as a nutrient source or where they serve as a carrier for a more effective antifoam.

The relative advantages of using oils as co-substrates as compared to other carbohydrates has been extensively reviewed by Bader *et al.*, (1984). They found that theoretically it was more energetically favourable to use an oil since the oxidation of a typical oil produces more than twice the amount of energy per kilogram than the oxidation of a simple sugar such as glucose (figure 3.2.). Therefore, on an energy basis and taking into account the relative cost of the substrates concerned, initially it would appear more economical to use oil as the main carbon source for industrial fermentations. However, oil is less readily oxidised than simple sugars and hence for a given energy input there is a higher oxygen demand of approximately 7% thus increasing running costs of the fermentation in terms of time.

Glucose oxidation

$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \rightarrow$ 3722Kcals energy/Kg

Oil oxidation

$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \rightarrow$ 8800Kcals energy/Kg

FIGURE 3.2 Comparison of oxidation of 1kg of glucose & 1kg of a typical oil

The use of oil has other advantages. For example, less of the substrate is required on a volume basis when compared to a sugar substrate in order to supply the amount of energy required during fermentation. It requires approximately 1.24 l of soybean oil to supply 10 Kcals of energy whereas it would require more than 5 l of glucose or sucrose to add the same amount of energy based on a 50% w/w solution. This again implies that it is more economical to use oil as a carbon source. A further advantage of using an oil is that it is cheaper in price relative to other carbohydrates such as the refined sugars.

There are disadvantages to using oils, the most significant of these is the presence of residual oil at the end of the fermentation process. This may be due either to a physical limitation of oil mass transfer in the fermenter or to an inability of the organism to metabolise certain components of the input material. The former is supported by the fact that the composition of the residual oil in the fermenter has been shown to be similar to that of the original input material in a number of studies (Stowell, 1987). A high level of residual oil is considered undesirable since it increases the viscosity of the fermentation broth which in turn decreases the rate oxygen transfer in the fermenter. It will also hinder the further process of the fermentation broth.

However, the presence of the oil may have a negligible viscosity effect relative to a non-Newtonian fermentation broth plus the situation can be improved by the addition of surfactants to improve the stability of the oil/aqueous emulsions. This in turn may affect the processing of the product further downstream since the residual oil and the surfactant must be removed further increasing the cost of the fermentation. For example, assuming that a typical fermentation delivers 100 tonnes of broth with a residual oil level of 2%, a plant running 100 fermentations per annum and using an oil at £400 per tonne, would put to waste oil with a value of £80,000 which could be minimised (Stowell, 1987). Therefore, although the relative advantages of using an oil in economic terms can be seen, other technical considerations must be taken into account when deciding on the final choice of material for a given process.

Chapter 3.1.1 Oils as antifoam agents

In the bioprocess industries foaming presents many problems particularly when the system has to be adequately agitated and aerated. Foaming is caused by the denaturation of proteins in the medium at the air-broth interface forming a skin which does not readily rupture. The foaming ability and type of foam produced as well as the stability and pattern of foam formation, are influenced by the initial medium components; the products of the process and the operating conditions of the bioreactor. If foaming is uncontrolled, it can lead to productivity losses. This is due to a reduction in working volume of the bioreactor; product and biocatalyst loss; lysis; enhanced gas hold up; decreased power dissipation and lower heat- and mass-transfer rates. This may also

cause contamination of the air exit-filters resulting in siphoning of the bioreactor contents (Vardar-Sukan, 1988). For these reasons antifoams are added which are surface active agents and hence reduce the surface tension in the foam resulting in its breakdown and dispersal.

Many types of antifoam are available commercially including alcohols, fatty acids and fatty acid esters, amines, amides, ethers, phosphate esters, silicones and both natural and mineral oils/fats. Natural oils and fats constitute the earliest type of antifoam and are still widely used, although their variable price and performance; their specificity of action and the availability of more efficient materials has resulted in a gradual decline in their use.

Studies have shown that the effectiveness of a natural oil in foam suppression varies greatly with the type and components of the medium since the physicochemical properties of the medium affect the surface phenomena in foam formation (Vardar-Sukan, 1988). For example, sugar-beet cosette, a cellulosic agro-industrial by-product, is known to contain an emulsifying agent, saponin, which enhances the dispersion of natural oil in aqueous solution increasing its efficiency (Sukan *et al.*, 1984).

Antifoams based on natural oils and fats, therefore, tend to be relatively inefficient and require addition of large amounts of antifoam to be effective. Oils may be metabolised as a carbon source, sometimes preferentially by the organism, which helps to explain their relative ineffectiveness. This can have both beneficial and detrimental effects on the growth of the organism. By contrast, employing a non-metabolisable antifoam may cause favourable changes in growth, pH pattern, rate of substrate consumption and an increased yield. For example, the addition of a silicone antifoam to a monomycin antibiotic fermentation delayed the onset of antibiotic production resulting a slightly improved yield (Hall *et al.*, 1973).

Another factor associated with antifoam additions, which causes concern, is their possible effect on oxygen transfer. Several studies have been undertaken with respect to the influence of antifoam on oxygen transfer in fermentation systems (Phillips *et al.*, 1961 and Deindoefer *et al.*, 1955). However, there are still many unsolved problems in the theory and methodology of oxygen transfer in fermentations in the presence of oil

antifoams (chapter 7.5). It has been reported that antifoams, including oil based antifoams, reduce the oxygen transfer rate in fermentation broth. This attributed to three factors. Firstly, the addition of antifoam causes the formation of larger bubbles resulting in a reduced interfacial area. Secondly, the addition of antifoam results in a reduction of contact time of the bubbles with the broth and finally, there is an increased gas-liquid interfacial resistance following the addition of a surface active material.

Although any oil, which is cheap and locally available, has probably been used at some time as an antifoam, the most common oils used are ground-nut oil, soybean oil, olive oil, rape seed oil and lard oil. They may be the used as the antifoam or as a carrier for another additive. However, the anphiphatic nature of these oils provided by the three ester linkages, is insufficient to confer good spreading ability. This factor, plus their high viscosities, limits the efficiency of the oils as antifoam agents and hence, they tend to be more often used where their antifoam characteristics are secondary to their value as a nutrient source or where they serve as a carrier for a more efficient antifoam.

Chapter 3.2 Types of oils used in industrial processes

A wide variety of different oils are used commercially and the most common oils are given in table 3.2. The bulk of the oils used are plant derived although some animal fats are favoured such as the edible animal fats, lard and tallow because they tend to metabolised at a faster rate due to preferential utilisation of unsaturated fatty acids. This is thought to be due to either better dispersion of the fat or due to some metabolic preference for one or more of the fatty acids which make up the oil (Stowell, 1987).

From the information illustrated in table 3.2, it becomes apparent that the majority of oils produced are derived from plants such as the vegetable oils. The most widely produced oil is soybean oil which is considered the major edible oil. Problems arise when using this oil due to its high linoleic acid content of approximately 5-18% which causes poor flavour stability and generation of unpleasant odours. Olive oil and corn oil are probably two of the most important edible oils produced.

TABLE 3.2 **The most common oils used commercially[a]**

Oil	Production tonnes (@1000)	% of total production
Soybean oil	13,154	22.6
Palm oil	5414	9.3
Rape seed oil	4115	7.1
Lard	3804	6.5
Olive oil	1249	2.1
Corn oil	525	0.9

[a] values taken from 1993/94 information

Chapter 3.2.1 Rape seed oil

The oil of prime importance for the purpose of this study is rape seed oil which accounts for approximately 10% of total oil production world wide. Rape seed oil, (RSO), also known as colza and canbra oil, is obtained from the seeds of the plants *Brassica napus,* (rape), and *Brassica campestris,* (turnip rape), and its composition is dependent on the plant and climate.

There are three types of RSO produced which vary in their fatty acid composition, (table 3.3), but are referred to as erucic acid oils. Each type of RSO is used for a different purpose such as edible or industrial uses. Traditionally, high erucic acid rape seed, (H.E.A.R.), which has an erucic acid content of approximately 25 - 55%, has been used exclusively in the oleochemical industry where a erucic content of greater than 50% is desirable.

However, low erucic acid rape seed, (L.E.A.R.), which has an erucic acid content of between 0 - 5% as a percentage of total fatty acids, is used entirely for edible purposes. The use of RSO with more than 5% erucic acid content in the manufacture of products for human usage is prohibited. There is one other type of RSO which contains zero erucic acid. This oil strictly no longer belongs to the erucic acid group of oils but to the linoleic group of oils and is characterised by its high linoleic and linolenic acid content of 35% and 3% respectively.

As well as the fatty acid content, other components vary with the type of rape seed oil used, such as the type of triglycerides present. In high erucic acid rape seed the triglycerides consist of 54% monooleodierucins, 28% dioleodierucins and 18% monosaturated monooleodierucins whilst low erucic acid rape seed triglycerides are made up of 20% tri-C18-unsaturated fatty acids, 15% monoeicoseno-di-C18-unsaturated fatty acids, and 35% monoenico-di-C18-unsaturated fatty acids.

TABLE 3.3 Composition and fatty acid distribution in various types of RSO

Species/Type	Range of Fatty Acids (WT %)			
	Oleic	**Linoleic**	**Linolenic**	**Erucic**
B.campestris				
Summer turnip rape[a]	17-34	14-18	9-11	24-40
Summer turnip rape[b]	48-55	27-31	10-14	0
B.juncea	7-22	12-24	10-15	18-49
B.napus				
Winter rape[a]	8-14	11-15	6-11	45-54
Winter rape[b]	40-48	15-25	10-15	18-49

[a] classical lines &[b] low erucic acid lines

The important component with respect to this study is the fatty acid content as certain types of fatty acid, such as linoleic and oleic acids, enhance antibiotic production. The type of oil used in this study is of low erucic acid content and high oleic acid, (50%), and linoleic acid, (30%), content which it is anticipated will be beneficial to the fermentation process with respect to antibiotic production.

Chapter 4 The effect of oils on antibiotic production

As previously mentioned, many industrial fermentations use mixed substrates such as complex sugars or oils as the main source of carbon. Oils are preferred because they tend to be cheaper than other more refined sugars and as recent studies have indicated, the use of oil substrates may lead to higher antibiotic titres. The microbial hydrolysis of oils leads to a mixture of fatty acids and glycerol. Early studies have reported either the stimulation or inhibition of microbial growth and/or product formation. For example, antibiotic, citric acid, glutamic acid, ethanol and fumaric acid fermentations in the presence of low concentrations of fatty acids or other surfactants (Jin *et al.*, 1981 and Tan & Gill, 1987).

Some of the earliest work with respect to the use of oils in fermentation was carried out in the 1940s and 50s. One of the first reports was made by Jarvis & Johnson, (1947) who found that under a given set of conditions, the major factor affecting the rate of fermentation was the nature of the carbohydrate used in the medium. They found that glucose was metabolised at a rapid rate but lactose was metabolised much slower in the initial stages of growth. The addition of oil as an antifoam increased the rate of growth of *Penicillium chrysogenum.*

Further studies by Ishida & Isono, (1952) found that the addition of either soybean oil or castor oil stimulated the production of penicillin when the oil was added to young cultures but when added to older cultures caused the lysis of the cells and a decrease in the antibiotic titre. A similar effect was observed by Goldschmidt *et al.*, (1950) and Yasuda *et al.*, (1952). They concluded that unsaturated fatty acids such as oleic, linolenic and linoleic acids were causing the increased antibiotic titre which could not be metabolised by the older more slow growing cells. Hence, the concentration of fatty acids increased to a toxic level resulting in the lysis of the cells.

More recent studies by Moller *et al.*, (1992) noted that penicillin V production by *Penicillium chrysogenum* was affected by the substrate used. A complex medium containing lard oil and lactose resulted in increased cell growth and product formation rate when compared to a complex medium containing lactose only. However, the use of the lactose only medium resulted in an increased amount of β-lactam compounds due to

the longer production time but there was a greater percentage decomposition of the products. Therefore, the final product concentration for both media was comparable and thus substitution of either substrate is a balance of the relative costs of the respective substrate and production time.

Many explanations for the observed increase in antibiotic titre have been proposed. Stefaniak *et al.,* (1946) suggested that the improved antibiotic titres were brought about by the oils acting as antifoam agents thus, a more abundant supply of oxygen was available to the cell after the removal of the foam. This however, would not account for the dramatic increases since if the increase in aeration actually was the sole cause, the addition of any non-toxic agent capable of breaking down foam should result in a similar increase. It was suggested that when certain oils were used as antifoams in fermentation, it protected the antibiotic formed from being inactivated in some way during the growth of the organism.

Further investigation showed that emulsification of the oils by a surfactant would increase the efficiency of oil as an antifoam but the presence of a low concentration of these surfactants could either stimulate or inhibit microbial growth and/or product formation. For example Sukan *et al.,* (1989) investigated the effects of different oils on growth and cellulase activity/production in *Trichioderma reesei* and *Sporotrichum pulverulentum* fermentations. It was observed that emulsification resulted in a higher cellulase activity in both cultures but there were variations in the enzyme levels depending on the presence or absence of Tween 80 and the type of oil used in the culture medium.

It was also demonstrated that saponin, another surfactant, had an inhibitory effect on both the enzyme activity/production and on the growth of the organism. At high levels the saponin interacted with the enzyme protein resulting in a decrease in enzyme activity whilst at low levels it interacted with the surfactant molecule resulting in inefficient emulsification of the oil and hence a decrease in growth of the organism. This suggests that there may be an actual physical interaction between enzymes in a secondary metabolite synthesis pathway resulting in a reduced antibiotic titre.

Earlier studies have suggested that the fatty acids produced by the hydrolysis of the oils affect antibiotic production (Goldberg *et al.*, 1985). It was proposed that since oils are metabolisable, the resultant fatty acids, such as linolenic, linoleic and oleic acids, would cause a drift towards acidic pH during the course of the fermentation altering the cell membrane permeability thus affecting diffusion of cellular components as well as the required product.

More recent work has also suggested that fatty acids are the contributing factor with respect to increased antibiotic production during fermentation. Paul *et al.*, (1997) found that the use of sesame oil increased both growth of *Cephalosporium acremonium* and antibiotic production when added during a cephalosporin C fermentation. Further studies to monitor the cellular fatty acid components found that oleic acid and linoleic acid, the fatty acid components of sesame oil, were present at all stages of growth. From these findings it was concluded that it was the fatty acids which enhanced antibiotic production. This supports previous work carried out by Paul *et al.*, (1994) which found that mustard oil, not rich in oleic and linoleic acid did not enhance antibiotic production. However, Park *et al.*, (1994) demonstrated that oleic, linoleic and linolenic acids present in soy bean oil had an inhibitory effect on cephamycin C.

The effect of the respective fatty acids appears to be dependent on the organism, the strain of the organism and/or antibiotic produced (Tan & Ho, 1991). It is also affected by the time at which the fatty acids are added to the fermentation (Khaoua *et al.*, 1992; Lee & Chou, 1994 and Lin *et al.*, 1996). Lee & Ho, (1996) demonstrated that olein and stearin fractions from palm oil stimulated growth and clavulanic acid production during *Streptomyces clavuligerus* fermentation whilst oleic and lauric acids were not utilised. Similar results were reported by Lee *et al.*, (1997). They found that palmitic acid had a beneficial effect on both growth of *Streptomyces fradiae* and tylosin production but oleic and lauric acid were not suitable for either growth or antibiotic production. These effects were only observed when the fatty acids or oils were added to the culture during the early stages of fermentation. Addition in the later stages of fermentation actually had a detrimental effect.

It has been proposed that the addition of fatty acids increases antibiotic titre by influencing the antibiotic synthetic pathway. For example, Huber *et al.*, (1985) suggested that the biosynthesis of factors in an antibiotic complex could be controlled by the addition of the appropriate fatty acid precursors whilst by changing fatty acid composition, they could be more readily assimilated (Gesheva *et al.*, 1997). Other reports have suggested that fatty acids may actually induce the synthesis of activation enzymes, in the biosynthetic pathway, at the time antibiotic is produced and therefore increase antibiotic titre (Laakel *et al.*, 1994).

From these studies it becomes apparent that oils play an important role in industrial fermentations due to their ability to increase antibiotic titre and enhance the growth of the organism although the mechanism by which this is achieved remains unclear. It may be due to the complex nature of the substrates resulting in slower growth thus removing catabolite repression associated with the use of a rapidly assimilated carbon source such as glycerol. It could be due to the fatty acids interacting with antibiotic synthesis as discussed. Further studies are required before a definitive reason can be given and even then this could well be fermentation specific.

Chapter 5 Lipases

In order to utilise oil, *Streptomyces clavuligerus* will have to produce a lipase. Lipase, or glycerol ester hydrolase, can be defined as an enzyme which catalyses the hydrolysis of lipids to their constituent fatty acids and glycerol as illustrated in figure 5.1. However, this hydrolysis occurs at varying rates depending on the specificity of the lipase involved.

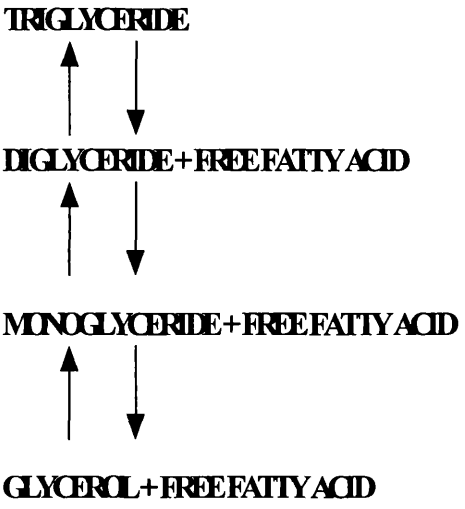


FIGURE 5.1 The lipase reaction

The natural substrates of lipase are triglycerides of long chain fatty acids which are insoluble in water. Therefore, a lipase can be characterised by its ability to rapidly catalyse the hydrolysis of ester bonds at the interface between the insoluble substrate phase i.e. the triglyceride and the aqueous phase in which the enzyme is soluble. Lipases are distinguished from other esterases by this ability to catalyse the hydrolysis of insoluble long-chain fatty acid esters although glycerides are their preferred substrate.

Lipases will only catalyse the hydrolysis of the substrates when they are present in the form of micelles, small aggregates or small particles since lipases require an interface in order to function, which is provided by the aggregated substrates. The interface is crucial in the reaction mechanism as lipolytic activity occurs only if the lipase is adsorbed at an interface. This would then imply that the interfacial area available for interaction, is a rate limiting step in the lipase reaction. The kinetics of lipolysis are reviewed in chapter 5.1.

Chapter 5.1 Kinetics of lipase activity

There has been much interest in lipolytic enzymes and their control mechanism due to their industrial importance. The chemical modification of lipids such as hydrolysis, esterification and inesterification reactions, all involve the use of lipases and the costs of producing the lipases necessary to catalyse the aforementioned amounts are often prohibitive. Hence, knowledge of the catalytic activity of lipases is important in order to improve process economics. The lipase is unique in that it has a requirement for an insoluble substrate namely the oil/lipid which results in the formation of an insoluble lipid-aqueous interface. It would appear that the lipase requires an interface in order to be activated and the water content of the reaction mixture controls the relative rates of reaction.

The kinetic activity of lipases has been extensively studied and their catalytic activity is affected by both the requirement for an interface and the structural properties of the lipase i.e. the proteinaceous nature of the enzyme. It should be noted that the physicochemical properties of lipases and the specificity characteristics also influence lipolytic activity. Various techniques have been employed in order to achieve these kinetic studies and involve the use of oils/lipids supplied in various forms such as monolayers, micelles and in an emulsified state.

Chapter 5.1.1 Activation of lipolytic enzymes by interfaces

Lipases are enzymes which are characterised by their need for an insoluble substrate and hence require an interface. Lipases can act on soluble monomeric substrates but practical utilisation of lipase-catalysed reactions are restricted to situations where the overall substrate concentration is higher than its solubility in the natural solvent, water. This characteristic provides a convenient criterion for differentiating lipases from conventional esterases which usually act on only soluble monomeric substrates.

This feature was first observed by Schϕnheyder & Volqvartz, (1945) and later by Sarda & Desnuelle, (1958). It was demonstrated that in contrast to esterase, which exhibits a normal Michaelis-Menten activity dependence on substrate concentration, pancreatic

lipase displayed no activity when a triacaproin substrate was in the monomeric state. However, when the solubility limit of the substrate was exceeded, there was an increased enzyme activity with the substrate in the emulsified state. The esterase appears to only be active on molecularly dispersed molecules whilst the lipase was capable of hydrolysing the substrate in an insoluble form. The observed rates of lipase-catalysed reactions were strongly influenced by the interfacial area available within the system (Desnuelle, 1961).

Many mechanisms have been proposed to explain this characteristic at either substrate and/or enzyme level but the actual pathway has yet to be elucidated. Theoretical interpretations of the activation of lipases by interfaces have also been attempted by a number of authors. These can be divided into two groups: (i) those which assume that the substrates are activated by the presence of an oil/water interface, and (ii) those which assume that the lipase undergoes a change to an activated form upon contact with the oil/water interface (Verger & Haas, 1976).

It has been proposed that interfacial activation may be due to an increased concentration of substrate molecules in the vicinity of the interface which would cause a more ordered structuring of the lipid molecules resulting in more frequent enzyme-substrate collisions (Brockman *et al.,* 1973). This would appear unlikely since esterases, which have a similar mode of action to lipases, behave differently. It has also been suggested that substrate aggregation and orientation into more suitable conformations for chemical reactions may influence enzyme catalysis. Substrate aggregation will cause a loss in rotational and translational energy resulting in a lower activation energy for lipolysis (Shah & Schulman, 1967; Mattson & Volpenhein, 1969; Brockerhoff, 1970 and Wells, 1974). Formation of substrate aggregates may also have a hydration effect on the lipid molecules which has been suggested may be required for lipase activation (Brockerhoff 1968).

The second theory involves the existence of separate adsorption and catalytic sites for the lipase such that the lipase can only become catalytically active after binding to the interface. The location and binding site has become known as the "Interface Recognition Site" and the active site as the "Classical Active Site". Associated with this is a conformational change to the lipase as it approaches the oil/water interface which aids

the activating effect of substrate aggregation due to a higher degree of order in this region (Desnuelle *et al.*, 1960; James & Augenstein, 1966 and Dawson, 1969). It has been suggested that the orientation of the enzyme is crucial since the lipase must orientate itself such that its active site is correctly located.

Orientation of the lipase has been considered of key importance with respect to both of the aforementioned theories. Studies by Mattson *et al.*, (1970) suggested that orientation of the lipase molecule at the oil/water interface and the specificity of the enzyme for its substrate were the main factors determining the rate of hydrolysis. They also demonstrated that the oil/water interface may adsorb other proteins increasing competition for lipase adsorption thus influencing the rate of hydrolysis.

Brockerhoff (1969) also investigated orientation and the specific adsorption of the lipase at the interface. It was proposed that the enzyme unfolds on adsorption which confers specificity by inducing conformational changes. It was also suggested that the Km, the Michaelis constant, may be the dissociation constant of the enzyme-interface complex and therefore, oil/water interfaces would behave as unspecific surfaces and adsorb proteins of the same affinity.

These differing results are probably justified since both the previous authors were using saturating interface concentrations where all the enzyme molecules are adsorbed onto the interface and hence, studied the affinity of the enzyme for its specific substrate in the interface. Therefore, the suggestion that orientation of the substrate molecules at the interface is important is probably valid.

At the oil/water interface, the lipid molecules will orientate in such a manner as to give rise to an ordered packing in which the polar heads are exposed to the water phase and the hydrocarbon tails are exposed to the organic phase. However, when lipid molecules are in true solution they orientate themselves randomly because they are completely surrounded by water molecules. This ordered continuum can also be achieved on the hydrophobic surface of a solid carrier. Rucka & Turkiewicz, (1990) concluded that only the immediate vicinity of the lipase should possess a hydrophobic character since the overall conformation of the enzyme and the local conformation of the active site depend

primarily on the intramolecular forces and the short range interaction of the protein with the solvent.

Similarly, Malcata *et al.*, (1990) proposed that the true oil/water interface is not likely to be in direct contact with the immobilised lipase for this reason and the existence of lipolytic activity requires a continuous ordered hydrophobic microenvironment. The surfaces of contact of two phases provide a continuum irrespective of whether these two phases are liquid phases e.g. oil and water or a liquid and a solid phase e.g. oil and an immobilised lipase. This fact seems to contradict the generally accepted theory that an oil/water interface is required for lipases to exhibit catalytic activity.

Chapter 5.1.2 Effect of surfactants on lipolytic activity

The action of lipases is complex in that each lipase can bind and catalyse each type of lipid molecule very differently as a function of the overall quality of the interface and independently of variable associations with the interface. Therefore, the kinetics of vary from system to system and for this reason several techniques have been employed in order to study this kinetic system. These methods involve the use of oils/fats supplied in various forms such as monolayers, micelles and in an emulsified state. Verger, (1980) gives a comprehensive review of these techniques.

Emulsified substrates have been commonly used and most closely resemble the situation in the fermenter environment where the oil is dispersed, by agitation, into an aqueous solution. However, the adsorption of the lipase and the level of activity present are dependent on the quality and quantity of the emulsion with respect to the degree of dispersion of the substrate and the emulsion droplet size (Benzonana & Desnuelle, 1965 and Mattson & Volpenhein, 1966). Therefore, these emulsified substrates have been adapted in order to improve the accuracy of these studies and this has resulted in the use of micellar substrates. However, inaccuracies can occur due to the enzyme molecules ability to penetrate the interface since the natural substrates for many lipases vary in chain length to those present in the micelles.

In order to study the kinetics of lipolysis in this way, surfactants and dispersion stabilising agents are added to the system which can affect the reaction of lipase in complex ways (Gargouri *et al.*, 1983 and Kawase *et al.*, 1985). During early studies, detergents were used as stabilising agents and were found to act as competitive inhibitors or substrate dilutors within the system (Kaplan & Teng, 1971 and Verger *et al.*, 1976). Later studies have demonstrated that surfactants may also act as non-competitive inhibitors dependent on the system used (Brown *et al.*, 1993; Marangoni *et al.*, 1993 and Tsai *et al.*, 1995). Therefore, it has been suggested replacing the detergents or removing them completely but this creates problems in that it involves the use of short-chain triglycerides which results in aggregate dispersion problems. This in turn will result in the slow hydrolysis of the lipid due to reduced aggregate formation and hence a lack of interfacial area (Entressangles *et al.*, 1968).

Lipase activity occurs at the oil/water interface and therefore a change the surface properties of the substrate by adsorption of surfactants will affect lipase activity. This may be due to shielding of the interface or, at higher concentrations of surfactant, obstructing the access between oil and water domains which indicates competitive inhibition of the enzyme (Kawase *et al.*, 1985). However, the effect on lipase activity will generally depend on the type of surfactant used. With the use of an anionic surfactant, such as sodium bis (2-ethyl hexyl) sulphosuccinate, lipase activity can be enhanced when compared to non ionic surfactants due to the accessibility of the lipid molecules to the enzyme (Skagerlind & Holmberg, 1994).

Surfactants and dispersion stabilising agents used in emulsion preparation can have a beneficial effect on lipase activity. Koshy *et al.*, (1988) found that the addition of surfactant resulted in increased lipase activity due to increased interfacial area. Reduction in the interfacial tension and generation of interfacial tension gradient across the droplet adding additional stress results in smaller droplets and therefore an increased interfacial area. Similar results were observed by Large *et al.*, (1999b) who found that the addition of SDS to *Streptomyces clavuligerus* fermentation resulted in higher levels lipase activity and oil utilisation due to increased oil dispersion and reduced oil droplet size. The study of lipolytic kinetics is complex since none of the methods employed are accurate in all situations for a number of reasons including the addition of surfactants and external factors such as surface pressure, pH, temperature and the type of substrate.

Chapter 6 Microbial lipases

To utilise oil *Streptomyces clavuligerus* produces a lipase. Several lipases have been isolated from a wide variety of sources, including plants, animals and micro-organisms although relatively few of them have been studied thoroughly. The isolation and purification techniques used for lipases are based on a multistep series of non-specific techniques such as ammonium sulphate precipitation; gel filtration and ion exchange chromatography and more recently affinity chromatography.

To date the lipases isolated have been acidic proteins of molecular weight ranging from 20,000-60,000 Daltons with specific activities varying from 500 -10,000 lipase units per milligram, (mg), of protein. The majority of the lipases are glycoproteins containing 2-15 % carbohydrate with the major glycoside residue of the enzymes being mannose. The carbohydrate portion of the glycoproteins is not required for catalytic expression but its true role remains unknown. It has also been suggested that lipases contain a high proportion of hydrophobic amino acid residues which are necessary for interaction with their hydrophobic substrates (Tomizuka *et al.*, 1966).

Microbial lipases have been extensively studied and the literature contains much information on the lipolytic activity of a wide range of micro-organisms. This is thought to be due to their stability and application in medicine and industry. Initial studies centred on the dairy and other food industries where lipases are produced in situ by the micro-organisms making the products more palatable. Lipolytic activity has been observed in micro-organisms which take part in cheese production such as *Streptococcus lactis* and *Penicillium roqueforti* as well as in micro-organisms used in the dairy industry such as *Lactobacillus* species (Stead, 1983).

The industrial demand for the highly active preparations of lipolytic enzyme continues to stimulate the search for new enzyme sources. Microbial lipases possess diverse enzymatic properties and substrate specificities which make them attractive for commercial applications. A large number of lipases have been screened for application as food additives (flavour-modifying enzymes); industrial reagents; detergent additives as well as medicine such as digestive enzymes and diagnostics. Industrial lipases have been extensively reviewed by Macrae, (1983) and Macrae & Hammond, (1985).

One of the first observations of lipolytic activity was made by Alford *et al.,* (1964) who studied the activity of microbial lipases on natural fats and triglycerides. He noted that the lipases demonstrated some form of substrate specificity. From his observations he proposed a classification scheme for lipases based on their specificity and divided the lipases into three categories. The first two groups he classified on the basis of their positional specificity whilst the third group was classified on the basis of its fatty acid specificity. This classification scheme is still used but has been extensively revised.

The first category is referred to as non-specific lipases. These lipases hydrolyse the triglyceride releasing fatty acids from all three positions of the glycerol moiety. This results in the complete breakdown of the triglyceride to free fatty acids and glycerol. The second group of enzymes will hydrolyse the triglyceride but releasing only fatty acids the outer 1 and 3 positions of the glycerol moiety such as the lipases from *Aspergillus niger* and *Rhizopus delemar* (Okumura *et al.,* 1976) and *Pseudomonas aeurignosa* (Gilbert *et al.*, 1991). This type of hydrolysis results in fatty acids plus the incomplete breakdown products, 1,2(2,3)-diglyceride and 2-monoglyceride. However, prolonged incubation will allow complete breakdown of the triglyceride due to chemical instability and acyl migration.

The final group of lipases identified were the so called fatty acid specific lipases which were observed to be the most difficult to identify. This type of lipase was first reported by Ota *et al.,* (1972) when moderate differences between the rates of hydrolysis of various saturated and unsaturated fatty acid esters were observed with *Candida paralipolytica* which demonstrated a preference for short chain fatty acids. Similar observations have since been reported with *Penicillium cyclopium* (Iwai *et al.,* 1975) and *Brochothrix thermosphacta* and *Lactobacillus curvatus* (Papon & Talon, 1989).

The specificity of the enzyme is controlled by a variety of factors such as the molecular properties of the enzyme; the structure of the substrate, which determines the identity of the digestion products used to identify the type of specificity, and the factors affecting the binding of the enzyme to the substrate or otherwise influencing the activity. Jensen *et al.,* (1983) gives a comprehensive review of the different types of specificity now recognised.

The fatty acid specificity of lipases has been widely studied (Alford & Pierce, 1961; Shimada *et al.*, 1992 and Obradors *et al.*, 1993). The nature of the fatty acyl group will affect the structure and physico-chemical properties of triacylglycerols (Brockerhoff & Jensen, 1974). The degree of unsaturation, the position of double bonds and their *cis*- or *trans*- conformation will affect the ability of the triacylglycerols to be emulsified and hence will be a determining factor in the rate of lipolytic activity since lipase catalysed reactions are strongly influenced by the interface (Malcata *et al.*, 1992).

It has been observed that lipases will preferentially hydrolyse triacylglycerols as opposed to aliphatic esters (Boutur *et al.*, 1995). Generally, esters of cis Δ9 unsaturated fatty acids with 16 or 18 carbon atoms are hydrolysed at the highest rates (Jensen, 1974 and Baillargeon *et al.*, 1991). For the same number of carbon atoms and degree of unsaturation, the presence of a Δ6 or Δ11 unsaturation has been shown to be unfavourable compared Δ9 with the preferred substrates being Δ9 unsaturated palmitoleic, oleic, linoleic and linolenic acids (Macrae, 1983; Sonnet *et al.*, 1993 and Boutur *et al.*, 1995). However, this may be dependent on the growth conditions employed (Jacobsen *et al.*, 1990).

Chapter 6.1 Factors affecting lipase activity

Microbial lipases can be identified and separated on the basis of their physical properties which vary considerably from one enzyme to another, such as thermal stability, temperature and pH optima. The location of the enzyme is also important, for example whether it is intracellular or secreted as an extracellular lipase or whether it cell-associated or cell-bound (Ota *et al.*, 1968; Gomi *et al.*, 1984; Jacobsen *et al.*, 1989 and Janssen *et al.*, 1994). The properties of the lipases tend to vary with the type of organism. Bacteria usually have pH optima in the alkaline region whilst fungal and yeast lipases tend to exhibit pH optima in the acid region and have a higher thermal stability than many of the bacterial lipases isolated (Suguira *et al.*, 1974).

Thermostability of lipases is a major concern in industry especially with respect to thermostable lipases produced by psychrotropic bacteria during the storage of heat processed commercially available sterile foods. The most commonly isolated bacteria is

Pseudomonas fluorescens, found in milk and milk derived products, which produces an extracellular, heat stable lipase. The lipase is active at alkaline pH. The lipase has a detrimental effect on the flavour of the products concerned (Adams *et al.*, 1981 and Iizumi *et al.,* 1990). Generally, lipases may be stable up to 65°C although this may not necessarily be the optimum for activity (Sugihara *et al.,* 1991).

As previously mentioned both pH and temperature are crucial for optimum lipase activity. The optimal ranges for each can vary considerably between organisms as well as species (Okumura *et al.,* 1976; Isobe *et al.,* 1988 and Phillips *et al.*, 1991). For example, the two lipases produced by *Geotrichum candidum* have different pH optima dependent on the isoform although the optimum temperature was the same for both (Heidrich *et al.*, 1991).

The majority of lipases isolated have been extracellular and are excreted through the external membrane of the cell into the culture medium. The culture environment can be optimised for lipase production by variation of the growth conditions which will affect the properties of the enzyme producer. This will also influence the ratio of extracellular to intracellular lipases produced. The actual amounts of the lipase produced is dependent on a variety of external factors such as temperature, pH, nitrogen composition, carbon and lipid sources, concentration of inorganic salts and the availability of oxygen. Therefore, the composition of the fermentation medium is of key importance with respect to lipase production (Rivera-Munoz *et al.,* 1991).

It has been demonstrated that lipase production is stimulated by lipids such as butter oil, lard oil, olive oil (Omar *et al.,* 1987; Tan & Gill, 1987 and Suzuki *et al.*, 1988) as well as certain fatty acids (Iwai *et al.*, 1973; Suzuki *et al.*, 1988 and Montesiros *et al.*, 1996). It has also been reported that a minimal level of lipid is required for lipase production which will be of great importance in this study where rape seed oil will be used as the main carbon source (Iwai *et al.*, 1973). It should be noted that other carbon sources such as glycerol, will support growth and lipase activity although again, this is species dependent (Petrovic *et al.*, 1990).

The carbon source is also important with respect to not only lipase activity but growth of the organism and secondary metabolite production (chapter 4). Gilbert *et al.*, (1991) showed that the lipase activity of *Ps. aeruginosa* was weakly induced by carbon and/or energy source limitations but was strongly induced by a wide range of fatty acyl esters including triglycerides and Tweens. However, lipase activity was repressed by long chain fatty acids. Further studies have reported that Tweens and other such anionic surfactants enhance lipase activity in a number of different organisms as well as stimulating the production of other enzymes (Reese *et al.*, 1969; Nahas, 1988 and Jacobsen, 1989).

The nitrogen source used in the culture medium as well as the presence of amino acids are important in achieving maximum lipase activity. It has been shown that lipase activity increases as the complexity of the nitrogen source used in the medium decreases, and when supplemented with various compounds such as amino acids arginine, lysine, aspartic acid and glutamic acid, lipase activity increases further (Alford *et al.*, 1963). Both peptone and ammonium sulphate have been used as nitrogen sources and have been shown to affect lipase activity (Narasaki *et al.*, 1968; Petrovic *et al.*, 1990 and Prabhakar & Raju, 1993). The use of either inorganic or organic nitrogen depends on the culture conditions; the source of the nitrogen as well as the organism being grown.

Besides the major medium components there are several co-factors which have been reported to enhance lipase activity and include albumin, lecithin and sodium chloride (Aisaka *et al.*, 1979). It should be noted that the lecithin does not actually increase the net synthesis of the lipase but accelerates the secretion of the enzyme formed into the culture medium. The addition of some salts such as potassium and sodium have been shown to increase lipase activity although at high concentrations have an inhibitory effect (Petrovic *et al.*, 1990). A similar effect has been observed with the addition of metal ions such as iron and magnesium.

The final consideration with respect to achieving the maximum lipase production possible, is the manner in which the organism is grown as well as the medium components are important (Rivera-Munoz *et al.*, 1991). In these studies several filamentous fungi for example, *Penicillium candidum* and *Penicillium camembertii* were

grown on both solid state and submerged culture. It was found that the lipase activity was higher in submerged culture than in solid media but the actual production of lipase occurred earlier in the solid state system and tended to be more stable. Similar observations were made by Maheura (1984).

One important factor to consider in the production of lipase by submerged culture is the effect of shear. Lee *et al.,* (1989) investigated the effect of shear inactivation of lipase produced by *Candida cylindracae*. It was found that the lipase was sensitive to denaturation by increasing shear-rate although the lipase did not appear sensitive to shear-stress. This indicated that the lipolytic activity was influenced by the length of time the enzyme was exposed to the shear forces.

However, Charm & Wong (1973) who conducted shearing experiments with a variety of other enzymes, such as catalase, reported a loss of enzyme activity as a function of shearing time and shearing rate. These findings are supported by Stahmann *et al.*, (1997) who found that by lowering stirrer speeds and removing baffles during *Ashbya gossypii* fermentation, lipase activity increased due to reduced shearing effects. From this and other studies it becomes clear that lipases can be induced but also inhibited by a number of parameters and hence careful manipulation of the environmental conditions is required to achieve optimum lipase activity. This in turn could be related to the growth and morphology of the organism.

Chapter 7 Rheology and mass transfer in filamentous fermentations

Chapter 7.1 The nature of fermentation broths

Streptomyces clavuligerus is filamentous in nature which causes problems when the organism is grown in submerged culture. The organism grows as long, thin branched hyphae which are linked in a three dimensional network creating a highly structured and viscous fermentation broth. This is further influenced by the morphology of the organism such as the geometry of the hyphae, the hyphal flexibility and hyphal-hyphal interactions. This has a significant effect on bulk mixing within the fermenter environment which will further influence the heat and mass transfer within the fluid. The situation is further complicated as the organism grows which not only leads to increased cell mass but also the accumulation of high molecular weight products such as extracellular polysaccharides. In addition the use of an oil substrate will enhance the viscous nature of the broth.

The diversity of fluid characteristics of fermentation broths varies greatly from one organism to another and the effect of operating variables on broth rheology cannot be generalised (Metz *et al.,* 1979). The rheology of fermentation fluids have been defined mathematically (table 7.1), the simplest equation being Newton's Law in which a fluid may be either Newtonian or non-Newtonian. A Newtonian fluid has a constant viscosity throughout a fermentation regardless of any mechanical forces to which the fluid may be subjected for example agitation.

A non-Newtonian fluid has a viscosity which will vary depending on the shear rate to which the fluid is exposed. Mycelial fermentation broths are non-Newtonian and have been found to show pseudoplastic or shear-thinning behaviour (Steel & Maxon, 1966) which means that they exhibit a decreasing viscosity with increasing shear rate. The Power Law model is also frequently used to describe the rheological behaviour of fermentation fluids (Richards, 1961; Taguchi, 1960 and Tuffile & Pinho, 1970) in which the viscosity of the fluid increases with increasing shear rate.

Filamentous cultures may also exhibit Bingham plastic rheology where the fluid develops Newtonian behaviour once a limiting shear stress has been overcome which is termed the yield stress, τo. Once this yield stress has been exceeded a linear relationship between shear stress and shear rate is exhibited. The slope of the line is termed the coefficient of rigidity or plastic viscosity, (η) (Deindorfer & West, 1960 and Solomons & Weston, 1961). However no studies have shown that yield stress actually exists in filamentous fermentations.

Finally, the Casson model for rheological properties, (Casson, 1959), described a type of non-Newtonian fluid which behaved as a pseudoplastic fluid in that the viscosity decreased with increasing shear rate but displayed a well defined yield stress resembling a Bingham plastic fluid. A few biological fluids such as *Penicillium chrysogenum* broth have been found to behave as Casson Body fluids (Roels *et al.*, 1974). The values of both the consistency and the flow behaviour index have been used as indicators of the nature of the filamentous broth although the consistency index is considered a more accurate estimation since the value of the flow behaviour index is affected by operational conditions and geometric design.

TABLE 7.1 Rheological models*

Category	Model	τo	n
Newtonian	$\mu = \tau/\gamma$	0	1
Power Law	$\tau = k(\gamma)n$	0	>1
Pseudoplastic	$\tau = k(\gamma)n$	0	<1
Bingham plastic	$\tau = \tau o + \gamma\eta$	>0	1
Casson Body	$\sqrt{\tau} = \sqrt{\tau o} + Kc\sqrt{\gamma}$	-	-

*where μ = viscosity (the fluids resistance to flow)

τ = shear stress (force applied to the fluid per unit area, N/m^2)

γ= shear rate (velocity gradient to which the fluid is exposed, s^{-1})

k = consistency index (equals apparent viscosity when = 1, N/m^2)

τo = yield stress (limiting shear stress ,N/m^2)

η = flow behaviour index

Kc = Casson viscosity, $(N/m^2s)^{0.5}$

Chapter 7.2 Experimental rheology of culture fluids

The rheological properties of most fermentation broths change appreciably during the course of a fermentation and have been extensively investigated. Initial studies were carried out with *Penicillium chrysogenum* which indicated non-Newtonian behaviour but there was debate as to the type of non-Newtonian fluid. Deindoerfer & West (1960) claimed that *P.chrysogenum* displayed pseudoplastic behaviour in the fermenter contrary to an earlier report which stated that the broth was plastic in behaviour (Deindoerfer & Gaden, 1955). Further work by Richards (1961) reported a correlation between broth rheology changes and changes in yield stresses and broth rigidity. It was concluded that the broth exhibited Bingham Plastic behaviour and that the methods used for the determination of the non-Newtonian nature of broths would account for the varying reports in the organisms' rheology.

However, Roels *et al.,* (1974) also investigated the rheology of fermentation broth in order to understand its effect on the fermentation process and concluded that the rheology of a penicillin broth may be described in terms of Casson's rheological equation. Both the power law and the Bingham plastic model failed to describe the behaviour in a sufficiently large range of shear rates. Further studies have indicated that there is no general relationship between biomass, viscosity and rheology since these factors will vary with the conditions within the fermenter (Ju *et al.*, 1991).

The rheology of *Streptomyces*, which tends to display non-Newtonian behaviour, has been studied at considerable length. Initial studies suggested that *Streptomyces* broth displayed Newtonian behaviour (Deindoerfer & West, 1960). During fermentation using *Streptomyces noursei* the broth displayed good Newtonian behaviour due to the lack of branched morphology associated with fungal and/or filamentous organisms. However, during fermentation using *Streptomyces griseus* the broth showed both plastic and Newtonian behaviour which they concluded was due to the physiological age of a fermentation broth and the growth circumstances. Similar behaviour has been observed during *Streptomyces aureofaciens* fermentation (Tuffile & Pinho, 1970).

The rheological properties of fermentation broths are affected by a number of contributing factors such as the concentration and the morphological state of the organism which in turn is affected by the operating conditions within the fermenter environment. Attempts have been made to correlate the biomass of the culture with the consistency index, k, as well as with η, the flow behaviour index (Blakebrough *et al.*, 1978 and Allen & Robinson, 1990). These factors in turn will be influenced by the viscosity of the fermentation broth and the operating/process conditions.

Morphology plays an important role in determining the rheological properties of fermentation broths and will be influenced by a number of process parameters such as growth, medium composition and mixing time (Olsvik & Kristiansen, 1992). This subject will be comprehensively reviewed in chapter 7.3. For several filamentous fermentations, it has been shown that 80-90% of the mycelium exists as aggregates although appearing as fully filamentous by visual examination (Packer & Thomas, 1990 and Tucker *et al.*, 1992). Therefore, relating broth rheology to morphology of only freely dispersed hyphae has been found to be of very little value. For this reason, image analysis techniques have been used to quantitatively determine the actual geometry of the organism (Thomas, 1992).

Studies by Roels *et al.*, (1974) using *P.chrysogenum* determined that the diameter of the mycelial aggregate, (dp), and the biomass concentration, (x), related to the rheological properties with respect to the power law. It was concluded that the hyphal character of the free mycelia influences the viscosity and rheology to a lesser extent than the aggregates and therefore rheological properties should be correlated to size and shape of the aggregates. Olsvik *et al.*, (1993) reported similar results during fermentation of *A.niger*. It was found that changes in roughness of the aggregate, (R), correlated with changes in the value of the consistency index, k as well as biomass. Hence, it was concluded that k was directly proportional to the biomass concentration within this particular system.

Various correlations between morphology and rheology have been proposed. For example, Metz *et al.*, (1979) defined a relationship between Casson parameters and the length of hyphae, L_e and the length of the hyphal growth length, L_{hgu} (equation 7.1).

$$Kc = 5.454 \cdot x^{1.0} \cdot L_{hgu}^{0.6} \qquad \textbf{(eqn 7.1)}$$

$$\tau_0 = 1.67 \times 10^{-4} \cdot x^{2.5} (L_e)^{0.8}$$

This correlation could not be applied to continuous culture due to variation in hyphal flexibility caused by differences in hyphal-hyphal interactions. Therefore, in later studies van Suijdam (1987) introduced a morphology factor, M_F, which was determined using viscosity data of the mycelial broth diluted to a biomass concentration where hyphal-hyphal interactions could be excluded (equation 7.2).

$$M_F = \lim_{x \to o} (\eta / \eta_o - 1) / x \qquad \textbf{(eqn 7.2)}$$

However, there are conflicting reports with respect to the correlation between rheological behaviour and morphology of the organisms and with the properties of the liquid medium in which the microorganisms are suspended.

Chapter 7.3 Effect of engineering variables on broth rheology & morphology

Substantial evidence has been presented that a number of factors influence the morphology and hence the rheology of microorganisms within the fermenter environment. The rheological properties of the culture are constantly changing during fermentation and becoming more complex. For industrial purposes, increasing the mixing and thereby increasing the oxygen transfer rate by reducing viscosity may prove the most profitable method of rheology control. Other attempts have been made to alter the rheology of the broth for example dilution (Sato, 1961; Taguchi & Myamoto, 1966 and Buckland *et al.,* 1987).

More recent studies have indicated however, that these improvements are only temporary. Olsvik & Kristiansen (1992) diluted a broth of *Aspergillus niger* with both water and a sugar solution. By diluting the broth the viscosity reduced and therefore the oxygen transfer rate increased but this in turn lead to increased cell growth and hence viscosity. It was concluded that the duration of this improvement in rheology was related to the initial broth viscosity.

There are various influential factors on broth rheology which include the oxygen availability in the fermenter; the growth rate of the organism and agitation. It is generally accepted that by changing the dissolved oxygen concentration within the fermenter, the mycelial properties of the organism will change and hence the viscosity of the broth will alter (Metz *et al.*, 1979). Early studies demonstrated that by aerating a fermentation broth with pure oxygen rather than air, resulted in a less viscous fermentation although the biomass concentration maybe higher (Dion *et al.*, 1954 and Zetelaki & Vas, 1968). It should be noted that carbon dioxide levels will also affect morphology as well as inhibiting cell growth (Smith *et al.*, 1985; Belmar-Beiny & Thomas, 1992 and Yang *et al.,* 1996).

Further work has reported that morphology is independent of the dissolved oxygen concentration provided the level is maintained above a critical level (Park *et al.*, 1993). Similar results were observed by Cui *et al.*, (1997). During fermentation the morphology of *Aspergillus awamori* changed very little whilst the dissolved oxygen concentration was maintained above 5% and below saturation, (equivalent to pure oxygen). However, above saturation dense pellets were formed with very little filamentous mycelia whilst below 5% oxygen loose fluffy pellets were formed. The pellets formed under increased oxygen concentration had higher intrinsic strength and therefore it was concluded that the porosity of the pellets is a function of the dissolved oxygen concentration. Braun & Vecht-Lifshitz, (1991) suggested that this induced pellet formation with high oxygen concentration was due to cellular aggregation caused by the hydrophobic interactions of the cell walls.

The growth rate of the organism will also affect morphology since it influences branching frequency of the mycelia. The rate of growth can be increased or decreased by altering the composition of the growth medium; by varying the temperature; by incorporating inhibitors into the medium or by changing the carbon source. However, the effect is dependent on the strain of the organism.

Various studies have been conducted to investigate the effect of growth rate on morphology. For example, Katz *et al*,. (1972) showed that there was a reduction in hyphal growth unit when the growth rate of an *A.niger* culture increased. Whilst Metz *et al.*, (1981) demonstrated that the hyphal length of a *P.chrysogenum* culture increased as the growth rate increased in a carbon limitation. Olsvik & Kristiansen, (1992) investigated the effect of growth rate on the morphology and viscosity of a culture of *A.niger* and found that the specific growth rate of the organism and the viscosity of the culture were dependent on each other. However, the consistency index of the broth, k, was dependent on the dissolved oxygen concentration.

There are a number of authors who found no correlation between growth rate and morphology (Katz, 1972). This is thought to be due to the fact that a survival instinct by the organism results in the production of hyphal tip expansion and vesicle production rather than changes in growth rate and hence morphology. The nutrient composition of the medium will also affect rheology directly. The addition of insoluble nutrients such as starch create a viscous medium initially but as it is utilised the broth becomes less non-Newtonian. The same can be said of oils and fats which are added as co-substrates in fermentation media.

Agitation is an important factor when considering morphology and rheology. The shearing forces to which a fermentation broth are exposed when agitated cause rupture of the cell wall, changing broth rheology and influencing cell morphology. It is generally accepted that by increasing the power input per unit volume i.e. the stirrer speed, the apparent viscosity of the broth will decrease (Reuβ, 1988; Smith *et al.,* 1990 and Sarra *et al.*, 1996). However, although there is a decrease in hyphal length with increasing power input per unit volume for many filamentous cultures, the effect of increasing shear stress on the effective hyphal length has been reported to be relatively small (Van Suijdam & Metz, 1981). In addition to the agitation intensity the type of impeller,

impeller geometry, the position of the impeller and the number of impellers will affect morphology since these parameters determine the mechanical forces to which the broth is subjected (Concerti *et al.*, 1993 and Justen *et al.*, 1996).

The morphology of an organism can change significantly due to fragmentation of the hyphae (Wiebe & Trinci, 1991 and Makagiansar *et al.*, 1993). Dion *et al.*, (1954) observed that *Penicillium chrysogenum* exhibited long filamentous mycelium if the agitation intensity was maintained at a low speed or short highly branched mycelium under higher agitation intensities. The filamentous type of morphology was only exhibited in the early stages of growth due to this fragmentation. A similar observation was made by Ujocova *et al.*, (1980) who found that thicker, more twisted, densely branched and septated hyphae were formed with increasing stirrer speed.

Some fermentation broths however, have been shown to be relatively insensitive to shear such as *Cephalosporin acremonium* which maintains rather short and fine hyphae regardless of agitation intensity whilst others have been shown to be extremely sensitive to shear such as *Cladosporium herbarum* which fragments considerably. This could be due to either strain dependency or the manner in which shearing forces influence the mycelial suspension. For a number of species it has been found that physical cell damage is caused by increasing the impeller speed and thus there is an upper limit to the agitator speed for growth and production. This damage results in the release of the cell contents including proteolytic enzymes and resulting in cell lysis and hence a reduction in viscosity. For example, Vadar & Lilly (1982) observed a shift from production of the desired product to cell constituents as agitation increases which would result in strengthening of the cell wall.

A final consideration is the age of the fermentation broth. Deindoerfer & Gaden (1955) studied the morphology of *Penicillium chrysogenum.* It was observed that the viscosity of the broth increased with the age of the culture although in the later stages of the fermentation, when the mycelial concentration was constant, the viscosity began to increase again due to changes in morphology and extracellular metabolite production. It was also found that *Streptomyces griseus* broth demonstrated similar changes in behaviour due to the development of an advanced hyphal network during the highly

aerobic growth phase. Similar results were observed by Sarra *et al.*, (1996) although the biomass concentration was also a significant factor with respect to rheological changes.

It can be said that mechanical disruption of mycelial hyphae due to agitation, growth rate and medium composition has a profound effect on the broth rheology although the effect on morphology appears to be strain dependent. The oxygen tension has a limited influence on the morphology of the organism whilst culture age will have a significant effect. The information in this chapter therefore, demonstrates the need for optimisation and control of process parameters during fermentation in order to maintain an efficient system.

Chapter 7.4 Effect of rheology & morphology on mass transfer

In non-Newtonian systems such as mycelial fermentations the limiting mass transfer resistance will be determined not only by the mass transfer coefficient, K_L, but also by the ratio of the cell surface area to the surface area of the gas phase which depends on the morphological characteristics and mixing conditions. Further complexity is introduced by the fact that the mixing conditions are influenced in turn by the rheological characteristics which is dependent on the culture morphology. Mass transfer is also dependent on scale since mixing states can vary dramatically with scale and anything that alters the state of mixing and the overall mixing conditions will affect the mass transfer rates.

A high degree of mixing is required in order to promote heat and mass transfer within a non-Newtonian broth. As the rheology of the broth changes during the course of the fermentation resulting in an increasing viscosity, mass and heat transfer as well as mixing rates will be adversely affected. The high viscosity of the these mycelial cultures will result in increased mixing times, heat, nutrient and chemical gradients as well as stagnant zones and hence reduced productivity. The influence of viscosity of the broth on mixing can be expressed as the Reynolds Number (eqn 7.3)

$$Re = \frac{Di^2 . N . \rho}{\mu} \qquad \textbf{(eqn 7.3)}$$

As previously mentioned the critical operation in an aerobic fermentation is the supply of oxygen to the cells. Since the solubility of oxygen in water is low, the culture may experience severe oxygen starvation. Transfer of oxygen from the gas to liquid phase is usually limiting but transport limitation may also result in the bulk liquid due poor bulk mixing or between the bulk liquid and the cell is crucial. This is further complicated due to shear rates and power dissipation being higher in the region near the impeller which results in oxygen transfer mainly taking place in this region. This means that there will be stagnant zones in regions away from the impeller.

Numerous studies have been done in order to correlate oxygen transfer rates and rheology of filamentous broths. In general correlations have been estimated using K_La in non-Newtonian fluids. In general a decrease in K_La has been observed as the viscosity of the broth increases (Deindorfer & Gaden, 1955; Chain *et al.*, 1966 and Gbewonyo *et al.*, 1992). However, the adverse effects on K_La are diminished if the organism assumes pellet morphology (Solomons & Perkin, 1958). One of the first studies was done by Deindorfer & Gaden (1955) who observed an 85% reduction in K_La as the viscosity of a *P.chrysogenum* broth increased during the course of a fermentation. This apparent decrease in oxygen transfer rate as the cell concentration increases has been reported by a number of other authors using different organisms.

Other conflicting studies have shown that K_La of fermentation broths can be increased by the presence of a higher biomass concentration (Tsao, 1968). This effect may be due to a number of reasons such as chemical enhancement by microorganisms, direct gas uptake by the organisms, hydrodynamical effects and an increase in K_La corresponding to an increase in ionic strength. Voigt & Schugerl (1981) also investigated the effect of increasing cell concentration on K_La using a culture of *A.niger*. They found that initially there was an increase in K_La but this is followed by a decrease due to the secretion of surface active compounds. The studies also showed that if dissolved oxygen concentration was in excess, the value of the consistency index (k) increased with increasing dilution rate but decreased if the broth is oxygen limited. This is thought to be due to the growing cells which may secrete surface active compounds or the cells acting as surfactants themselves.

There has been a variety studies to determine methods for measurements and evaluation of the oxygen transfer coefficient in fermenters since oxygen transfer rates determine the production capacity of the fermenter. A number empirical correlations for estimating K_La in non-Newtonian fluids have been formulated, for example Ruy & Humphrey (1972).

$$K_La = k \cdot (P/V)^a \cdot (Vs)^b \cdot (a)^c \qquad \textbf{(eqn 7.4)}$$

where a,b,c, & k = constants

However, the use of these types of correlations are not totally accurate since they are scale dependent because they do not incorporate explicit measures of mixing qualities such as mixing time. Other problems have been encountered with the use of these correlations since experimental data in real fermentations is difficult to obtain and hence, model polymer solutions have been used to simulate broth behaviour which are inaccurate since the polymer solutions are homogeneous.

Therefore, it becomes apparent that morphology of the organism is a major determinant of the flow properties of the broth. Accurate determination of the actual effects of the broth on mass transfer and rheology is difficult due to variations in measurement techniques and properties encountered during measurements caused by the nature of the broth itself. This is further complicated by the use of certain medium components such as oils which is discussed in the next section.

Chapter 7.5 Oil utilisation and broth rheology

As with this study, fermentation using a dispersed oil phase as a carbon source is complex since there is a higher oxygen demand during metabolism and oil tends to remain at the end of the process. In order to be utilised the oil requires dispersion within the system. Therefore, the oil droplet size and size distribution are important parameters which determine not only the stability of the dispersion but also the efficiency of cell/oil contact and hence oil utilisation (Clarke & Sawistowski, 1978).

During a filamentous fermentation there is a complex field flow occurring in the vessel and changes in broth rheology critically influences flow conditions in the vessel and hence the mechanism of drop breakage and dispersion (Boye *et al.*, 1996).

Chapter 7.5.1 Effect of oils on mass transfer

The growth of an organism on a carbohydrate substrate such as rape seed oil creates a four phase system which will consist of the continuous phase which is generally the aqueous phase containing soluble medium components and three dispersed phases which tend to be within a turbulent environment. The first of these is the solid phase which consists of the microorganism itself and varies in size between 0.5 and 5μm. The second is the gaseous phase which consists of air bubbles with diameters varying between 0.5 and 5μm and the final dispersed phase is the liquid phase which consists of the oil with a droplet size of between 0.5 and 40μm. The dispersed oil phase can influence the transfer of oxygen by absorbing oxygen or supplying it to other phases, or by influencing the fluid mechanics of the continuous phase, thereby influencing the mass transfer coefficients and interfacial areas of the continuous phase. This system is schematically represented in figure 7.1.

There are four possible routes for oxygen transfer. The first is gas-liquid-solid transfer, (**1**), in which the gas molecule is dissolved into the aqueous phase and is then used directly by the organism. The second option involves gas-solid transfer, (**2**), and relies on the organism using the oxygen at the interface of the gas bubbles. The third option is that of gas-liquid-liquid-solid transfer, (**3**), where the gas is absorbed into the oil phase which is then transferred to the aqueous phase where it can be utilised by the cells. With the final option, (**4**), the cells are located at the surface of the oil droplets and use the oxygen directly which depends on the affinity of the cells for the oil and on the size of the emulsified oil droplets in the aqueous phase. The actual interaction between air bubbles and oil droplets is not well established although Roque *et al.,* (1987) proposed that the relationship varied according to the spreading coefficient of the oil.

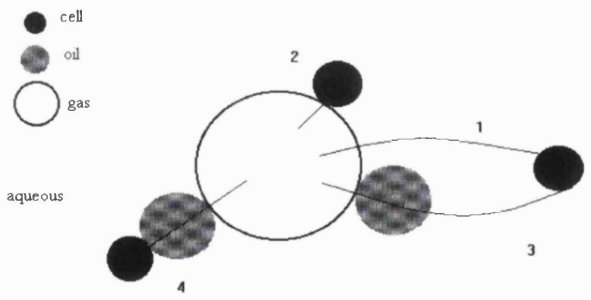


FIGURE 7.1 4-Phase transfer system

There are conflicting reports on the actual effect of oils in fermentation media with respect to oxygen transfer rates. The oil concentration used in media for antibiotic fermentations varies from 1-20%(w/v). When higher concentrations are used, antibiotic production has been seen to sharply decrease, apparently due to the decreased oxygen transfer (Kralovcova *et al.,* 1984 and Rezanka *et al.*, 1984). Whilst McMillan & Wang, (1990) showed that oil may have a beneficial effect on the oxygen transfer rate. Since oxygen solubility in oil is higher than in water, oil droplets located within the boundary layer of the gaseous dispersion can increase the oxygen permeability in this layer thus increasing oxygen transfer.

Further studies by Rols & Goma, (1989) also demonstrated that the presence of the oil actually enhanced the oxygen transfer capacity. The presence of 19%(v/v) of soy bean oil enabled a 1.85-fold increase of the K_La value calculated on a per litre aqueous phase basis. For smaller oil fractions, the K_La increased linearly with oil loading. This was thought to be due to the oil acting as an oxygen vector. However, when Adler *et al.,* (1980) investigated the use of soy bean oil in a range of 0.5 to 1.5% (v/v), there was very little influence on the value of K_La. This suggests that there is a critical concentration range in which the use of oil has a beneficial effect. Similar results were observed by Ho *et al.*, (1990) and Rols & Goma, (1991).

For an oil to influence the oxygen transfer coefficient, the oil must act at the gas-water interface or within the water boundary layer of the gaseous dispersion. Rols *et al.*, (1990) proposed that the oil phase was an active intermediary for oxygen transport from gas bubbles to water and that the oxygen transfer rates were increased by the layering of the oil as a thin film at the gas-liquid interface. The coefficient which governs this phenomena is the spreading coefficient defined for a spreading oil droplet on the water surface at dynamic equilibrium (equation 7.5).

$$Sp = {}_{wg} - ({}_{og} + {}_{ow}) \qquad \textbf{(eqn 7.5)}$$

When $Sp \geq 0$, oil will spread on the water surface but when $Sp \leq 0$, oil will simply form droplets within the system. The value of Sp will be dependent on the oil used. For example, soy bean oil has a Sp value of + 37.4 dynes/cm which suggests that the oil droplets would spread as a thin film at the gas-liquid interface. This could explain the enhancement of the oxygen transfer coefficient when certain oils are utilised and a reduction when other types of oil are used.

Oils have been widely used as antifoaming agents due to their surface active properties (Chapter 3.1.1). Therefore, oils migrate towards and concentrate at the gas-liquid interface, thereby creating surface tension gradients which influence the gaseous specific interfacial area. This will impact on oxygen transfer (Linek & Benes, 1976 and Kawase & Moo-Young, 1990). The overall change in the value of K_La depends on the relative magnitudes of the effect on K_L and a respectively.

Early studies demonstrated that the addition of the oil based antifoam decreased the value of K_La although the interfacial area for oxygen transfer increased (Benedek *et al.*, 1971). The study also showed that K_La was independent of the agitation intensity although it was influenced by bubble diameter. The addition of an oil based antifoam resulted in a linear relationship between K_La and bubble diameter over a range of 0.1 to 0.4cm. Linearity of the equation is assumed to result from the simultaneous reduction of mass transfer and of coalescence by the damping of interfacial area.

Similar observations were reported by Yagi & Yoshida, (1974) when they investigated the effects of medium composition and cell concentration on oxygen absorption during fermentation. The presence of an oil based antifoaming agent changed the air bubble distribution size which in turn lowered the gas-liquid interfacial area and hence reduced the oxygen transfer rate. From this they proposed an empirical correlation for bubble size distribution based on a bubble column (equation 7.6).

$$a \,.\, Dc = 1/3 \,.\, (g.\, Dc.\, \rho / \sigma)^{0.5} .\, x \,.\, (g.\, Dc^3 / v^2)^{0.1} .\, \varepsilon g^{1.13} \qquad \textbf{(eqn 7.6)}$$

Therefore, it would appear that the bubble size distribution is a key factor in determining the rate of oxygen transfer (Schugerl *et al.,* 1978 and Kawase & Moo-Young, 1990) as is the oil droplet size (Rols *et al.*, 1990).

Therefore, it has been demonstrated that it is possible to increase the K_La within a fermentation by the addition of oil in the aqueous phase, without changing the conditions of agitation and aeration. However, to maintain a culture using oil rather than sugar requires a higher oxygen uptake rate. Generally, a typical oil requires 2.7 times the oxygen for oxidation reactions compared to glucose although it contains more carbon on a weight basis. This can result in the oxygen uptake rate becoming excessive, the dissolved oxygen concentration being depleted and hence antibiotic production being hampered as reported by Bader *et al.,* (1984). It would therefore seem that oils offer potential to improve fermentations by increasing both antibiotic production as well as in some cases improving oxygen transfer rates but is dependent on the system and the type of oil utilised.

Chapter 8 Size distribution analysis of oil/water dispersions

For oil to be utilised during fermentation, it has to be dispersed within the fermentation broth. This is achieved by means of agitation. How readily the oil is dispersed together with the size of the oil droplets and the stability of the emulsion formed, will affect the efficiency of oil uptake due to dependency on the formation of the oil/water interface as well as cell/oil contact. Therefore, the oil droplet size distribution was measured, in a model system, to investigate whether this could be a limiting factor to oil uptake during fermentation.

Chapter 8.1 Experimental materials

Distilled water was used as the continuous phase to which was added 0.3 % w/w of sodium dodecyl sulphate, (SDS). SDS is added in order to stabilise the dispersion. Rape seed oil was used as the dispersed phase at a constant concentration of 2.5% w/v whilst other components essential in the growth medium for *Streptomyces clavuligerus*, such as soya flour at 1% w/v and dextrin at 1% w/v, were added during later experimentation. The amount of oil added was calculated as detailed below (calculation 8.1).

Calculation 8.1 2.5% oil concentration in 1.4 l of distilled water

$0.025 \times 1.4 = 0.035$ l of oil per 1.4 l of distilled water.

Taking the density of the oil as $\rho = 915$ Kg/m^3 (Perry, 1989)

Volume × Density = Mass

$\therefore$ $0.035 \times 0.915 = 0.032025$ Kg

Therefore, 32.03g of oil are required in a total volume of 1.4l for an oil concentration of 2.5% w/v.

Chapter 8.2 Experimental mixing equipment

The mixing vessel was a round bottom glass cylinder, 12.5cm diameter, 14cm high and a 1.5l volume, with an outer jacket for cooling water circulation. The agitator was a Bench Equipment Company unit ELB EXP agitator driven by a 240 volt DUTY MASTER a.c. motor drive with interchangeable gearheads allowing variable speeds up to 2875 revs per minute, (rpm). Exact mixing speeds were determined using a SOLEX TA250 photo/contact tachometer.

The impeller used was a standard six bladed Rushton turbine, 5cm in diameter with a blade height to impeller diameter ratio of 1/8. The impeller was located 1/3 of the distance from the tank bottom to the liquid surface. The temperature of the emulsion was maintained using a thermostatically controlled water bath set at 26^{o}C +/- 0.5^{o}C.

Chapter 8.3 Sample analysis equipment

Droplet size distribution of the dispersed phase, oil, depends on the condition of agitation. In this case the most important factor to consider is the interfacial area which can be determined using the mean droplet size. This is usually expressed as the Sauter mean diameter and can be measured using laser diffraction. This is a highly advanced and automated technique using the principle of Fraunhofer theory in measuring and interpreting the angular distribution of light diffracted by the droplets. This technique has a number of advantages which are listed below. However, the technique has one major disadvantage, as it cannot be used for on-line measurement. Other problems include sample dilution during analysis and maintaining the sample cell which must be kept clean of any impurities.

Advantages of the Malvern Laser Diffraction Particle Sizer

•No calibration is required and it can be used for both liquid and solid phase dispersions.

•Measurement is completed in a few seconds enabling analysis to be done quickly and efficiently.

•Only a small volume of sample is required.

•Smaller drop diameters are easily measured whereas with other techniques such as photography, the smaller drops are not easily distinguishable.

Chapter 8.3.1 Mode of operation of the Malvern Laser equipment

The instrument uses a low power laser transmitter to produce a monochromatic beam of light that illuminates the sample droplets or particles flowing in an appropriate cell. The illuminated particles diffract the incident light, the degree of diffraction being inversely related to the size of the particles i.e. the smallest particles diffract the most amount of light, (figure 8.1).

A continuous flux of particles through the illuminated area is then collected on a series of concentric detection rings and integrated over a suitable time period, giving a diffraction pattern representative of the bulk sample. An analogue signal proportional to the incident light intensity is produced by the Fourier transform lens focusing the diffraction pattern onto a multi-element photoelectric detector. This detector is directly interfaced to a computer which reads the diffraction pattern and performs the necessary integrations digitally.

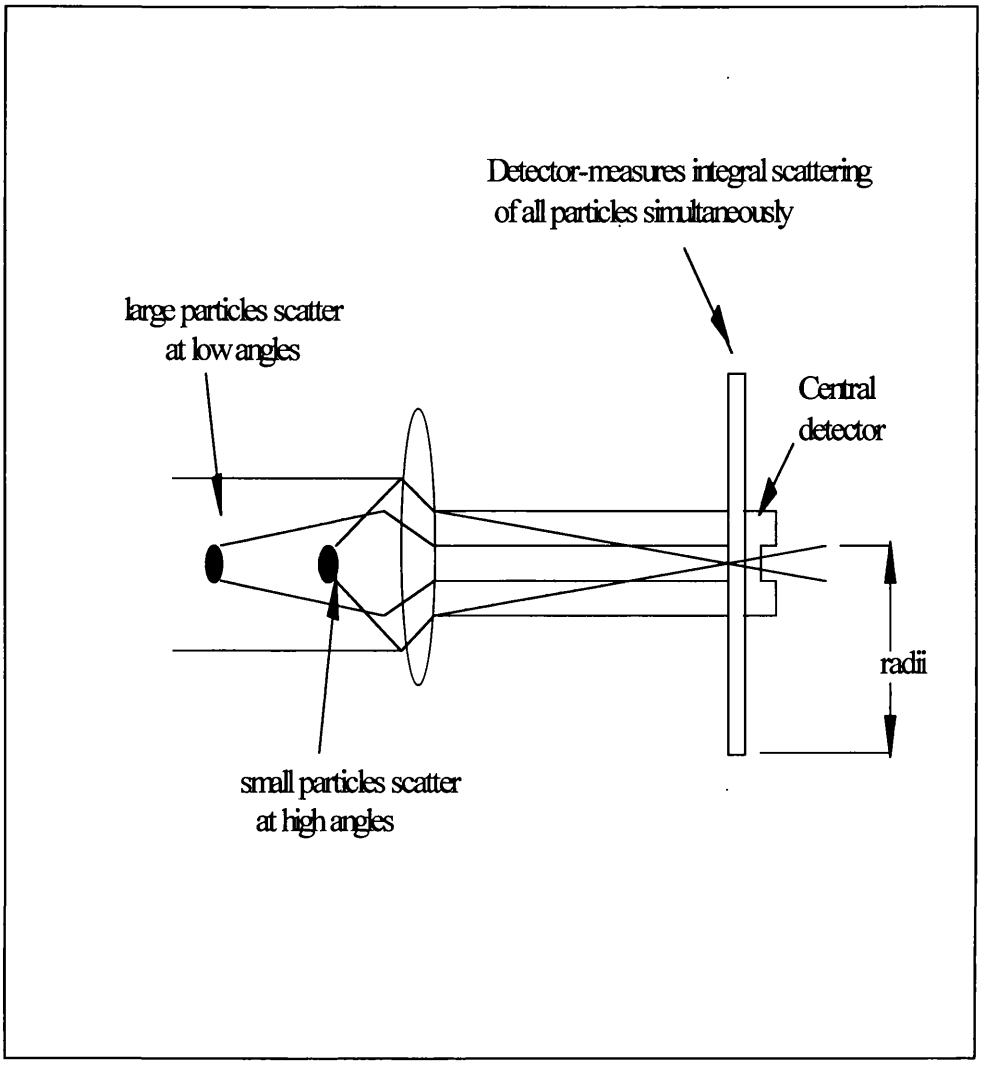


FIGURE 8.1 The principle of laser diffraction

Having measured a diffraction pattern, the computer uses non-linear least squares analysis to find the size distribution which gives the most closely fitting diffraction pattern. The machine produces 31 channels of data, which are presented on a cumulative undersize basis. The machine is capable of measuring particles of sizes ranging from 5μm to 564μm in diameter.

Chapter 8.4 Mixing procedure

A model system of rape seed oil and water, at a constant volume fraction, was used as the dispersed and continuous phases respectively and agitated in the described vessel at impeller speeds ranging from 250rpm to 750rpm. The impeller speed was set and then

appropriate aliquots of the continuous phase, distilled water, were transferred to the clean, dry vessel. The surfactant used to stabilise the dispersion, SDS, was added at a concentration of 0.3%w/w and allowed to dissolve in the distilled water before adding the dispersed phase, rape seed oil. The appropriate amount of the dispersed phase was then added which was dependent on the volume fraction required, (calculation 8.1). The reaction mixture was mixed for one hour so that a state of dynamic equilibrium was reached.

Chapter 8.5 Sample analysis procedure

The particle size measurements were carried out using the Malvern Laser equipment and associated software, as described previously. The sample cell was rinsed using a buffer solution of 0.3% w/w SDS in order to clean the cell. The cell was then filled slowly, to prevent bubble formation, with the buffer solution to the required level and placed in the machine within a few millimetres of the detection lens.

Having set the equipment parameters, the laser was then aligned to ensure even distribution of the light and to maximise light intensity. With the laser light illuminating the central region of the cell above the detector port, the magnetic stirrer was switched on and the cover housing shut. The buffer intensity was then measured. The reading should be in the upper region of the scale, (GOOD), on the screen and the channel output should read LOW which relates the clarity of the actual reading in relation to the background level. This gives the background noise level. Once a steady background reading was obtained, approximately 50µl of the sample was added, using an open aperture syringe, to obtain an obscuration rate reading of between 0.28 - 0.30.

The laser diffraction technique has a certain range of acceptable scattering intensities over which the accuracy can be maintained. Since intensity is a function of particle size and concentration, the sample concentration limits depend on the size of the material. At lower concentrations, poor signal level and large random error exist, whilst at high concentrations multiple effects may introduce systematic error.

In all the analyses performed, the lens focal length was set at 300mm. Particles in the size range from 6μm to 564μm were detected with an accuracy of ~4% on the basis of the volume median diameter. Typical results generated include cumulative size distribution, volume fraction within each band, Sauter mean diameter and volume mean diameter.

Chapter 9.1 Analysis of lipase activity

To utilise oil during fermentation, *Streptomyces clavuligerus* has to produce lipase. Lipase activity can be detected and measured by various methods and this chapter details the assays selected for assessment for this study.

Chapter 9.1.1 Agar plate assay

This technique is based on a method determined by Arima *et al.*, (1972). 7.5g of agar and 5g of potato dextrose broth were dissolved in 495ml of water. To this was added 5ml of substrate which was then emulsified by stirring vigorously for several minutes. The mixture was autoclaved for 20 minutes at 121°C and then allowed to cool before pouring into Petri dishes at 5mm layer height. Once set, a hole of 5mm depth and 6mm diameter was bored aseptically. The hole was then filled with 50µl of sample and the plates were incubated at 25, 30 or 37°C for 72-120 hours after which time the diameter of the clearance zone around the well was measured. Solutions of standard lipase concentrations were assayed initially to obtain a standard curve and then fermentation samples were tested.

Chapter 9.1.2 Titrimetric lipase assay

Chapter 9.1.2.1 Materials

•Reagents

The substrates used for the initial optimisation studies were tributyrin, olive oil, a triolein/Tween mixture and rape seed oil. The oils were selected based on their structure with respect to fatty acid composition and in particular, chain length. These were emulsified using gum arabic in an emulsion containing sodium chloride, phosphate and glycerol. The titrant used was 0.01M sodium hydroxide.

•Equipment

A Titrilab (Radiometer-Copenhagen) pH-stat comprising: VIT 90Video Titrator, ABU 93 titraburette and SAM 90 Sample station was used for the assays which was supplied by V.A. Howe & Co. Ltd (London, England). A thermostatted reaction vessel was also used in order to ensure a constant temperature was maintained throughout the analysis.

Chapter 9.1.2.2 Method

•Emulsification Reagent

17.9g of sodium chloride plus 0.41g of potassium dihydrogen orthophosphate were added to a 1 l beaker followed by 400ml of distilled water plus 540ml of glycerol. Under vigorous stirring, 6.0g of gum arabic were added and the solution was then transferred to a 1 l volumetric flask and the volume made up to 1 l with distilled water. This may be stored for up to one month at 4°C.

•Substrate Emulsion

15ml of substrate plus 50ml of emulsification reagent plus 235ml of distilled water were added to a 1 l beaker. This was then blended at maximum speed for 20 seconds and then left stirring until required. Fresh emulsion must be prepared every day.

•Sample Analysis

20ml of substrate emulsion were placed in the thermostatted reaction vessel and allowed to equilibrate at 26, 30 or 37°C. The pH of the mixture was then adjusted to pH 6.6, 7.0 or 7.2 using 0.01M NaOH. 1ml of sample, (chapter 12.6), was then added to the substrate emulsion and the analysis started. Automatic titration with 0.01M NaOH was allowed to continue for 10 to 20 minutes and the lipase activity was calculated from the rate of addition of alkali, corrected for the rate in the absence of enzyme as illustrated below.

•Calculation of Lipase Activity

The consumption of 1ml of 0.01M NaOH is equivalent to the liberation of 10μmole of fatty acid i.e.

$$\text{moles of NaOH} = M \times V/1000$$
$$= 0.01 \times 1/1000$$
$$= 1 \times 10^{-5} \text{ moles} = 10\mu\text{moles of NaOH}$$

From this it follows that for the activity of the lipase activity:

$$\text{Volume activity} = \frac{\text{volume of NaOH consumed}}{\text{time period of assay}} \times 10^3 \times 10 \text{ (U/ml)}$$

Chapter 9.1.3 p-Nitrophenyl palmitate assay

Enzyme activity with p-nitrophenyl palmitate, (pNPP), as a substrate was measured according to the method of Winkler & Stuckmann, (1979) and is detailed below.

30mg of pNPP dissolved in 10ml propan 2-ol were emulsified in 90ml 0.05M sodium acetate, pH 5.6, containing 200mg Triton X-100. 0.1ml of enzyme solution was mixed with 2ml of the pNPP-containing emulsion and incubated at 26, 30 and 37°C. The reaction was stopped by adding 0.15ml of 0.1M sodium carbonate solution and the absorbance was measured at 410nm. A blank solution was used with water instead of enzyme solution. One unit, (U), was defined as the amount of enzyme that liberated 1μmol p-nitrophenol per minute under the given conditions.

Standard lipase solutions with known lipase activities were assayed using all of the methods to give an indication of their sensitivity and reliability. Fermentation samples were then assayed using all the methods and on the basis of these results, the best assay was determined for the study.

Chapter 9.2 Lipid Analysis

To monitor oil utilisation during fermentation two main methods were used, chromatography and a biochemical assay. Both of these methods are detailed in this chapter.

Methods for the separation and identification of lipids generally involve the use of chromatography in some form although biochemical test kits may be used. Chromatography involves the separation of components of a mixture by virtue of the differences in the equilibrium distribution, (K), between the mobile and stationary phases. Chromatography was used in this study namely, Thin Layer Chromatography, (TLC), as well as an assay based on a biochemical testing kit.

Chapter 9.2.1 Thin layer chromatography

In TLC the mobile phase is usually a non-polar or less polar solvent than the lipid itself and the stationary phase is a solid such as silica gel or alumina. However, silica gel may be used as a support material which can be coated with a non-polar liquid and hence the mobile phase can be a more polar solvent i.e. reversed phase TLC.

Chapter 9.2.1.1 Materials & Equipment

The TLC plates used were Pre-coated silica gel plates, (20cm ×20cm), with 0.25mm thickness of coating and fluorescent at 254nm which allows the identification of the components by absorption at 254nm. A developing tank, which could be vacuum sealed in order to create a saturated environment was used, with a mobile phase containing 200ml of petroleum ether; diethyl ether; acetic acid at a ratio of 150:50:1. The plates could be identified and separated using visualising agents which in this case were bromophenol blue for identification of the fatty acids and iodine for the identification of the triglycerides, diglycerides and monoglycerides.

Chapter 9.2.1.2 Method

•Lipid Extraction

A broth sample of a known volume, (approximately 2ml), was transferred to a polypropylene tube. For each ml of the broth, 7ml of a chloroform:methanol, (1:1v/v), solution were added and mixed. The sealed tube was then placed in a water bath at 65°C for 5 minutes to destroy any lipase, then cooled to room temperature and left for one hour. The mixture was centrifuged at 2000rpm for 10 minutes and the supernatant decanted into a fresh tube. The pellet was then resuspended in 7ml of chloroform:methanol:water solution, (3:3:1 v/v/v), and left to stand at room temperature for one hour. The centrifugation procedure was then repeated.

The supernatant was decanted and added to the previous supernatant and to this was added 5ml of chloroform and 5ml of water. This results in the formation of two separate phases and the accumulation of a substantial amount of material at the interface. The next stage of the extraction involves the removal of the upper aqueous layer leaving the interface intact. After evaporating it to dryness, the residue was redissolved in 1ml of chloroform:methanol, (1:1 v/v). The material at the interface will not dissolve but may be removed by centrifugation. This chloroform:methanol extract was then assayed for lipid content by TLC after having been filtered using a 0.45μm filter to remove any contaminating particles.

•TLC Assay

The plates were first activated by incubating at either 100°C for 2.5 hours or 110°C for one hour taking care not to darken the plate. The solvent mixture was then poured into the developing tank and sealed to allow a saturated environment to be formed. After activating the plates, lines were scored through the coating of each plate, 10mm from the top and sides. This helps in reducing the edge effects during the development of the plates. A line was drawn approximately 20mm from the bottom of each plate and a mark put where each sample was to be spotted.

Each spot was loaded with 5μl of sample in 1μl aliquots, allowing the spot to dry between each addition. Once dry, the plates were put into the developing tank and left to run for approximately 1.5 hours. The plates were removed and allowed to dry. The fatty

acids were visualised by spraying the plates with a 0.5% solution of bromophenol blue in 0.2% citric acid and the lipid by staining with iodine. Initially, standard oils and fatty acids were run and then fermentation samples were tested.

Chapter 9.2.2 Total lipid assay

This assay was based on the Boehringer test combination kit, (catalogue number 124 303), and was an alternative method to TLC and/or HPLC for calculating lipid present in the sample. The principle of this assay is based on the fact that lipids react with sulphuric and phosphoric acids and vanillin to form a pink coloured complex. The amount of lipid present was proportional to the intensity of the pink colour observed.

Chapter 9.2.2.1 Materials

The reagents for the assay were based on the Total Lipid Boehringer kit and the Lipid Standard used was part of this kit. The reagents were vanillin and phosphoric acid which were mixed together as follows. 1.98g of vanillin were dissolved in 40ml of ethanol and made up to 100ml using distilled water. This was then made up to 1 l using concentrated phosphoric acid and stored in a light proof container.

Chapter 9.2.2.2 Method

Broth samples were warmed to 30°C and then mixed vigorously. 50µl of broth was taken from each tube immediately after mixing and placed in large clean test tubes. 50µl of lipid standard was also added to individual tubes to be used as the control standards for the assay. 2ml of concentrated sulphuric acid were added to each tube and mixed well. Each tube orifice was covered with a small plastic marble and placed in boiling water for 10 minutes. These were then allowed to cool and once cool, 100µl of each sample were transferred to clean tubes and 2.5ml of vanillin reagent were added. The tubes were then mixed thoroughly and left to incubate at room temperature for 30 minutes, mixing occasionally. The samples were assayed using a spectrophotometer at

536nm. The blank used was sulphuric acid/vanillin mixture. The amount of lipid was calculated using the calculation below:

$$\text{Lipid concentration} = \frac{\text{absorbance of sample}}{\text{absorbance of standard}} \times 10$$

Chapter 9.3 Biomass determination

Samples from fermentations were assayed for growth using both dry weight measurements as well as using a Boehringer test kit for DNA analysis.

Chapter 9.3.1 Dry weight analysis

20ml broth were filtered through glass micro-fibre filters, (Whatman, Grade GF/A 4.7cm diameter), using a Sartorius SM vacuum filter holder. The mycelia were washed with 1M hydrochloric acid before oven drying at 100°C for 24 hours.

Chapter 9.3.2 DNA assay

SAFETY STATEMENT – DIPHENYLAMINE IS HIGHLY TOXIC & HARMFUL TO THE ENVIRONMENT. THEREFORE, THIS ASSAY MUST BE CARRIED OUT IN A FUME CUPBOARD AND SUITABLE PROTECTIVE CLOTHING WORN.

2.2ml of trichloroacetic acid were added to 2ml of broth sample which was then heated to 90°C for 10 minutes. After cooling, the samples were centrifuged at 2500rpm for 10 minutes using a MSE Chilspin Labtop Centrifuge. 0.5ml of supernatant were then added to 0.1ml of 70% perchloric acid and 2.9ml of diphenylamine reagent, (DPA). This was incubated for 12 hours at room temperature and the absorbance read at 600nm.

Chapter 9.4 "CobasBio" analysis

The "CobasBio" V8326, (1983; F. Hoffman-La Roche & Co., Basle, Switzerland), is a single unit self contained centrifugal analyser which offers different procedures for determination of substrate concentration or enzyme activities. The measuring and calculation procedures are adapted to the kinetics of each chemical reaction with respect to the assay required.

The unit allows the use of lower reagent volumes since absorbance measurements are made horizontally relative to the light path which also allows variation in the photometric range. Temperature may also be controlled to within 0.1°C over a range of 25 to 40°C. The system is controlled by a 64K Intel 8080A micro-processor into which the assay parameters may be programmed. Clavulanic acid, dextrin, phosphate, ammonia and protein concentrations throughout the fermentations were determined in this way.

Chapter 9.4.1 Dextrin/total glucose assay

The assay is based on the CentrifiChem Glucose Test, (ref.; 27-010-800), in which glucose and ATP are converted to glucose-6-phosphate and ADP by hexokinase which may be activated by magnesium. Then, glucose-6-phosphate dehydrogenase converts the glucose-6-phosphate and NAD to 6-phosphogluconate and NADH. The amount of glucose present is measured by the increase in absorbance at 340nm of the NADH.

•Sample Preparation

To 1ml of broth sample 2ml of 1M hydrochloric acid were added in a glass universal bottle and the mixture was then solubilised by autoclaving for 10-15 minutes. After cooling, the sample was then made up to 10ml in a volumetric flask thus creating a 1 in 10 dilution. This can then be assayed directly.

•Assay method

The sample rotor was loaded with prepared samples and a glucose standard with a concentration of 1mg/ml of glucose. The reagent boat was filled with glucose reagent from the CentrifiChem test kit which had been reconstituted with 13.1ml of distilled water. The assay was then started and after approximately 5 minutes the results were automatically printed out.

Chapter 9.4.2 Protein assay

Both total and soluble protein were assayed. The assay is based on a modified version of the Lowry method which is a colormetric assay. The sample was prepared as described and added to the working reagent which reacts with the peptide bonds in the protein to yield a purple/blue complex, the colour of which can be intensified by the addition of the phenol reagent, Folin-Ciocalteu's reagent. The intensity of colour corresponds to the amount of peptide bonds present. The absorbance is read at 550-750nm and a standard of known concentration is used to determine the actual concentration.

•Sample Preparation

For total protein determination the sample was prepared in the same manner as for total glucose/dextrin. To 1ml of broth sample 2ml of 1M hydrochloric acid were added in a glass universal bottle and the mixture was then solubilised by autoclaving for 10-15 minutes. After cooling, the sample was then made up to 10ml in a volumetric flask thus creating a 1 in 10 dilution. The sample diluted by 1 in 20 making a total dilution of 1 in 200. For soluble protein determination the broth sample was centrifuged and then the supernatant diluted by 1 in 200.

•Assay method

The sample rotor was loaded with the diluted samples plus one standard containing 100μg/ml of Bovine Serum Albumin, (Sigma, UK). The reagent consisted of four solutions. Solution A consisted of 2% sodium carbonate in 0.1N sodium hydroxide, (20g of sodium carbonate plus 4g of sodium hydroxide in 1 l of water). Solutions B and C were made up of 1% copper sulphate and 2% sodium potassium tartrate respectively. Solution D, which is used for the actual assay, consists of 50ml of solution A plus 0.5ml

of solution B plus 0.5ml of solution C. Solution D was then placed in the reagent boat along with Folin Ciocalteu's phenol reagent, (BDH, UK).

Chapter 9.4.3 Phosphate assay

The assay is based on inorganic phosphate reacting with molybdate to form a stable, yellow heteropolyacid complex which has an absorption maximum in the ultraviolet region of the spectrum. The complex is quantitated at 340nm by quantifying the unreduced phosphomolybdate heteropolyacid. The assay used is based on the CentrifiChem Inorganic Phosphate Assay which is a modification of the Daly Ertingshausen method described above.

•Sample Preparation

Sample preparation was dependent on whether assaying for soluble or total phosphate. For soluble phosphate assays, the broth samples were centrifuged and the supernatants diluted 1 in 5. The diluted supernatants were then assayed directly. For total phosphate assays, the sample preparation was more complex. 0.1ml of whole broth sample was incubated with 0.1ml 2.5M sulphuric acid in a drying block at 190°C for 15 minutes. This was carried out in a fume cupboard. To the digested pellet 2.5ml of distilled water were added and incubated at room temperature for 30 minutes. This was made up to 10ml using distilled water and mixed well. This was then filtered through 0.8μm GF/F Whatman Filter paper using a Sartorius SM vacuum filter holder. The filtrate was assayed directly which corresponds to a sample dilution of 1 in 100.

•Assay method

The sample rotor was loaded with the samples and a standard of 1mM phosphate, (either sodium or potassium). The working reagent consisted of 0.5ml surfactant solution plus 13ml of inorganic phosphorous reagent mixture. The phosphorous test solution was made up of 1.1mM/l, of ammonium molybdate in 0.45M sulphuric acid. The surfactant solution was made up of 10ml of Tween 80, (polyoxyethylenesorbiton monooleate), dissolved in 20ml of distilled water.

Chapter 9.4.4 Ammonia assay

This assay is based on a reductive amination.

•Sample Preparation

Ammonia estimations were carried out on broth supernatants that had been diluted by 1 in 50. (This assay can be used on frozen samples which must be thawed, centrifuged and then diluted.

•Assay method

The rotor was loaded with the samples and a standard containing 18μg/ml ammonia concentration which was made up of 0.0165g ammonium sulphate dissolved in 250ml in deionised distilled water. Two reagents were required for the assay. An alkaline hypochlorite solution containing 5.0g of sodium hydroxide dissolved in 5.38ml sodium hypochlorite solution, made up to 500ml with distilled water and a phenol solution containing 15.5g of phenol plus 0.0625g of sodium nitroprusside dissolved in 500ml of deionised distilled water. 10μl of sample were diluted with 40μl of phenol solution to which were added 30μl of hypochlorite solution. This was incubated at 40°C and then read at 625nm.

Chapter 9.4.5 Clavulanic acid assay

Clavulanic acid levels were measured using the method described by Foulstone & Reading, (1982) as modified by Bird *et al.*, (1982). This is a spectrophotometric titration of the reaction product with imidazole.

•Sample Preparation

4ml of shaken whole broth were measured into Technicon cups and 25μl of concentrated acetic acid were added to each cup. The samples were centrifuged for 15 minutes at 3000rpm. The supernatant was then diluted 1 in 4 and assayed.

•Assay method

The rotor was loaded with the samples and a standard potassium clavulanate solution at a concentration of 600μg/ml. The reagent, 5% imidazole solution, was added to the reagent boat which was then incubated at 30°C for 4 minutes. The reading was taken at 313nm.

Chapter 9.5 Determination of morphology

Morphological measurements were made using image analysis according to the method described by Packer *et al.*, (1988), except that a semi-automatic method involving both automatic and manual manipulation was used. The microscope stage was moved automatically and for each field an editor was used in order to erase small particulate matter i.e. background noise which could obscure measurement. The main hyphal length, number of tips and branching length and frequency were measured in each sample. For each culture sample 10 slides were prepared and 5 fields were measured per slide.

The slides were prepared by first diluting the broth samples by 1 in 50 using distilled water. 100μl of diluted sample were then mounted on a clean microscope slide and left to air dry. Once dry, the sample was heat fixed and then stained with either methylene blue or Sudan black for approximately one hour. After this time the stain was washed off using distilled water and the slides were allowed to dry and heat fixed.

Chapter 10 5l batch fermentation of *Streptomyces clavuligerus*

Chapter 10.1 Fermentation materials

Chapter 10.1.1 Organism

An isolate of *Streptomyces clavuligerus* ATCC 27064, supplied by SmithKline Beecham Pharmaceuticals (Worthing, West Sussex, UK), was used for this work.

Chapter 10.1.2 Chemicals

The suppliers of the major medium components and chemicals are listed in table 10.1. All other chemicals were Analar grade and were obtained from Fisons Scientific (Loughborough, Leics., UK); BDH Chemicals (Merck art. 1076, Poole, Dorset, UK) and Sigma Co. (Poole, Dorset, UK). Chemicals were also the generous gift of SmithKline Beecham Pharmaceuticals, (Worthing, West Sussex, UK).

Chapter 10.2 Fermentation equipment

Initial fermentations were done using shake-flasks of 250ml and 2 litres. The shake flask studies were used as a means of developing and then optimising assays capable of monitoring the fermentations. Larger scale fermentations were carried out using 7l fermenters, with a 5l working volume, and the broths were assayed for growth, lipid/oil and lipase as well as dextrin, protein, phosphate, ammonia and clavulanic acid.

Chapter 10.2.1 Shake flask fermentation

Preliminary studies were carried out using 250ml shake flasks of 50ml medium volume. The major components of the medium, based on basic growth medium supplied by SmithKline Beecham, were 2.0% soya flour, 1.0% dextrin, 0.5% rape seed oil (w/v) and trace elements. The flasks were inoculated with 10µl of a *S.clavuligerus* spore

suspension at a concentration of 8.7×10^8 spores/ml. The cultures were incubated at 26°C for up to 96 hours on orbital shakers at 200rpm. Samples of broth were taken every 24 hours and assayed for lipase activity, lipid concentration and growth. Further experiments were conducted using 2l shake flasks containing 200ml medium volume.

Chapter 10.2.2 5l batch fermentation

Fermentations were carried out using a 7l LH fermenter series 210, (LH Fermentation Ltd, UK), using a working volume of 5l. The top plate contained eight openings for probes, acid/base addition, antifoam addition, air inlet/outlet and a sample line. Two Rushton turbines were mounted in the stirrer shaft and the vessel contained four baffles. The geometric ratios were:

$\frac{H_L}{T} = 1.86$ $\frac{D}{T} = 0.45$ $\frac{H_I}{T} = 0.76$

The fermenter instrumentation consisted of an Ingold steam-sterilisable dissolved oxygen tension probe with the following specifications: oxygen electrode, 320mm, with 4 pole screw cap, cap nut and connecting cable with 4 pole plug in socket. The pH was measured with an Ingold steam-sterilisable insertion probe (type 764-31). The collection, storage and processing of the on-line data and derived data from the fermenter and the mass spectrometer was carried out using a computer system called RT DAS.

The gas analysis was either a VG Gas Analysis Ltd MMG-80 or Prima mass spectrometer which was used to sample carbon dioxide (CO_2), oxygen (O_2), nitrogen (N_2) and argon (Ar) in the fermenter off-gas every 15 minutes. The mass spectrometer was connected to the aforementioned computer system. The data was processed and the oxygen uptake rate, (OUR, mmolO_2/l/h), carbon dioxide evolution rate, (CER, mmolCO_2/l/h), and the respiratory quotient, (RQ), calculated.

TABLE 10.1 **Suppliers of media components and chemicals**

Constituents	Suppliers
Sodium Dodecyl sulphate	BDH (UK).
Dextrin	Sigma (UK)
Rape Seed Oil	SmithKline Beecham (UK)
Soya Flour	SmithKline Beecham (UK)
MOPS	Sigma (UK)
Magnesium Sulphate	Sigma (UK)
Zinc Sulphate	Sigma (UK)
Potassium Dihydrogen Orthophosphate	Fisons (UK)
Soluble Starch	Fisons (UK)
Bacteriological Peptone	Oxoid (UK)
Potato Dextrose Broth	Oxoid (UK)
Technical Agar No1	Oxoid (UK)
Triolein	BDH (UK)
Tween-80	BDH (UK)
Olive Oil	Fisons (UK)
Tributyrin	Sigma (UK)
Gum Arabic	BDH (UK)
Sodium Chloride	Fisons (UK)
Glycerol	Fisons (UK)
Sodium Hydroxide	BDH (UK)
Petroleum Ether	BDH (UK)
Diethyl Ether	BDH (UK)
Acetic Acid	BDH (UK)
Chloroform	BDH (UK)
Methanol	BDH (UK)
Vanillin	Sigma (UK)
Orthophosphoric Acid	Fisons (UK)
Trichloroacetic Acid	BDH (UK)
Diphenylamine Reagent	Sigma (UK)

Chapter 10.3 Fermentation methods

Chapter 10.3.1 Spore production and storage

An isolate of *Streptomyces clavuligerus* was grown on an agar slant in a Thompson bottle of solid growth and sporulation medium with the composition shown below and the pH was adjusted to 7.0 before sterilisation at 121°C for 20 minutes.

Dextrin	1.0% w/v
Phosphate	0.1% w/v
Magnesium sulphate	0.1% w/v
Ammonium sulphate	0.1% w/v
Sodium chloride	0.1% w/v
Calcium carbonate	0.4% w/v
Trace elements	0.1% w/v
Agar	3.0% w/v

After incubation at 26°C for 7 days, the spores were harvested using the following procedure. 2.0g of sterile glass beads of 5mm diameter were added to the slants and gently rolled over the surface of the slants to remove the spores onto the beads. A few millilitres of a solution containing 0.05% w/v of Triton X-100 and 0.85% w/v of sodium chloride were added, in order to wet the spores. The slant was then flooded with 200ml of 10% sucrose solution in order to suspend the spores. Then, 2.5-3.0 ml aliquots were aseptically into sterile Bijou bottles. These can be stored as frozen stocks at -70°C.

Chapter 10.3.2 Spore suspension sterility control

Spore suspensions were serially diluted in ten fold dilutions to a final dilution of 1 in 10^8 using distilled water, and were then plated onto Petri dishes containing growth and sporulation medium, (chapter 10.3.1). The Petri dishes were incubated at 26°C for 3 days. Microscopic observation at this stage, with staining, allow viability checks to be made.

0.1ml aliquots of spore suspension were also used to inoculate Petri dishes with nutrient agar, (28g/l; pH adjusted to 7.0 before sterilisation at121°C for 15 minutes). The Petri dishes were incubated at 37°C for 3 days. The spore suspension could be classed as sterile if there was no contamination in either the growth/sporulation medium or the nutrient agar.

Chapter 10.3.3 Seed preparation

The inoculum used for the 5l fermentation was a culture of *S. clavuligerus* grown in a seed medium with the following composition: (%, w/v):

Soya flour	2.00
Dextrin	1.00
Rape seed oil	0.50
Phosphate	0.06

The seeds were grown in 2l shake flasks with 200ml medium volume and were inoculated with 100µl of a 8 x 10^8 spores/ml spore suspension which had already been tested for sterility. The cultures were incubated at 26°C for 48 hours to allow the organism to reach maximum growth phase and therefore, actively growing biomass seed was used. The broths were assayed for lipase before inoculation into the fermenter.

Chapter 10.3.4 Fermentation media

Three different media were used in order to observe variations in growth and lipid utilisation rate depending on composition. The composition of the media used was based on the industrial process medium and each is outlined below. The quantities are given in % w/v.

- **Complex medium**

Soya flour	3.50
RSO	2.30
MOPS	1.05
Dextrin	1.00
Trace elements	1.00
Phosphate	0.12

- **Soluble medium**

Dextrin	3.40
RSO	2.30
Bacteriological peptone	2.20
Soluble starch	1.30
MOPS	1.05
Trace elements	1.00
Magnesium sulphate	0.25
Phosphate	0.12

- **Oil medium**

  -the same as the complex medium except that the dextrin was replaced by RSO on a weight for weight basis.

Following initial fermentations, the composition of each media was altered by variation of oil concentration by +/-10g/l. The media were adjusted to pH 7.2 before sterilising at 121°C for 20 minutes and after sterilisation the pH was approximately pH 7.0-7.1. Steam sterilisation was carried out in situ.

Chapter 10.3.5 Fermentation conditions

The conditions for the fermentation were the same as those for shake-flask experiments and the same irrespective of the medium in use. The temperature was maintained at 26°C. The pH was maintained at 6.8 initially using a 30% solution of ammonia, which was changed to 0.01M potassium hydroxide during more detailed metabolic studies.

Initial experiments were carried out using a stirrer speed of 500rpm but this was found to inappropriate for the fermentation since increasing the stirrer speed was necessary in order to maintain a sufficient oxygen concentration for growth. Therefore, various other speeds were tried and the speed was set at 625rpm. At speeds higher there was a significant foaming problem which could not be controlled using an antifoam feed. The antifoam, as supplied by SmithKline Beecham (UK), was aseptically added in batch at the start of the fermentation at a concentration of 1ml per litre working volume.

Having tested a range of air flow rates from 0.25vvm to 1.5vvm, the air flow rate was set at 1vvm. At this level, the oxygen concentration could be maintained for the duration of the fermentation (Chapter 9). Although there was slight foaming, this could be controlled by the addition of an antifoam The culture was grown until no further increase in growth was observed/nutrient exhaustion, (approximately 50 hours), determined by dextrin and lipid analysis. Broth samples were taken and assayed as described in the next chapter. In later experiments both the stirrer speed and the air flow rate were monitored and controlled in order to observe the effect of both dissolved oxygen concentration within the fermentation broth and shearing on the expression lipolytic activity.

Chapter 11 Oil droplet size distribution in oil/water dispersions

Oil-liquid phase dispersion is of key importance throughout this study since it involves fermentation in which a dispersed oil phase is utilised as a main carbon source. The size and size distribution of the droplets will determine not only the stability of the dispersion and the efficiency of contact but will also influence the utilisation of oil since lipase activation requires the presence of the oil/water interface.

Stamatoudis & Tavlarides, (1987) measured the droplet size distribution during the initial period of liquid-liquid dispersion in mechanically agitated vessels using a xylene/water system. These studies showed that following the step change in agitation the droplet size distribution changes gradually and the equilibrium droplet size is reached over several minutes. Figure 11.1 shows the measurements of droplet size distribution carried out during the study for oil/water dispersion for an oil concentration of 2.5% v/v.

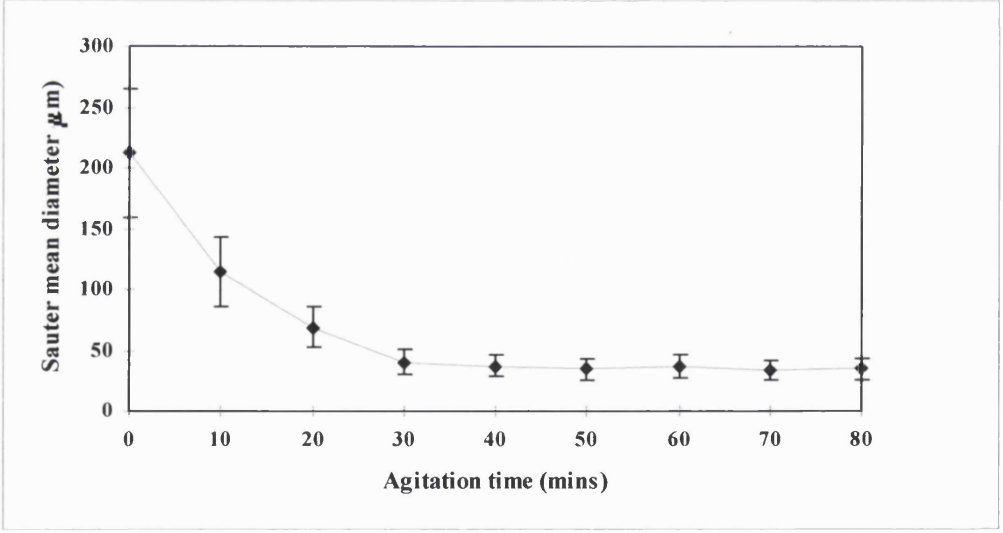


FIGURE 11.1 Average transient droplet size distribution for a 2.5% v/v oil/water dispersion at varying impeller speeds and an impeller diameter of 0.05m

The plot shown in figure 11.1 indicates that the equilibrium droplet size distribution is established after approximately thirty minutes of agitation irrespective of the degree of agitation. During the first twenty minutes agitation there is a rapid decrease in the oil droplet size with the Sauter mean diameter decreasing up to three fold from >200μm to approximately 70μm. After thirty minutes, when the dispersion has established equilibrium, the measured Sauter mean diameter was approximately 40μm. After forty minutes there is no further change in the oil droplet size distribution and the Sauter mean diameter is relatively constant over the following 30 minutes. Based on these observations a minimum period of sixty minutes of agitation was considered sufficient for the establishment of the final droplet size distribution and was therefore used in all of the experiments reported in the present study.

Chapter 11.1 Oil/water dispersion stability

Oil and water do not readily mix in the absence of surfactants and for the purpose of the study SDS, an anionic surface active agent, was added in order to stabilise the dispersion. On stopping agitation, samples were taken every twenty minutes over a period of 3 hours and the oil droplet size determined for a dispersion with an oil concentration of 2.5% v/v at an impeller speed of 450rpm. In this way the stability of the dispersion may be determined.

On ceasing agitation the dispersion does not immediately separate out into two distinct phases of oil and water but forms an emulsion where the excess water settles on the bottom of the tank. The emulsion formed was relatively stable and showed no significant change in structure for one-two hours after stopping agitation. The presence and stability of the emulsion was made possible by the presence of the surfactant, SDS, acting on the dispersion.

Figure 11.2 illustrates the variation in droplet size of triplicate samples over the time stated. The plot shows that the overall mean oil droplet size increases with time having ceased agitation although there appears not to be a direct linear relationship between time and droplet size. Over the first 50 to 60 minutes there was very little change in the Sauter mean diameter of the oil droplet, only increasing from 20μm to 23μm. However, after this initial period there is a rapid increase in the Sauter mean diameter until after 150 minutes there is a two fold increase in droplet size.

By 180 minutes the Sauter mean diameter of the oil droplet has increased to 47μm. This increase in size is caused by coalescence of the oil droplets. This result demonstrates that although the addition of SDS will help to stabilise the dispersion, its' effect is limited and therefore, samples should be analysed within a maximum time of 20 minutes in order to achieve an accurate droplet size distribution.

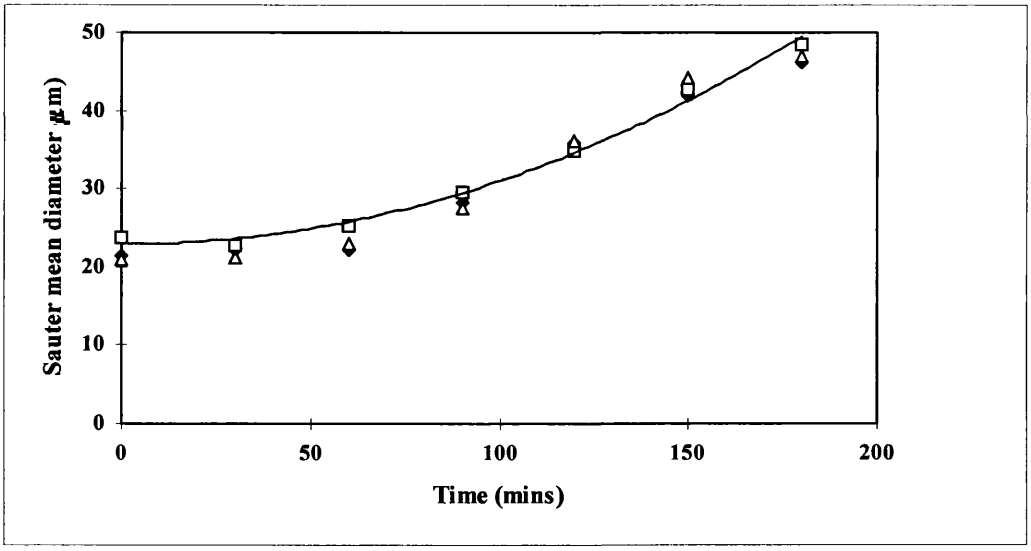


FIGURE 11.2 Stability of oil/water dispersion with respect oil droplet size at an oil concentration of 2.5% v/v and an impeller speed of 450rpm

Chapter 11.2 Oil droplet size distribution of a 2.5% v/v oil/water dispersion

The oil droplet size distribution of a 2.5% v/v oil/water dispersion was measured, after 60 minutes agitation, at 250, 350, 450, 550, 650 and 750rpm respectively. The resultant distribution curves are shown in figures 11.3 and 11.4. Each graph shows the oil droplet size against the proportion of readings that were measured at that particular size. In this way a size distribution curve is obtained. From the data obtained the Sauter mean diameter, D_{32}, can be calculated which can be used to determine the average interfacial area per unit volume.

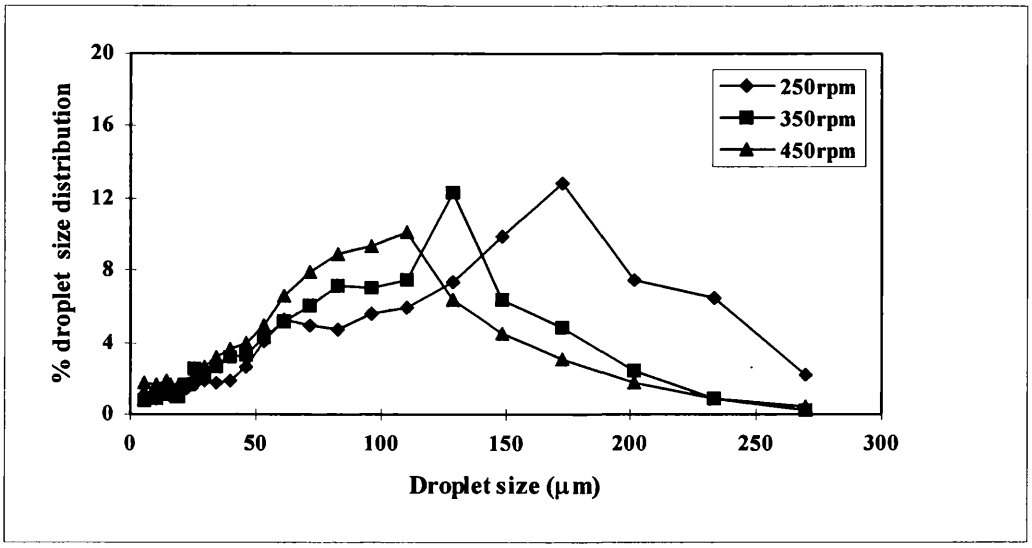


FIGURE 11.3 Oil droplet size distribution in an oil/water dispersion at an oil concentration of 2.5% v/v and impeller speeds of 250, 350 and 450rpm

The plots show an overall trend of decreasing droplet size with increasing impeller speed with the modal oil droplet size decreasing from 160μm at 250rpm to 15-20μm at 750rpm. The results also illustrate the various stages in droplet breakage that is signified by the non-uniform distribution with respect to oil droplet size. This is most clearly demonstrated in figure 11.3 where each of the plots appear to have three distinct phases of size distribution.

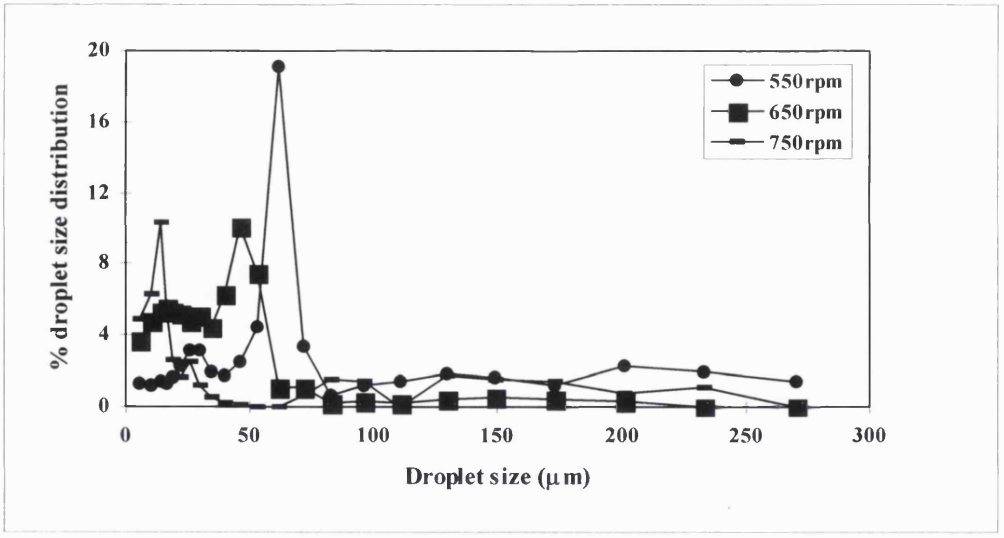


FIGURE 11.4 Oil droplet size distribution in an oil/water dispersion at an oil concentration of 2.5% v/v and impeller speeds of 550, 650 and 750rpm

The oil droplet size distribution at an impeller speed of 250rpm has an initial peak in droplet size at 160μm with a frequency 12%. There is then a rapid decrease in droplet size until a steady state is reached between 110μm and 60μm, (7%), signified by a plateau in the distribution curve. This is followed by a second decrease in droplet size reaching a steady state between 40μm and 20μm, (3%). The oil droplet size remains relatively stable after this second breakdown phase. A similar pattern can be seen at 350rpm and 450rpm with initial peaks in droplet size at 125μm and 110μm, followed by a decrease in size reaching plateaus between 110μm and 90μm and 100μm and 80μm respectively. The droplets are then further broken further until reaching a steady state between 20μm and 40μm irrespective of impeller speed.

Figure 11.4 demonstrates a similar trend at higher impeller speeds although to a lesser extent. The plot shows a definite bimodal distribution at both 550 and 650rpm with initial peaks in distribution at 60μm and 50μm respectively followed by droplet breakage resulting in a final droplet size of between 20μm and 30μm irrespective of impeller speed. At 750rpm there is only one peak distribution between 15μm and 20μm. However, the plot shows a significant increase in the droplet size percentage value at 550rpm. This suggests that oil droplet breakage is stirrer speed dependent such that increasing the Reynolds number induces the next breakage stage.

These results show that the only variation in oil droplet size distribution between agitation rates appears to be the initial peak in droplet size which decreases with increasing impeller speed. When the oil is dispersed in water the oil undergoes instantaneous breakage, or primary breakage, due to mixing effects which results in the formation of large oil droplets. This initial breakage is dependent to a certain extent on impeller speed employed as well as the properties of the two phases.

The droplets are then subjected to deformation due to rotational directions resulting in further droplet breakage and after continued mixing the droplets are broken down even further due to local shearing action and/or pressure fluctuations. These pressure fluctuations arise from the instantaneous turbulent velocity differences acting on the oil droplet and may be either shear or normal depending on the direction of the velocities influencing the oil droplets (Shamlou *et al.*, 1994). The secondary breakage stages occur at a slower rate and the number of droplets remains constant which accounts for the plateau regions seen in the distribution curves. Droplet breakage will continue until a dynamic equilibrium is reached.

The droplet breakage will be dependent on the physical properties of the dispersed phase as mentioned previously, such as the oil viscosity, density and surface tension. The cohesive forces which are dominant, are viscosity of the droplet and the interfacial tension. If the viscosity of the dispersed phase is greater or equal to the viscosity of the continuous phase, then the viscous forces over a distance less than or equal to the droplet diameter, are minimal. Therefore, surface tension is the dominant force holding the oil droplet together and will oppose droplet breakage.

The force around the circumference of the droplet, F_d, resisting breakup can be defined as;

$$F_d = 2 . \Pi . a . \sigma \qquad \textbf{(11.1)}$$

where a is the radius of the droplet and σ is the interfacial tension. Whilst the pressure holding the droplet together, P_d, can be defined as;

$$P_d = \frac{2 . \Pi . a . \sigma}{\Pi . a^2} \qquad (11.2)$$

$$P_d = \frac{2 . \sigma}{a} = \frac{4 . \sigma}{d}$$

where d is the droplet diameter. The droplet is stable when the pressure fluctuations causing break up of the droplet are less than or equal to

$$\frac{4 . \sigma}{d}$$

Droplet coalescence must also be considered when discussing the oil droplet size distribution and can be influenced by both random collisions and gravity effects (Nishikawa *et al.*, 1987). Each dispersion will have a maximum stable droplet size, Dc. If the initial droplet size, D_0, is greater than the stable droplet size, the oil droplet will undergo breakage until D_0 = Dc. In regions of higher turbulence breakage results in a larger number of oil droplets and due to increased collision frequency, the oil droplets will coalesce. In turn, coalescence will result in droplets which are greater in size than Dc and therefore undergo breakage once again. This results in a localised coalescence-breakage equilibrium and can be linked with the secondary breakdown stages of droplet breakage.

Further analysis of the distribution curves allows the calculation of the Sauter mean diameter, as mentioned previously. Figure 11.5 shows the Sauter mean diameter plotted against the rotational speed for a 2.5% v/v oil/water dispersion. There appears to be a linear relationship between impeller speed and oil droplet size which decreases with increasing agitation rate. This suggests that the flow regime in the tank was sufficiently turbulent and maintained Newtonian flow properties.

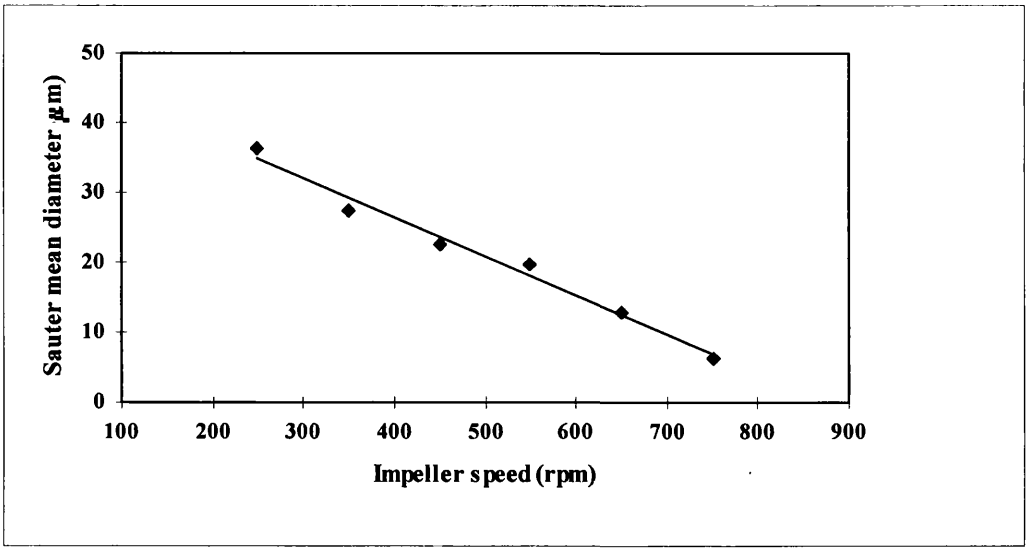


FIGURE 11.5 Effect of increasing impeller speed on oil droplet size in a 2.5% v/v oil/water dispersion with an impeller diameter of 0.05m

By comparison with the power input, P/V, a different trend is observed (figure 11.6). The power input can be calculated according to;

$$\frac{P}{V} = \frac{4 . k . \rho_c . N^3 . Di^5}{g . \Pi . T^3} \qquad (11.3)$$

where k is the proportionality constant, (6.3 for a Rushton turbine); ρ_c is the density of the continuous phase; N is the impeller tip speed; Di is the impeller diameter; g is the gravitational force and T is the tank diameter. This demonstrates that there is a non-linear relationship between droplet size and power input and therefore, drop breakage would appear to be dependent solely on impeller speed and impeller diameter but also on physical parameters such as interfacial tension. This also supports droplet distribution data as illustrated in figure 11.4.

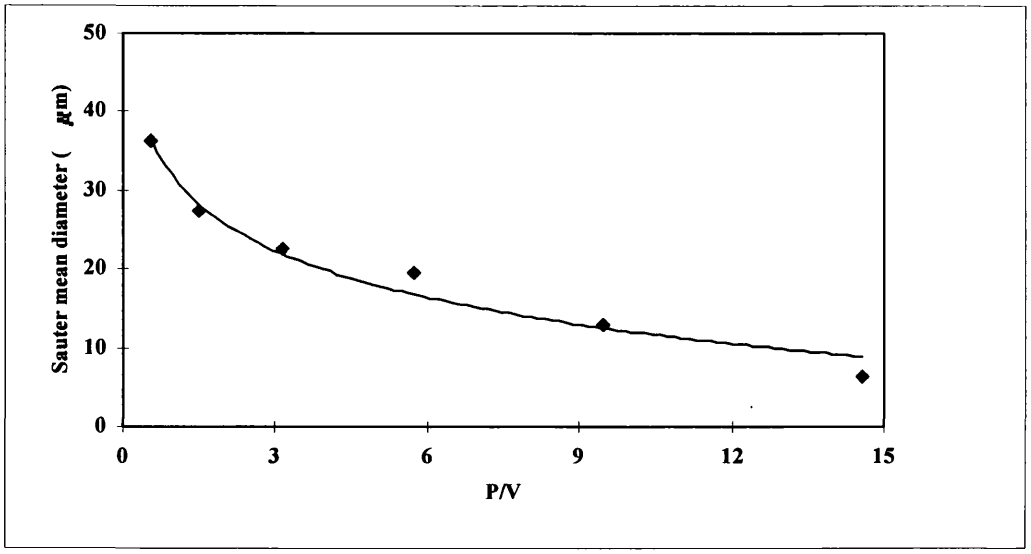


FIGURE 11.6 The relationship between oil droplet size in a 2.5% v/v oil/water dispersion and power input, with an impeller diameter of 0.05m

Chapter 11.3 The effect of medium composition on oil size distribution in oil/water dispersions

The oil droplet size distribution in an oil/water dispersion will be affected by many factors such as the concentration of oil; the impeller speed and power input as well as the interfacial forces resisting oil droplet breakup (chapter 11.1). The presence of other particulate matter within the dispersion, as is the situation within the fermentation broth, may also affect the oil droplet size distribution for a variety of reasons. For example, there may be droplet/particulate interaction resulting in an increase in Sauter mean diameter or the particulate matter may have surfactant effects which would affect the interfacial forces resisting breakup and reduce the Sauter mean diameter of the oil droplets (Ohta *et al.*, 1995).

Any changes in the viscosity within the mixture will also affect droplet size, (Nishikawa *et al.*, 1987),. The effect of viscosity of the continuous phase on the droplet size, where the viscosity of the dispersed phase is fixed, can be correlated by;

$$D_{32} \propto (\mu_d/\mu_c)^{1/5} \qquad \textbf{(11.4)}$$

where μ_d is the viscosity of the dispersed phase and μ_c is the viscosity of the continuous phase. However, if the viscosity of the dispersed phase changes, whilst the viscosity of the dispersed phase remains constant, the relationship changes (Yamaguchi *et al.*, 1963).

$$D_{32} \propto (\mu_d/\mu_c)^{1/8} \quad \textbf{(11.5)}$$

Therefore, the presence of other matter such as medium components, whether soluble or insoluble, may affect the efficiency of oil uptake in the fermenter environment.

The effect of major fermentation medium components, soya flour and dextrin, on oil droplet distribution were investigated. Initial experiments were carried out using the equivalent medium concentrations but extensive foaming occurred even at low impeller speeds. Although the addition of an antifoam would have reduced the level of foaming it was thought that this would also have affected the oil droplet size distribution as well as oil dispersion due to changes to the interfacial tension between the oil and water. Therefore, dextrin was used at a concentration of 5% and soya flour at1% w/v.

Initially, particle size distribution was determined for dextrin/water dispersions and soya flour/water dispersions at a range of impeller speeds. Each component was then added separately to a 2.5% v/v oil/water dispersion in order to observe the effect on oil droplet size distribution. The addition of dextrin, one of the main carbon sources used in the fermentation medium, appeared to have very little effect on droplet size. The dextrin was completely soluble in the water and hence there was direct contact between the oil droplets and dextrin particles reducing possible interfacial interactions.

Figures 11.7 and 11.8 show the particle size distribution of a 1% w/v soya flour/water dispersion at a range of stirrer speeds. At 250rpm stirrer speed, due to the insoluble nature of the soya flour, there was incomplete dispersion of the soya flour resulting in clouding of the lens and noisy measurements. After 90 minutes agitation an equilibrium had still not been reached and therefore no results are represented on either of the plots.

The plots show, as observed with oil/water dispersions, a bimodal particle size distribution with an initial peak in particle size at 30 to 35μm equivalent to the Sauter mean diameter and a modal particle size of between 60 and 65μm irrespective of stirrer

speed. However, at the higher impeller speeds there is a shift in overall particle size distribution. This can best be observed at 650rpm and 750rpm where there is a rapid decline in the percentage of particles above the modal particle size of 60μm and less than 10% of the particles being greater than 150μm diameter. At the lower speeds there is a broader distribution with more than 50% of the particles being 60μm or greater although this shift in distribution has very little effect on the average particle size of 30-35μm.

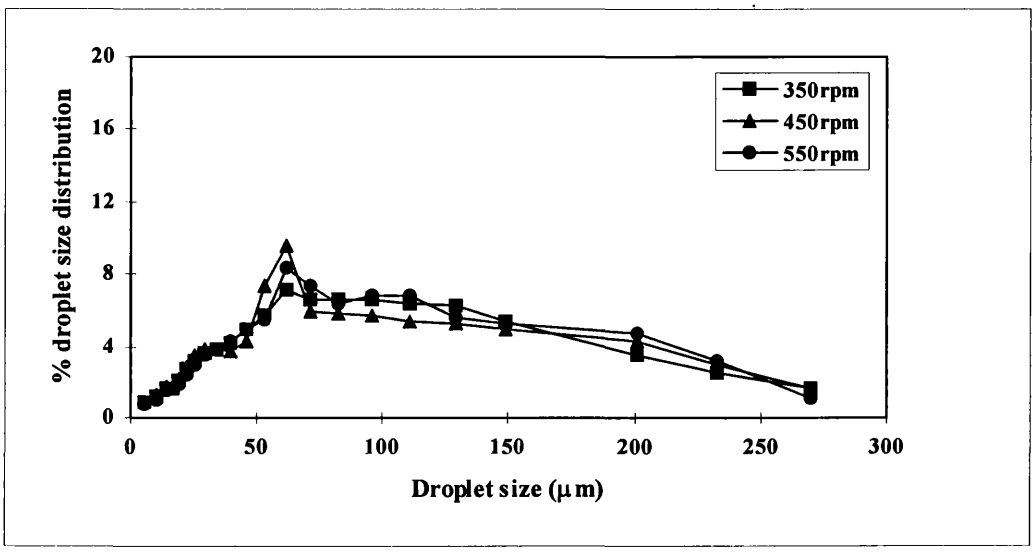


FIGURE 11.7 Soya flour particle size distribution in a soya flour/water dispersion at a concentration of 1% w/v and at impeller speeds of 350, 450 and 550rpm

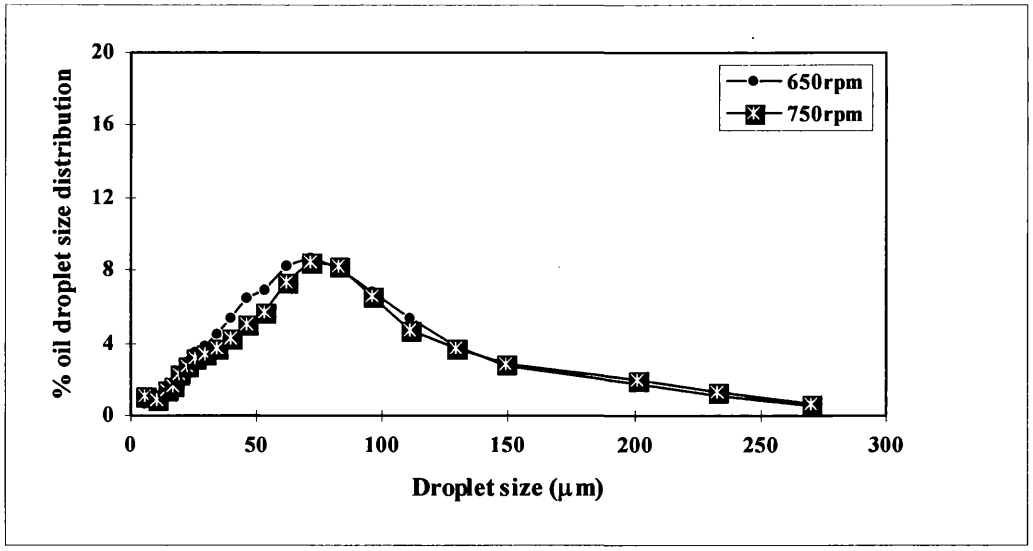


FIGURE 11.8 Soya flour particle size distribution in a soya flour/water dispersion at a concentration of 1% w/v and impeller speeds of 650 and 750rpm

This demonstrates that although the soya flour particles are initially subject to deformation due to rotational directions, at lower stirrer speeds further particle breakage due local shearing and pressure fluctuations is limited which may be partially due to incomplete dispersion of the soya flour. Bell & Dunnill, (1982) demonstrated that soya protein precipitate particle size was influenced by the extent and duration of agitation. However, they also determined that the dominant particle breakup mechanism was fragmentation and the size of the fragments was also dependent on the agitation and shear rates. Therefore, the size distribution of soya flour particles is influenced by the flow regime but this in turn is less dependent on the agitation rate and geometry of the vessel and more by the properties of the soya flour particles.

Figures 11.9 and 11.10 show a comparison in particle/droplet size distribution between oil/water and oil/soya flour/water dispersions at 350rpm and 450rpm respectively. Size distribution measurements at higher impeller speeds were once again not possible due to extensive foaming and hence no results are illustrated.

The plots demonstrate a change in particle size distribution in the presence of soya flour. At both 350rpm and 450rpm, although more pronounced at 450rpm, there is a peak at approximately 60μm, as previously observed in the soya flour/water dispersions, which represents the soya flour particle size. A second peak at 150μm can also observed at

both impeller speeds which has not been seen previously and does not correspond to either oil droplets or soya flour particles individually. However, this second peak would appear to equal the sum of the modal particle sizes for oil and soya flour, respectively, at both impeller speeds.

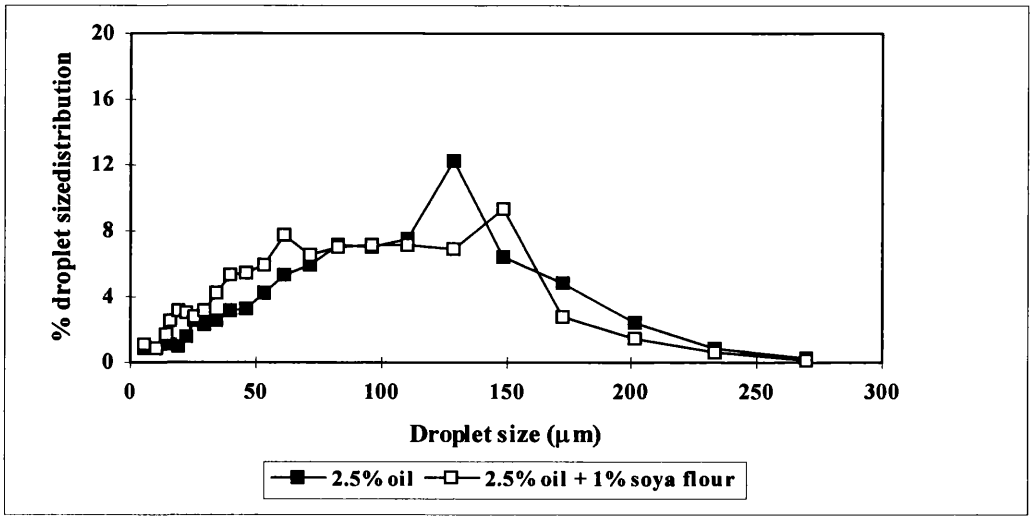


FIGURE 11.9 Effect of 1% soya flour on oil droplet size distribution in an oil/water dispersion at an oil concentration of 2.5% v/v and an impeller speed of 350rpm

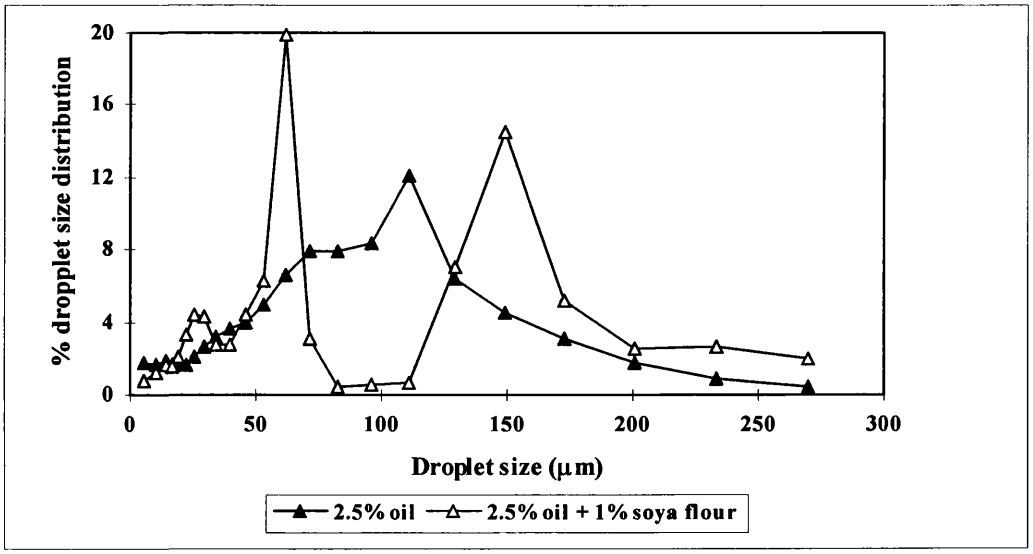


FIGURE 11.10 Effect of 1% soya flour on oil droplet size distribution in an oil/water dispersion at an oil concentration of 2.5% v/v and an impeller speed of 450rpm

As observed previously, there is a significant change in the droplet size percentage value at 450rpm (figure 11.10). This reiterates the theory that droplet breakage is dependent on stirrer speed and that a "threshold" speed induces the next or different breakage stage.

There does not appear to be a peak in particle size equivalent to the oil droplets which indicates a change in oil droplet size distribution in the presence of soya flour. These results suggest that there is an interaction between the soya flour particles and the oil droplets which is more pronounced at higher stirrer speeds. This may be due to increased dispersion and hence particle/droplet contact.

One explanation for this observed increase in droplet size could be due to a physical interaction between the oil and the soya flour. For example, the oil may be coating the soya flour particle forming a thin layer surrounding the particle which would result in the formation of a larger particle. This would depend on the affinity between the oil droplet and the soya flour particle i.e. the surface properties of the two components. This would also provide barrier to further fragmentation or breakup of the soya flour particles as well as making the oil less susceptible to agitation effects. It is possible that this would alter the interfacial forces of the particles thus increasing resistance to breakup. It is possible that the reverse could be occurring. The soya flour particles could adhere to the surface of the oil droplets, as with colloidal solids such as mustard, again resulting in the formation of larger particles/droplets. Further photographic measurements of the particles could help to determine this effect since it has been observed that duplex structures can be formed (Brooks & Richmond, 1991 and Matsumoto, 1993).

The second possibility is that the proteinaceous nature of the soya flour is promoting the coalescence of the oil droplets resulting in the increase in droplet size. However, this would seem unlikely since studies have shown that soya flour actually plays an important role in the emuslification of oil in the fermenter environment resulting in an increased level of oil consumption (Ohta *et al.*, 1995). Therefore, smaller oil droplets would have been observed during these experiments although this is not an accurate representation of the situation occurring during fermentation.

Either of these interactions would have a significant effect if transferred to the fermenter environment. If the oil were to adhere to the soya flour particles it would make the oil inaccessible for lipase activation and hence reduce the level of oil utilisation. This may be counteracted by the emulsification effects of the soya flour or other medium components. Therefore, these initial findings suggest that the composition of the fermentation medium will affect not only the mixing characteristics and the dispersion of the oil within the fermenter environment but the actual size of the oil droplets formed and hence lipolytic activity.

Chapter 12 Lipase determination

Lipases catalyse the hydrolysis of lipids/oils to their constituent fatty acids and glycerol. During the fermentation of *Streptomyces clavuligerus* using rape seed oil, lipases are produced to enable the metabolism of the oil and therefore, in order to monitor the progress of the fermentation, it is necessary to measure the amount of lipase present. Various methods available for assaying lipolytic activity were assessed in order to determine the most suitable method for this study. The methods were then to obtain consistent results and the effect of various parameters, such as pH, temperature and reaction substrates, investigated. The results obtained are detailed in the forthcoming chapter.

Chapter 12.1 Agar plate method

The lipase activity toward olive oil, tributyrin, rape seed oil and triolein/Tween 80 was determined according to the method described by Arima *et al.*, (1972). The oils were selected based on structure with respect to fatty acid composition i.e. chain length. The agar plate assay relies on the lipase present in a sample utilising oil in a solid agar medium which results in clear zones around the sample point. The clearance zones are proportional to the lipolytic activity of the sample. Initially, standard solutions of known lipase concentrations were assayed in order to obtain a standard graph on which to base lipolytic activities. The activity was measured after the plates had been incubated for 72-120 hours at 26, 30 and 37°C. The standard lipase solutions were made from a purified lipase from *Candida cylindricae* as supplied by Sigma.

Figure 12.1 illustrates the effect of substrate and temperature on lipolytic activity indicated by the relative clearance zones after 96 hours incubation. The results from the use of rape seed oil substrate are not given since after incubation for 120 hours no distinct clearance zones were formed. This was due to problems with emulsification of the oil into the agar medium and hence it remained as large globules and was not evenly distributed throughout the agar medium. This demonstrates that the use of rape seed oil as a potential substrate for lipase determination, with this method, is not possible.

Results are not shown either for plates incubated at 37°C for a number of reasons. When agar plates with tributyrin and triolein/Tween 80 substrates were incubated at 37°C, the rate of clearance was significantly faster than at lower temperatures and resulted in indiscrete clearance zones which varied little in size with increasing lipolytic activity. Therefore, the determination of lipolytic activity, especially at lower lipase concentration, was relatively inaccurate. When agar plates with olive oil substrate were incubated at 37°C there was no change in the rate of zone clearance which suggests that the substrate used is the key parameter in this assay.

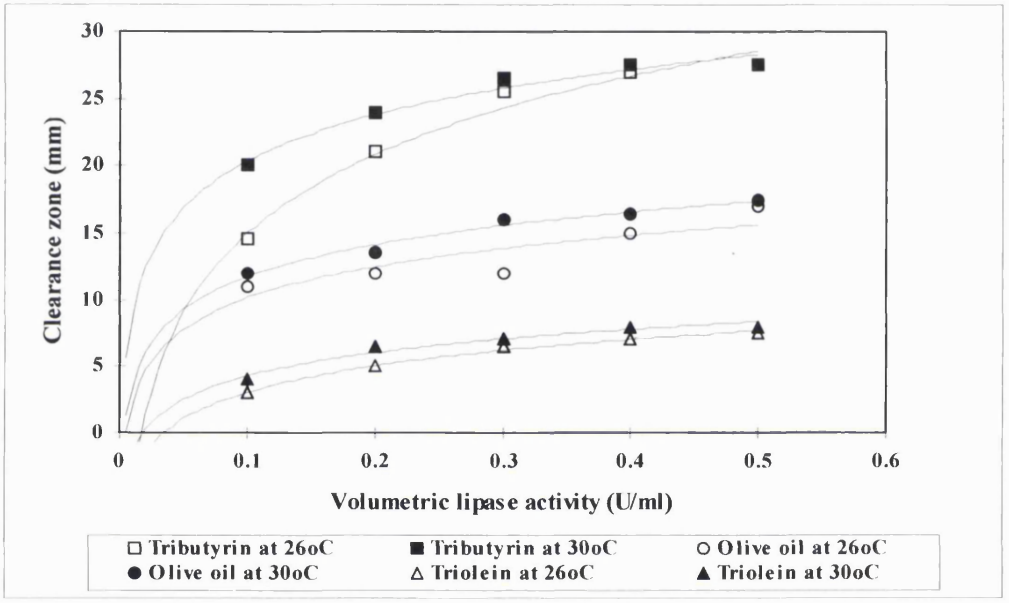


FIGURE 12.1 The effect of substrate and temperature on lipolytic activity with the agar method for lipase determination

Figure 12.1 indicates that there is a non-linear relationship between the size of clearance zone and lipase concentration which is to be expected since the assay relies on diffusion which is a log function. Between 0 an 0.1U/ml lipase activity there is a lag period before the appearance of the clearance zone regardless of substrate or temperature. The zones which are formed are irregular and indiscrete. This may be due to the manner of substrate emulsion which results in a delay in activation of the lipase or the use of each specific substrate requires the lipase to be induced. Therefore, determination of lipase activities below 0.1U/ml are inaccurate which has the overall effect of reducing the sensitivity of the assay.

From the plots it would appear that the temperature at which the plates were incubated had a minimal effect on the final result of the assay since there was very little variance in the clearance zones formed. The plates incubated at the higher temperature initially resulted in the faster development of clearance zones but at lipase concentrations greater than 0.2U/ml the difference in the clearance zones was minimal. This trend was observed regardless of the substrate used.

The results do, however, demonstrate that the choice of substrate for the assay is crucial with respect to both the accuracy and sensitivity of the assay. The use of a substrate of tributyrin resulted in the formation of the largest and most distinct clearance zones but below a lipase concentration of 0.1U/ml the clearance zones were indiscrete and difficult to measure accurately. The use of olive oil resulted in discrete measurable clearance zones but as the lipase concentration increased the difference in clearance zone was less significant and therefore, determination of activity became less accurate at higher lipase concentration. Again the measurement of lipase activity below a lipase concentration of 0.1U/ml was inaccurate. However, with the use of triolein lipase activity could be measured at concentrations between 0.05 and 0.1U/ml but above 0.2U/ml measurement became less accurate due to smaller clearance zones which varied little between lipase concentration.

The effect of the substrate is dependent on emulsification. The tributyrin was completely emulsified in the agar and was evenly dispersed through the agar whereas when triolein was added large droplets were formed in the agar despite the addition of Tween 80, an emulsifier. Emulsification problems were again encountered with olive oil although to a lesser extent. Therefore, the results suggest that this method could be used to determine lipase activity if a suitable substrate is used such as tributyrin or olive oil at 30 or 37°C.

Having obtained the standard plots for lipase activity, samples of *Streptomyces clavuligerus* fermentation broth were assayed after 96 hours growth on rape seed oil. In order to be utilising the oil the organism would have to produce a lipase and therefore the broth samples were assumed to be lipolytically active. The samples were assayed, in a minimum of triplicate, using tributyrin, olive oil and triolein/Tween 80 at 26 and 30°C

and the lipase activity determined after 96 hours incubation. The results obtained are given in table 12.1.

TABLE 12.1 Temperature and substrate effects on the assay of *S. clavuligerus* fermentation broth for lipase activity using the agar method

Temperature (°C)	Substrate	Clearance zone (mm)	Lipase activity (U/ml)
26	tributyrin	24	0.25
26	olive oil	14	0.2
26	triolein	3	0.1
30	tributyrin	26	0.4
30	olive oil	16	0.35
30	triolein	4	0.12

The results demonstrate that the lipase produced by *S.clavuligerus* could be detected using the agar method but as with the lipase standard the actual activity measured was dependent on the temperature and the substrate used. This effect is amplified due to the unknown specificity of the lipase produced by the organism. The lipase showed maximum activity of 0.4U/ml toward tributyrin when incubated at 30°C but when incubated at 26°C there was a 35% decrease in measured lipase activity. The lipase was also active toward olive oil although to a lesser extent and demonstrated a similar temperature effect. However, the lipase exhibited very low activity towards triolein/Tween 80 which could be due to either lipase specificity or substrate emulsification problems.

In summary, the agar method could be used for the determination of lipase activity in fermentation broth with a tributyrin substrate at 30°C. However, the method lacks accuracy and reproducibility and therefore, would be best used as an indicator for the presence of lipase i.e. a quantitative method as opposed to a qualitative method.

Chapter 12.2 p-nitrophenyl palmitate assay

The assay of lipase activity with p-nitrophenyl palmitate, (pNPP), as a substrate was measured according to the method of Winkler & Stuckman, (1979). This is a spectrophotometric method in which a pNPP containing emulsion reacts with a lipase enzyme to produce p-nitrophenol resulting in the formation of a pink complex. The intensity of the colour formed is proportional to the amount of enzyme present. One unit of lipase activity was defined as the amount of enzyme that liberated 1μmol p-nitrophenol per minute at 37°C under given conditions.

Initially, standard solutions of known lipase concentrations were assayed in order to obtain a standard graph with a comparison of measured lipase activity with known concentration. The standard lipase solutions were made from a purified lipase from *Candida cylindricae* as supplied by Sigma. The results of the assay are shown in figure 12.2.

The plot demonstrates a linear relationship between the measured lipase activity and the standard lipase concentration but also indicates that there is a reduction of 35-45 % in measured lipase activity when compared to the standard activities. An average loss of 5-10% loss would be expected due to normal experimental error and the accuracy of the actual assay. However, the loss of lipolytic activity measured using this method was significantly greater than experimental error. The loss of activity was most likely due lipase substrate specificity. The assay relies on the lipase produced by *S. clavuligerus* being active towards palmitate whilst the lipase may also have positional or fatty acid specificity.

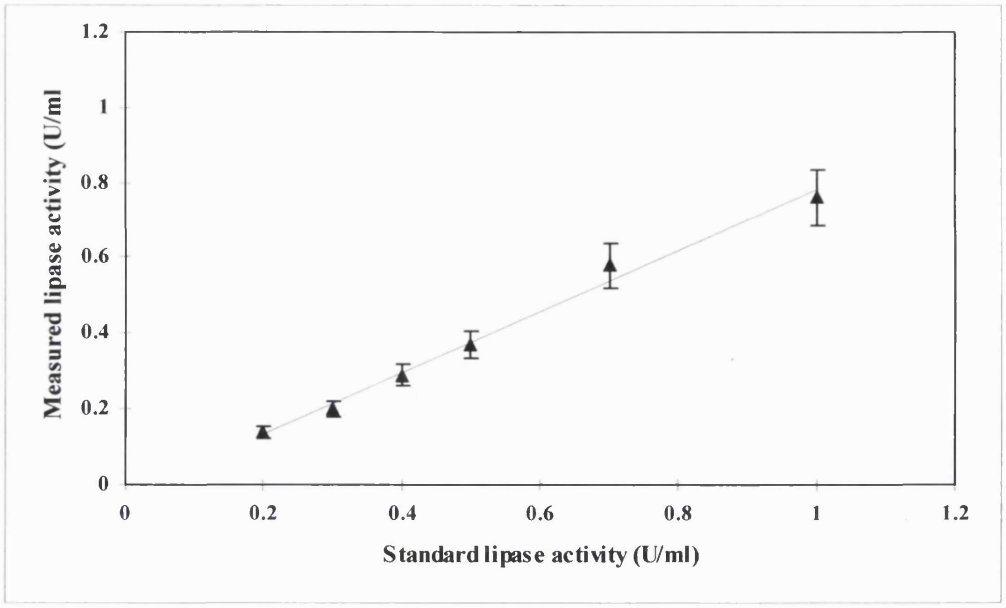


FIGURE 12.2 Determination of lipase activity using the pNPP assay method

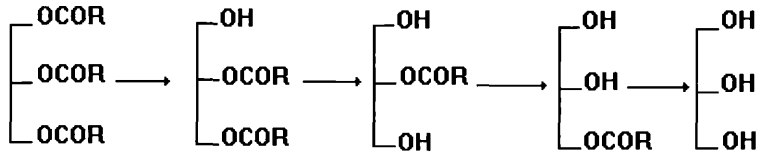
Samples of *S.clavuligerus* whole broth were assayed using this method after 96 hours growth. Less than 0.02U/ml of lipase activity could be detected under the given conditions although when the same samples were analysed using the agar method lipase levels between 0.1 and 0.4U/mlwere detected, dependent on assay conditions. This assay however, proved to be a poor technique for the system under study as the *S.clavuligerus* lipase demonstrated a very low activity towards the substrate due to enzyme specificity, measuring only approximately 10% the activity toward tributyrin using the agar method.

Chapter 12.3 Titrimetric assay

The principle of this assay is based on lipase hydrolysis of emulsified triglycerides of long chain fatty acids according to the reaction below:

triglyceride + H_2O $\Rightarrow$ diglyceride + fatty acid anion

In each step catalysed by lipase, one fatty acid is liberated as illustrated below:



By titration at the pH optimum, the formation of free fatty acids per unit time is measured. The number of fatty acid ester bonds hydrolysed per unit time, as determined by the amount of sodium hydroxide required to maintain a constant pH, is a measure of the lipase activity. As the active concentration of the substrate depends on the oil/water interface, the substrate must be prepared as an emulsion which will be stable for the period of the assay. Gum arabic is the most commonly used protective colloid for the emulsion in the titrimetric determination of lipase activity. There may be slight alkaline hydrolysis of the gum arabic which in the lower range of sensitivity of the method can lead to errors resulting in a non-linear curve in the first few minutes of the assay. Hence a time period of 10 to 30 minutes incubation for the assay is used to overcome this problem.

As with previous experiments, the effects of environmental conditions, such as pH and temperature, as well as substrate specificity were investigated. The assays were carried out using olive oil, rape seed oil, tributyrin and triolein/Tween 80 as substrates at temperatures of 26, 30 and 37°C and pH values of 6.6, 7.0 and 7.2. Again the oils were selected based on their fatty acid composition. A standard lipase solution of 1U/mL from *Candida cylindricae* was used initially to assess the various parameters and the average results obtained are shown in figures 12.3 and 12.4. *S.clavuligerus* fermentation broth samples were assayed in triplicate afterwards (figures 12.7 and 12.8).

It should be noted that no activity was observed at 26°C with any of the substrates at any pH and therefore the results are not represented. The use of the triolein/Tween mixture resulted in negligible levels of lipolytic activity being detected under any of the conditions investigated when the standard *C.cylindricae* lipase was used. Therefore, these results are not presented either. This is again due to problems emulsifying the substrate mixture.

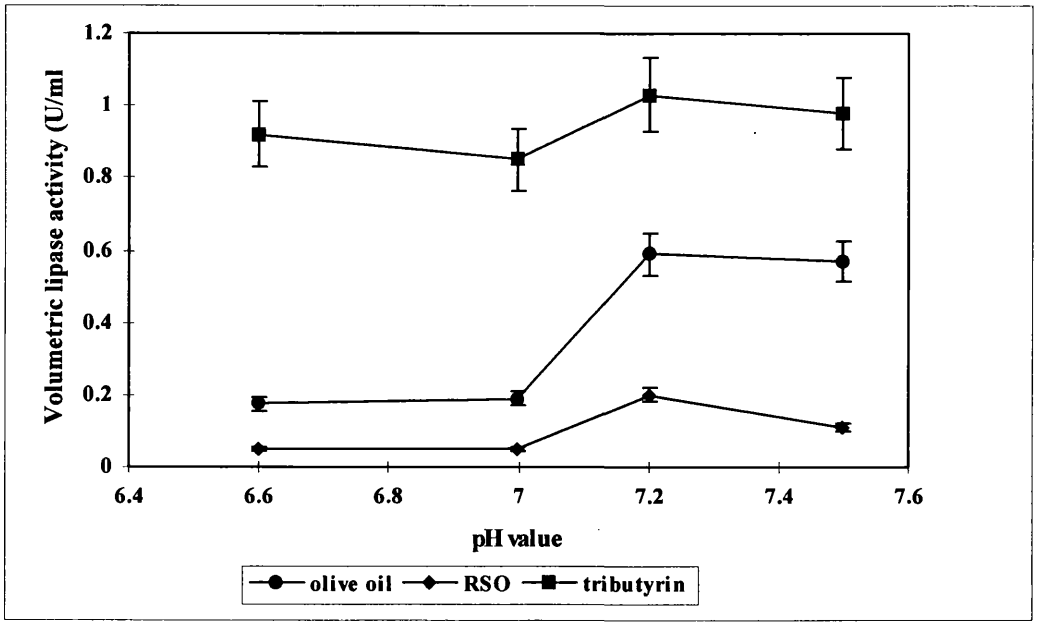


FIGURE 12.3 The effect of pH & substrate on lipase activity using the titrimetric assay at 30°C

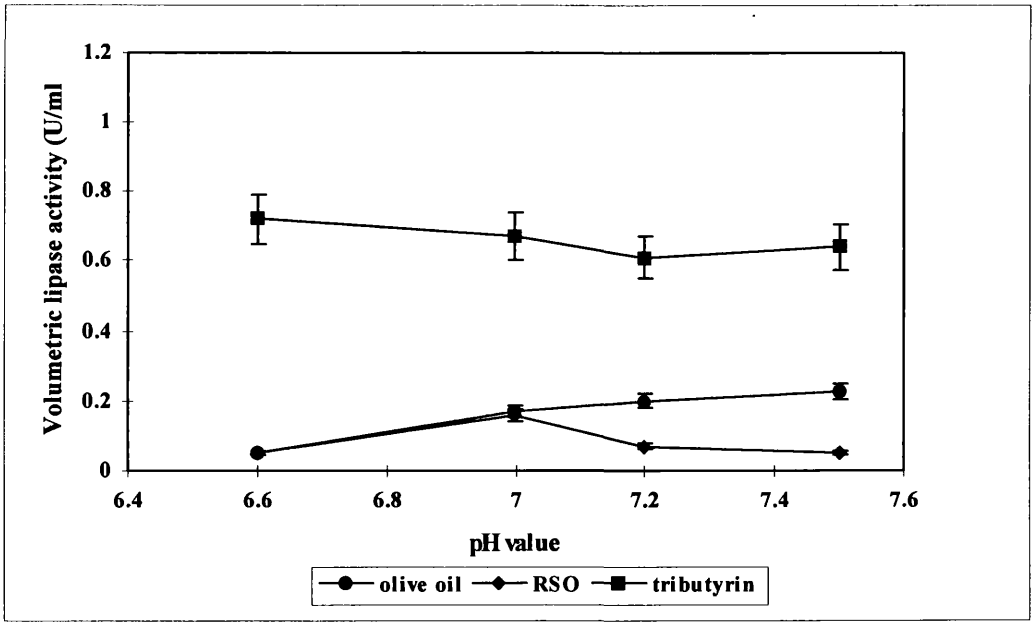


FIGURE 12.4 The effect of pH & substrate on lipase activity using the titrimetric assay at 37°C

In general the plots show that the use of both olive oil and rape seed oil as substrates exhibited a degree of lipolytic activity although the levels measured showed a loss in activity between 50 to 90% when compared to the activity of the standard solution of *C.cylindricae* lipase at 1U/ml, dependent on assay conditions. However, when tributyrin was used as the substrate for the levels of lipase detected were within 80 to 90% of the standard lipase activity, dependent once again, on the assay conditions.

Temperature appears to significantly affect the result of the assay, at 37°C the levels of lipase activity measured vary from the levels detected at 30°C. Where olive oil was used as the substrate, the levels of lipase activity detected using samples of the standard lipase solution were approximately 50% less at 37°C than at 30°C. A loss in the detectable level of lipolytic activity of approximately 30% less was also observed with the use of tributyrin as the substrate. In the case of rape seed oil, the levels detected were similar regardless of temperature but varied significantly with pH.

All the levels of lipase activity detected, regardless of substrate, were below the level of activity of the standard lipase at 37°C. However, at 30°C, in the case of the olive oil substrate and the tributyrin substrate, the levels of activity measured were approximately 60% and 90% that of the standard lipase activity respectively, although this was dependent on the pH value used.

The pH value used had a significant effect on the level of lipase activity measured. At 30°C, (figure 12.3), the profiles of the lipase activity detected show a similar trend irrespective of the substrate used. The level of lipase activity measured at both pH 6.6 and pH 7.0 are similar but the activity level detected increases significantly in each case when the pH is increased to pH 7.2. The level of lipase activity then decreased slightly the pH was then further increased to pH 7.5. This was most significant with the olive oil substrate where the measured lipase activity increased from 0.2U/ml at pH 6.6 to 0.6U/ml at pH 7.2, a three fold increase. The lipase level then decreased slightly to 0.57U/ml at pH 7.5. With the tributyrin substrate the measured lipase activity increased from 0.89U/ml at pH 6.6 to 1.03U/ml at pH 7.2 and fell to 0.95U/ml at pH 7.5.

At 37°C the effect of pH on lipase activity varies depending on the substrate used, (figure 12.4). In the case of the olive oil substrate the lipase activity increases from 0.05U/ml at pH 6.6 to 0.19U/ml at pH 7.5. However, the use of a tributyrin substrate results in a steady decline in the levels of lipase activity with the increase in pH, from 0.73U/ml to 0.63U/ml. Where rape seed oil has been used as the substrate for the assay there appears to be a defined optimum pH value of 7.0.

From the results it was concluded that the assay conditions are of key importance when determining lipase activity using this method. The most accurate and reproducible assay involved the use of a tributyrin substrate at a temperature of 30°C and pH 7.2. This also resulted in the least loss in measured lipase activity when compared to the lipase standard and therefore, these were the selected conditions for the titrimetric method.

Chapter 12.4 Comparison lipase determination methods

From the methods assessed, one assay had to be selected for the purpose of this study and developed and optimised for the given conditions and the specific lipase produced. A summary of the results obtained from measurement of lipase activity during the batch growth of *S.clavuligerus* using each of these methods is given in figure 12.5. Tributyrin was used as the substrate for the agar plate method and the plates were incubated at 30°C. The titrimetric assay again used a tributyrin substrate at a pH of 7.2 and a temperature of 30°C whilst the pNPP assay was carried out at 37°C.

Comparing the activity towards pNPP with that toward tributyrin, it becomes evident that general proportionality of both activities does not exist. The rate of hydrolysis of pNPP was only in the range of 0 to 0.05U/ml and approximately 5 - 15% of the activity towards tributyrin irrespective of the method used. This is similar to the results obtained with the standard lipase solution and would suggest that pNPP is a poor substrate for the *S.clavuligerus* lipase.

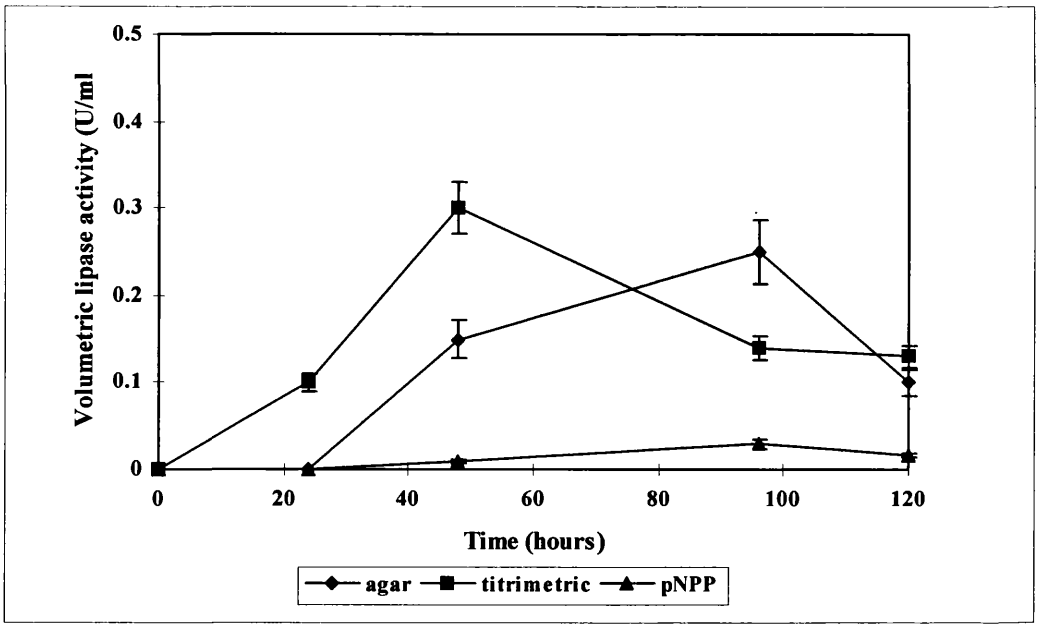


FIGURE 12.5 Comparison of methods for lipase determination using whole broth samples from *Streptomyces clavuligerus* batch culture

The agar plate method using tributyrin detected significant levels of lipase activity in the broth after 48 hours but at 24 hours growth no lipase was detected. This is due to the reduced sensitivity of the method at lower enzyme concentrations as observed previously. However, the titrimetric assay, under the given conditions, demonstrated the presence of lipase after 24 hours culture growth at a level of 0.1U/ml and detected lipolytic activity throughout the fermentation at reproducible levels.

From the results of the studies it has been shown that the pNPP assay was a poor technique for system being studied since the *S.clavuligerus* lipase developed a very low activity toward the substrate. The use of the agar method for lipase determination was accurate at lipase concentrations above 0.2U/ml and provided a good indication of the presence of lipase but lacked sensitivity at lower lipase activities. Therefore, having assessed these three methods, it was concluded that the titrimetric assay would be the most suitable assay for the study since it was relatively simple and was more accurate and reproducible than the other methods.

Chapter 12.5 Titrimetric assay development

Having selected the titrimetric method for lipase determination, the assay was developed and optimised for the fermentation conditions and the lipase produced by *Streptomyces clavuligerus*. Broth samples, known to be active, were assayed for lipase activity using the assay under varying conditions of pH and temperature and alternative substrates. The results are shown in figures 12.6 to 12.8.

Initial studies centred on the suitability of the substrate to be used for the assay, (figure 12.6), and tested lipase activity, at 30°C and pH 7.2, towards firstly, rape seed oil, (RSO), which is chosen carbon source during *S.clavuligerus* fermentation. Tributyrin and olive oil were also tested since the lipase produced by the organism demonstrated a level of activity toward both the oils with the agar method for lipase determination.

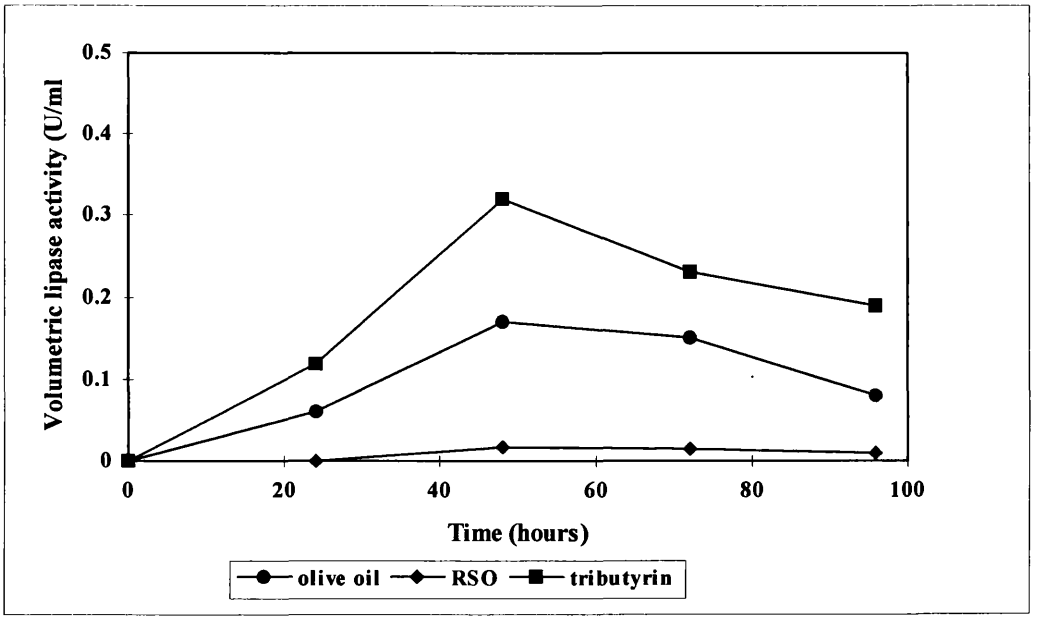


FIGURE 12.6 The effect of substrate on the determination of lipase activity of *Streptomyces clavuligerus* broth samples using the titrimetric assay at 30°C and pH7.2

The plot demonstrates that rape seed oil was not a suitable substrate for this assay with the lipase exhibiting very low activity toward the oil. The result is a little surprising since the organism had been growing on the oil in the fermentation broth and therefore, the lipase produced by *S.clavuligerus* must be capable of hydrolysing rape seed oil *in vivo*. This was thought to be due to the low emulsification ability of the oil thus making RSO unsuitable for use in this method.

The use of both olive oil and tributyrin resulted in the measurement of significant levels of lipase activity, comparable to the levels detected with the agar method, and varied with the time course of the fermentation. The activity detected with an olive oil substrate were approximately 40% lower than those with tributyrin and it is supposed that lipase demonstrated the greatest sensitivity toward tributyrin due to the increased emulsification of the substrate. This would suggest that tributyrin would be the most suitable substrate for this assay.

Figures 12.7 and 12.8 show the effect of varying pH and temperature on measured lipase activity. The assay was carried out at a pH range of 6.6 to 7.5 and figure 12.7 shows lipase activity could be detected at all the pH values within the tested range although the activity measured increased with increasing pH value from 0.05U/ml at pH 6.6 to 0.46U/ml at pH 7.2. The level of lipase activity detected at pH 7.4, 0.41U/ml, was lower than that at pH 7.2 and therefore, the lipase demonstrated an optimum pH of 7.2 under the given conditions.

A similar trend could be observed with temperature variation (figure 12.8). The effect of temperature on measured lipase activity was determined at pH 7.2 using a tributyrin substrate. The active broth samples were analysed at a range of temperatures between 26°C and 37°C and significant lipase activity was detected irrespective of the temperature used. There was an overall trend of increasing lipase activity measured with increasing temperature although temperature appeared to have lesser effect than varying pH with only a 25% variation between the levels detected. A maximum activity of 0.41U/ml was detected at 30°C with the level decreasing slightly to 0.39U/ml at 32°C. Therefore, the lipase exhibited a temperature optimum of 30°C under the given conditions.

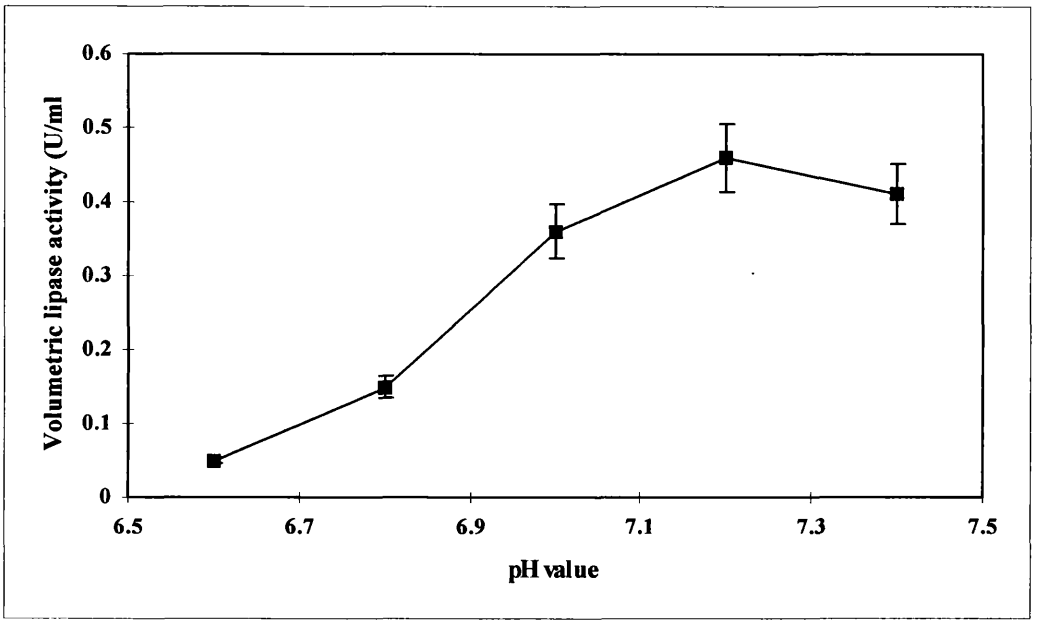


FIGURE 12.7 The effect of pH on the determination of lipase activity of *Streptomyces clavuligerus* broth samples using the titrimetric assay at 30°C

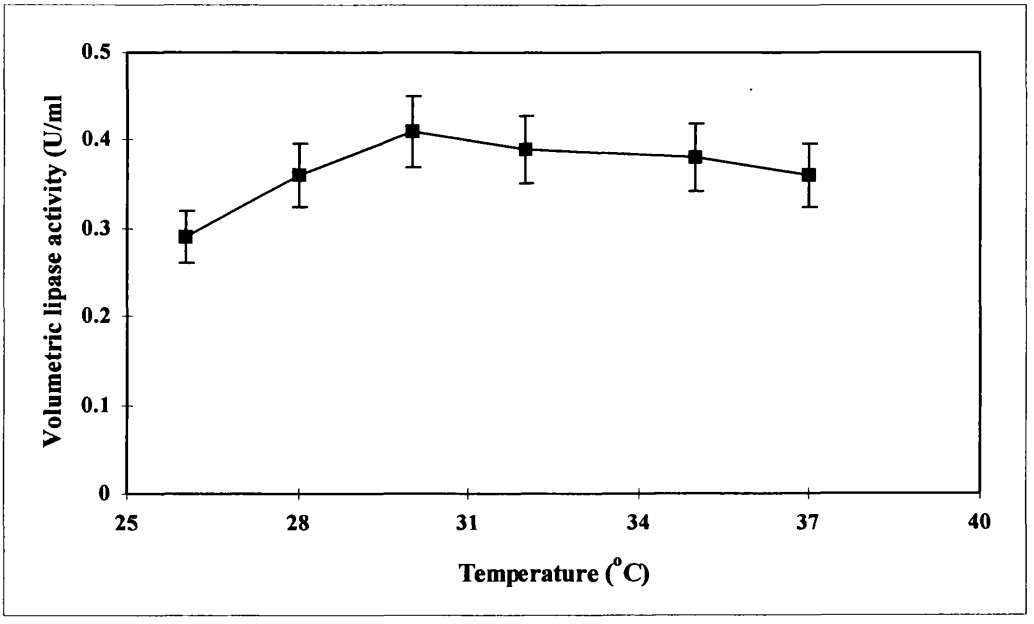


FIGURE 12.8 The effect of temperature on the determination of lipase activity of *Streptomyces clavuligerus* broth samples using the titrimetric assay at pH7.2

Therefore, to increase the sensitivity of the assay during this study, samples were assayed at an optimum pH of 7.2 and temperature of 30°C using a tributyrin substrate. It should be noted that the fermentation was maintained at pH 6.8 and 26°C due to growth and productivity constraints.

Chapter 12.6 Sample preparation and lipase location

As discussed in chapter 6.1, lipases may be either cell associated or extracellular and thus, the manner in which a broth sample is prepared for analysis will have a significant effect on the lipase activity detected. Therefore, optimum assay conditions were used in order to develop a greater understanding as to the location of the lipase enzyme in *Streptomyces clavuligerus*. The results are shown in figure 12.9.

Figure 12.9 shows that the sample preparation significantly affects the level of lipase activity detected. The highest level of lipase activity, of 0.46U/ml, was detected when whole broth was assayed after 48 hours fermentation. When the broth supernatant was assayed no lipase activity was detected and therefore, no results are shown in figure 12.9. The fact no activity could be detected in the broth supernatant provides the first indication as to the location of the lipase and suggests that the lipase present is not extracellular but intracellular or cell associated. However, when the pellet was resuspended and the sample analysed there was an 80-90% recovery in lipase which would indicate that it may be cell associated and not extracellular. On resuspension, the resultant loss in activity could be due in part to the actual processing of the sample causing deactivation of the lipase and part to the accuracy of the measurement.

This theory of the lipase being cell associated was supported by the results obtained from samples of sonicated whole broth. Sonication of the whole broth sample resulted in a greater than 80% reduction in detectable lipase activity. Sonication disrupts the cell membrane and therefore, if the lipase is cell associated its activity would be significantly affected by cell disruption. If the lipase was intracellular there would have been a significant increase in detectable lipase activity. However, a low level of activity of less than 0.05U/ml, could be measured after 48 hours. This is thought to be due again to the

disruption of the cell membrane releasing intracellular cell components such as lipases and esterases which could be detected using the assay.

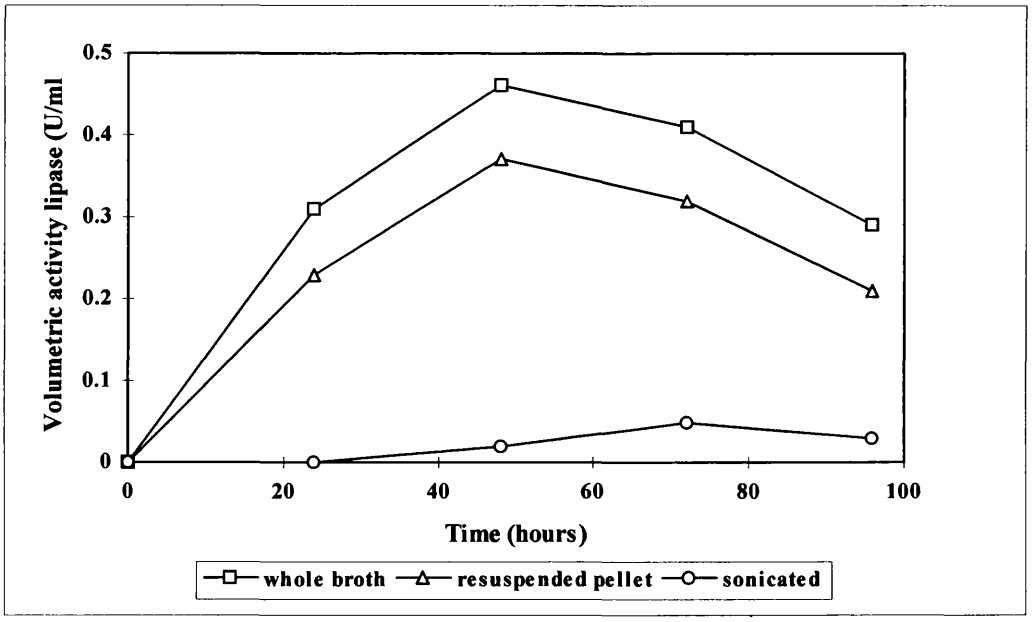


FIGURE 12.9 The effect of sample preparation on the determination of lipase activity of *Streptomyces clavuligerus broth* samples

To investigate further whether the lipase was cell associated, Triton X-100, a non ionic detergent, was added to an active sample of whole broth at a concentration of 0.01% (v/v). This mixture was then placed on a magnetic stirrer in an ice bath for 45 minutes in order to disrupt the cell membrane (Jacobsen *et al.*, 1989). Whole broth, supernatant and resuspended pellets, (in deionised water), were analysed. The results obtained are detailed in table 12.2.

The addition of Triton X-100 resulted in a decrease in lipase activity detected in whole broth when compared to untreated samples whilst lipolytic activity could be detected in the supernatant sample following treatment with Triton X-100. However, the level of lipase activity in the resuspended pellet sample was dramatically reduced and was virtually undetectable, within the sensitivity range of the assay, in a proportion of samples. Again a reduced level of lipase activity was detected after sonication due to not only cell disruption but also deactivation of the lipase.

TABLE 12.2 Comparison of levels of lipase activity detected in fermentation samples treated with Triton X-100 and untreated fermentation samples

Sample preparation	No Triton X-100 addition	Triton X-100 addition
Whole broth	+ +	- +
Supernatant	- -	+ -
Resuspended pellet	+ +	- +/- -
Sonicated whole broth	- + (low levels)	- +

(+ + activity detected, - - no activity detected, - + reduced activity detected, + - increased activity detected)

Triton X-100 has the effect of disrupting the cell membranes, similar to sonication, but does not denature protein structure. The results demonstrate this property by the fact that the supernatant sample shows a detectable level of lipase. When the membrane is disrupted in this way intracellular proteins, such as lipases or esterases, leak or are released from the cell into the supernatant and hence will be detected during the assay. The reduced level of activity measured in the whole broth and resuspended pellet samples, due to the disruption of the cell membranes, again suggests that the lipase is cell associated.

Chapter 12.6.1 Effect of reaction mixture on lipase activity

During the course of optimising the titrimetric assay, lipase activity could not be detected under certain assay conditions such as sample preparation method. To ensure that the reaction mixture was not affecting the level of lipase activity detected. When no lipolytic activity was observed, the reaction mixture containing the inactive sample, was spiked with a standard lipase solution of known concentration. This was the same as that used during initial assay development. The results are indicated, in triplicate, in figure 12.10.

Figure 12.10 shows that when a non active reaction mixture is spiked with a standard lipase solution of 1U/ml, after 10 minutes reaction time, there was a 95-100% recovery in lipase activity. The lipase activity could still be detected after a further 20 minutes of the assay. This would suggest that the reaction mixture had no significant effect on the measurement of lipase activity under the conditions given.

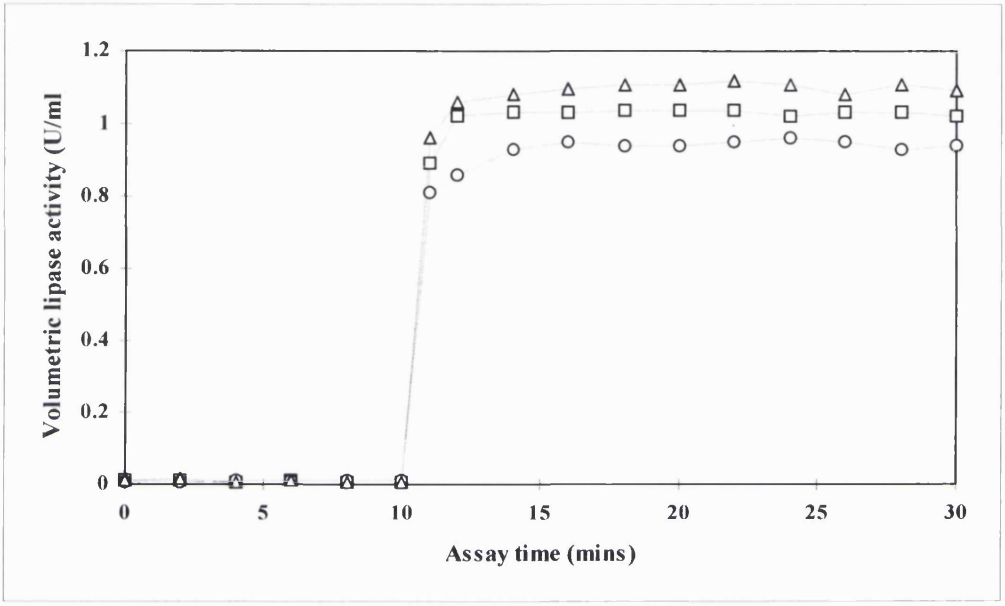


FIGURE 12.10 Effect of reaction mixture on detected lipase activity using a tributyrin substrate at 30°C and pH 7.2

In conclusion, these experiments suggest that the manner in which the sample is prepared for assay, the substrate used and the assay conditions have a significant effect on the measured lipase activity. However, the reaction medium had little or no effect. Therefore, it can be concluded that the most accurate and reproducible way of measuring the lipase activity present in a fermentation sample, is to assay using the titrimetric method, with tributyrin substrate at 30°C and pH 7.2, and using a whole broth sample assuming that the lipase is cell associated.

Chapter 13 Lipid analysis

Analysis of the oil content of the fermentation broth was necessary in order to monitor the progress of the fermentation and therefore, an analytical technique to assay the lipid content of the fermentation samples was developed. Two main methods were investigated which were Thin Layer Chromatography, (TLC), and a biochemical assay based on a Boehringer test combination kit catalogue number 124 303 (chapter 9.2.2).

Chapter 13.1 Thin layer chromatography

Thin layer chromatography, TLC, is a simple technique based on one underlying principle. A lipid mixture is applied to an adsorbent coated onto a thin layer support. The point of application of the sample is termed the origin. The mixture is then resolved into its components by differential migration as a stream of solvent passes through the layer of adsorbent by capillary action. In a given solvent system each lipid component has a characteristic mobility, its Rf value. The Rf value can be defined by the equation given below (equation 13.1).

$$\mathbf{Rf} = \frac{\text{Distance run by the solute}}{\text{Distance run by the solvent}} \qquad \mathbf{(13.1)}$$

In order to establish the migration characteristics of the different oils and oil components involved in the study, TLC plates were run using standard oils, olive oil, tributyrin, triolein and rape seed oil. The fatty acid components of rape seed oil, oleic acid, linoleic acid and linolenic acid, were also analysed. Glycerol was also studied since this is the final breakdown product of the oil hydrolysis. From these initial plates the respective Rf values were calculated which could be used as standards for comparison with later fermentation samples. The results obtained are detailed in table 13.1.

TLC plates produced when standard oils were run over a period of one hour, were visualised by staining with iodine. Copies of the plates are not available since the stain intensity diminished over time and hence reproduction was of poor quality. The plates showed that the oils appear to have similar Rf values between 0.8 and 0.75 except for tributyrin. This indicates that they have migrated at similar rates and distances through

the plate which is to be expected since the oils have similar polarity being neutral oils consisting of glycerol and fatty acids. In the case of tributyrin, the Rf value is 0.54 which is lower than with the other oils. This is due to differences in physicochemical properties of the lipid compared to the other oils such as surface charge. This results in a more polar compound which binds more strongly to the adsorbent and hence slows migration.

TABLE 13.1 Typical Rf values for standard components

Component	Average Rf value
Rape Seed Oil	0.80
Olive Oil	0.77
Triolein	0.75
Tributyrin	0.54
Oleic Acid	0.53
Linoleic Acid	0.51
Linolenic Acid	Unable to run
Glycerol	Remains at the origin

Some colouration by the stain also occurred around the origin in the case of RSO, olive oil and triolein. This appears to be most pronounced with triolein where intense colouration occurs between the origin and a Rf value of 0.25. This indicates that there either may have been some breakdown of the oil resulting in the formation of diacylglycerols and/or monoacylglycerols or that there were free fatty acids in the standard oils. In the case of glycerol, there is no migration through the plate and the spot remains at the origin which is to be expected.

TLC was also used to separate fatty acids as mentioned previously. Again, as with the oils, the fatty acid fronts ran similar distances. Oleic acid had an Rf value of 0.53 whereas linoleic acid had an Rf value of 0.51. The fatty acids did not migrate as far as the oils since fatty acids tend to be more polar then the oils and hence bind more strongly to the silica gel. This is thought to be related to the fatty acid composition. Linolenic acid however, showed no movement from the origin.

These results indicate that TLC could be used during analysis of fermentation samples to monitor the progress of oil hydrolysis by demonstrating the presence of oil as well as the oil breakdown products such as fatty acids and glycerol. The method, however, does not quantify the actual concentration of each compound except by the intensity of the stain used such that the more oil present, the darker the stain. It therefore, provides a qualitative technique for lipid analysis.

Chapter 13.2 Lipid analysis using biochemical assay

The spectrophotometric biochemical assay for lipid concentration is based on the reaction of lipids with vanillin and sulphuric and phosphoric acids to form a pink complex. The intensity of the colour of the complex is directly proportional to the amount of oil present in the sample. Figure 13.1 illustrates the results, in triplicate, for a standard curve for the assay which plots the standard lipid concentration against the read absorbance.

The oil standards were obtained directly from Boehringer or by dilution of the standard solutions purchased. These manufactured standards were also used as a control during the future assay of fermentation samples. The assay was found not to be accurate at oil levels below approximately 5g/l since there was very little variation in the absorbance reading making the assay inaccurate with respect to determination of lipid concentration. Hence, the results are not shown on the plot. This was not thought to be a problem with respect to the fermentation analysis since the concentration of oil used in the medium was up to seven times this level.

The graph shows that a sigmoidal plot is produced when standard samples were assayed. However, at higher oil concentrations, between 10 - 15g/l, the plot demonstrated a more linear relationship although at the upper concentration limit there was a greater deviation in the absorbance readings obtained, up to 10 - 15%, as compared to 5% at lower concentrations. This is due to the sensitivity to the assay.

Broth samples of known oil concentration were then analysed to determine the accuracy of the assay. The samples consisted of complex medium with the addition of known amounts of rape seed oil. These were agitated for a minimum of one hour in order to disperse the oil sufficiently. The "broth" samples were then prepared as described in chapter 12.6. The results of the lipid analysis are shown in figure 13.2.

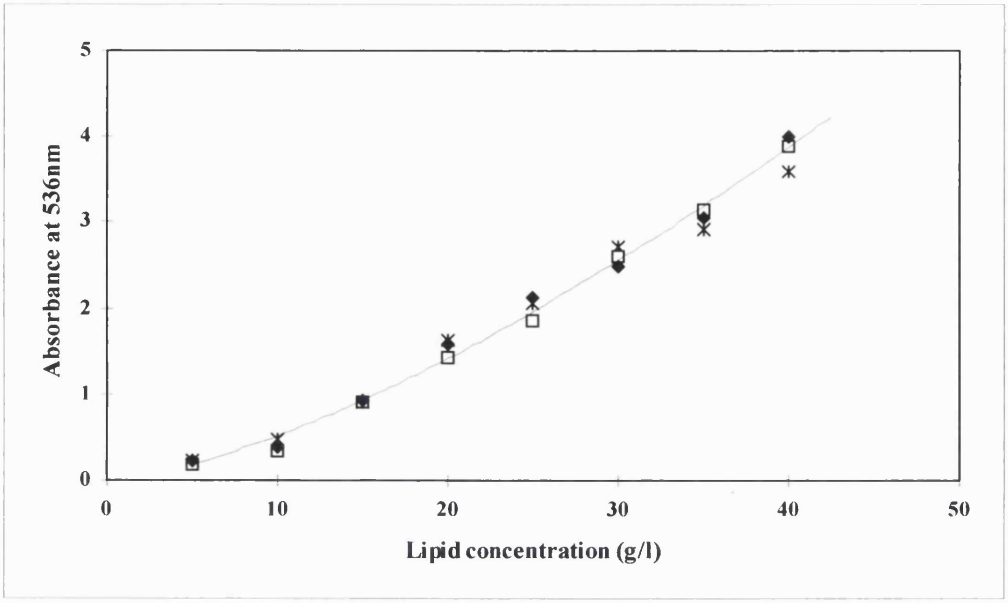


FIGURE 13.1 Standard curve for lipid determination

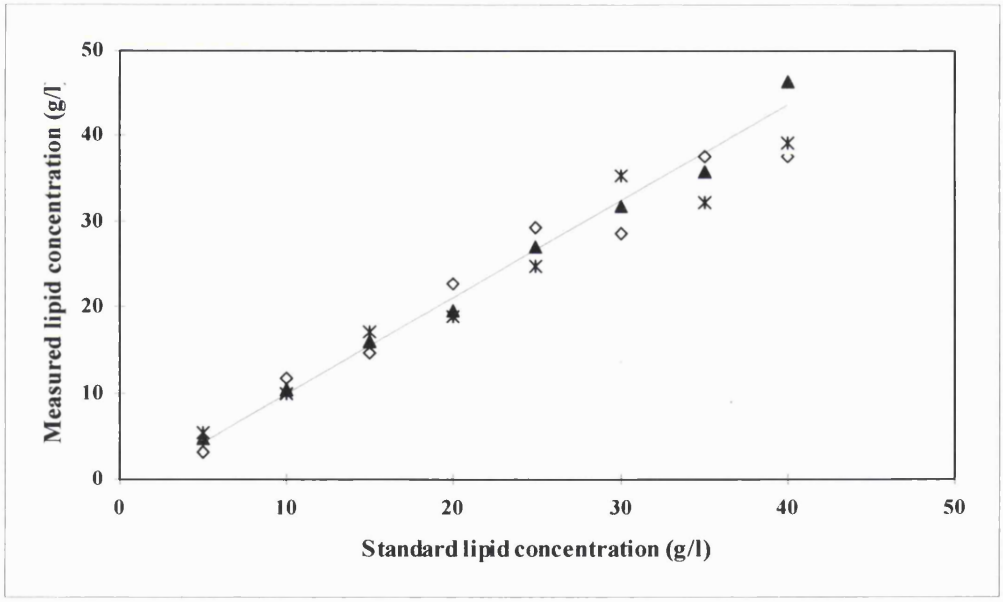


FIGURE 13.2 Comparison of broth samples of known oil concentration with lipid standards

The plot shows a linear relationship between the measured oil concentration and the lipid standards although the fit of the line deviated dependent on the oil concentration. At concentrations up to 15 - 20g/l there was a deviation of 5 - 10% in the measured oil level and as the oil concentration increased the deviation became greater reaching a

maximum of 25 - 30% at levels above 30g/l. It is thought that this was due to mixing problems of the oil in solution, particularly at higher oil levels, where there may have been incomplete dispersion of the oil resulting in inaccuracies in the measurements read. For this reason, when using this assay method, the samples must be assayed in at least triplicate.

Chapter 14 *Streptomyces clavuligerus* fermentation - shake flask trials

Initial fermentation studies of *Streptomyces clavuligerus* were carried out in shake flasks in order to establish firstly, that the organism was capable of utilising rape seed oil and secondly, to obtain a preliminary understanding of the growth kinetics of the organism on the selected substrate(s). During these initial studies a complex medium containing both dextrin and rape seed oil was used.

The preliminary experiments were done using 250ml and 2l baffled shake flasks containing 50ml and 200ml medium volume respectively. The volumes were selected in order to provide the optimum proportions for aeration and growth. The flasks were inoculated with 10µl of a *S.clavuligerus* spore suspension at a concentration of 8.7×10^8 spores/ml. The cultures were incubated at 26°C, the optimum for growth, for up to 170 hours on orbital shakers at 200rpm. Samples of broth were taken every 24 hours and assayed for lipase activity, lipid concentration and growth. In later experiments samples were also analysed for dextrin. The results obtained are illustrated in figures 14.1 to 14.3.

Figure 14.2 demonstrates that lipolytic activity was directly detectable in shake flasks at both 250ml and 2l scales but the levels measured were dependent on scale At 250ml scale, lipase activity was detected late in the growth phase reaching a maximum specific activity of 0.042U/mg after 48 hours. The activity then decreases and is undetectable by the stationary growth phase at 120 hours. At 2l scale, lipase activity was again detected during the growth phase reaching maximum specific activity of 0.086U/mg after 100 hours. The lipase activity decreased after this time and was negligible by the stationary growth phase.

The actual growth rates achieved were similar irrespective of the scale although a higher biomass was achieved in the 250ml fermentations (figure 14.1). This would also account for the higher specific lipase activity. The lag phase of growth lasts approximately 40 hours at 250ml scale and 60 hours at 2l scale respectively with a growth phase of 24 hours at both scales. Stationary phase occurs at 96 hours and 120 hours for 250ml and 2l fermentations respectively. The increase in lag phase at 2l scale was thought to be due to

the number of cell generations whilst the reduced biomass concentration was attributed to agitation and aeration.

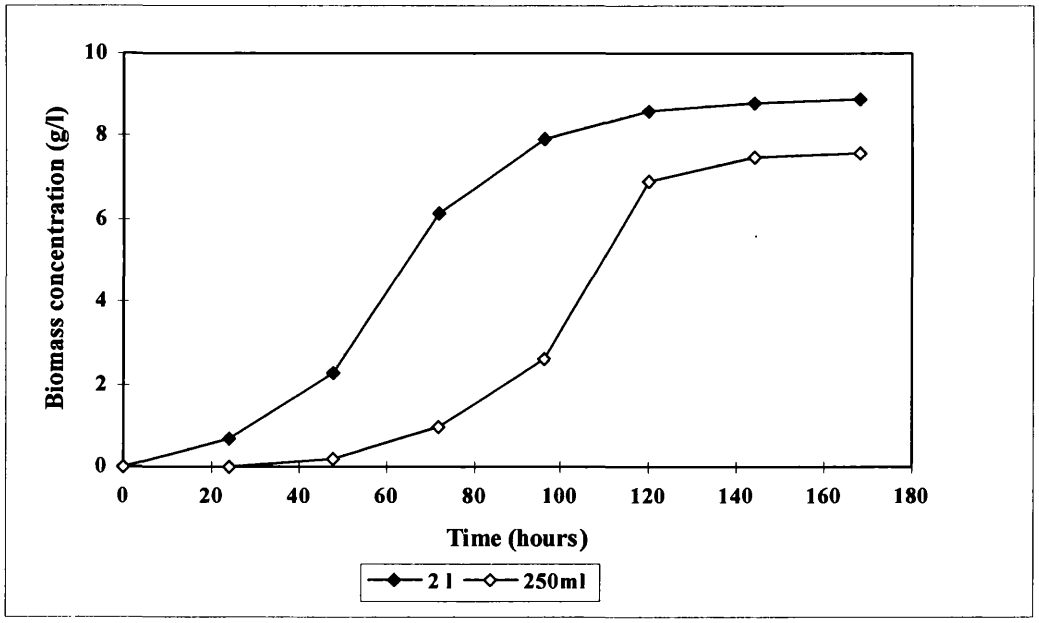


FIGURE 14.1 Growth profiles for 250ml & 2l shake flask fermentations on complex medium at 26°C

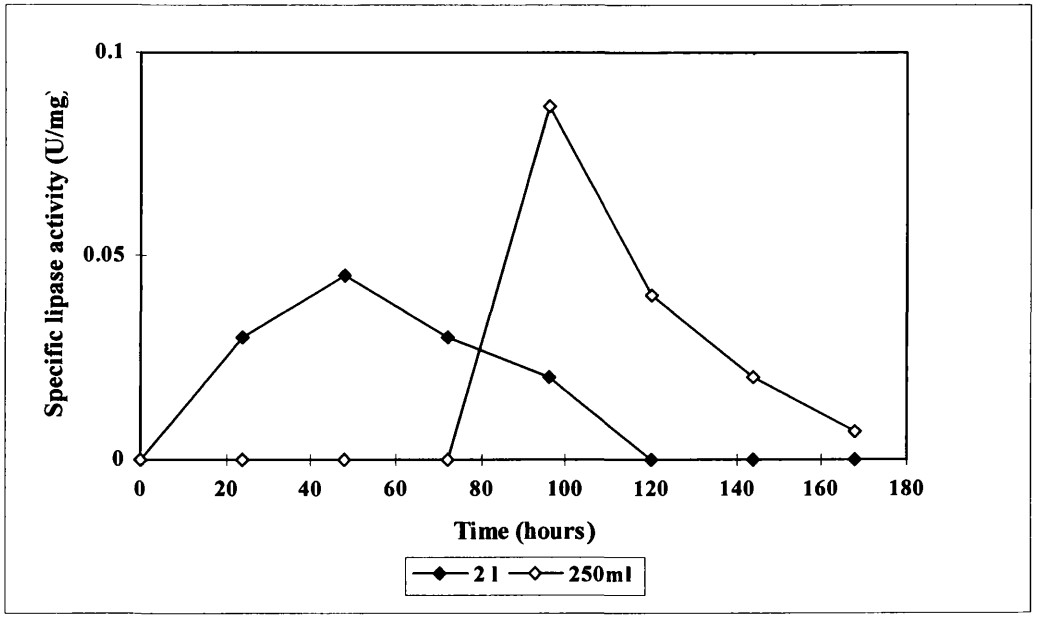


FIGURE 14.2 Lipase activity profiles for 250ml & 2l shake flask fermentations on complex medium at 26°C

Initially, the oil levels were determined using the TLC method in order to observe whether the oil was actually being utilised over the time course of the fermentation. The TLC plates showed the gradual disappearance of the lipid over the course of the fermentations which is indicated by a decrease in the intensity of the lipid stain. However, there was no significant change in the intensity of staining of the oil until 96 hours and after this time colouration was observed between an Rf value of 0-0.5 indicating the presence of diacylglycerols and monoacylglycerols. This indicates that the oil was being utilised but oil was still detectable at the end of the fermentation. Having established the gradual reduction in lipid concentration, the biochemical lipid assay was then used to provide definitive amounts of oil present in the broth sample and hence determine an initial rate of degradation. Results from analysis of the 2l shake flask culture are shown in figure 14.3.

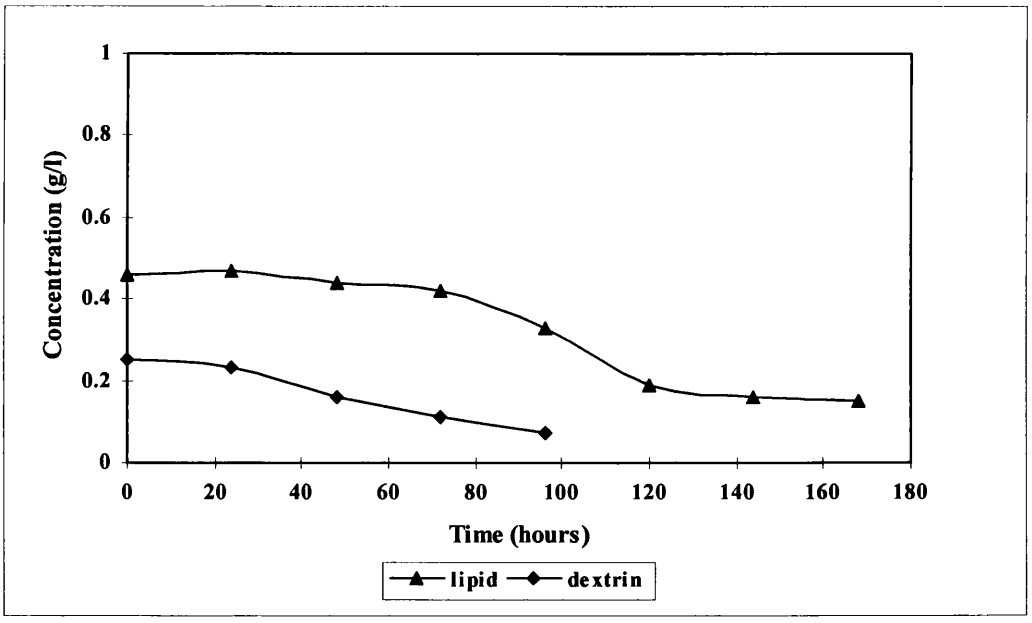


FIGURE 14.3 Dextrin and lipid utilisation profiles for 2l shake flask fermentation on complex medium at 26°C

Figure 14.3 shows the lipid and dextrin utilisation profiles during the 2l shake flask experiment. It would appear that the dextrin is utilised at the start of the fermentation, after 24 hours, in preference to the oil. There is a 50% decrease in dextrin concentration during the growth phase, 24-60 hours, but the degradation rate decreased by 72 hours.

The oil utilisation profile indicates that although little lipase activity is detectable, there is some triglyceride consumption in the initial stages of growth. However, the utilisation rate increased significantly after 80 hours when maximum activity was detected. Therefore, it would appear that the rate of lipid utilisation is directly related to the lipolytic activity. The results also demonstrate that *Streptomyces clavuligerus* has a preference for dextrin as a carbon source and hence only metabolises the oil once the sugar source has been depleted.

In conclusion it can be stated that *Streptomyces clavuligerus* is capable of utilising a rape seed oil carbon source but if an alternative carbon source, such as dextrin, is available this will be use in preference to the oil. In order to utilise the oil, the organism produces a lipase which can be detected in the broth sample. The rate of oil utilisation is dependent on the activity of the lipase produced. The next stage of the study was to scale the fermentation to gain a better understanding of the kinetics of the fermentation and the relative benefits of utilising oil with respect to antibiotic production (chapter 4).

Chapter 15 5l batch fermentation of *Streptomyces clavuligerus*

Following the shake flask fermentation trials, studies were scaled up to 7 l fermentations of *Streptomyces clavuligerus*, with 5 l working volume. Three different media, (chapter 10.3.4), were used in order to investigate the effect of medium composition on oil utilisation and lipase activity. The first medium was the complex medium, used for the shake flask studies, which is based on the minimal medium used during the SmithKline Beecham production process. A soluble medium was also used in which the insoluble nitrogen source, soya flour, was replaced with a more soluble peptone. The third medium was an oil based medium which had the same composition of the complex medium except the dextrin was replaced with rape seed oil on a weight for weight basis.

Preliminary batch fermentations were carried in order to optimise the fermentation conditions for the system involved. The environmental conditions were determined to ensure comparability with the industrial and published data with respect to optimum growth and antibiotic production. The temperature was set at 26°C and the pH at a value of 6.8. Biomass growth and clavulanic acid titre have been shown to be stable over a pH range of 6.2 - 7.5 but each parameter has an optimum range. A maximum clavulanic titre is produced at pH 6.5 but with reduced growth and the optimum for growth is pH 6.8-7.2 although above pH 6.8 clavulanic acid production falls significantly. Therefore, a pH of 6.8 was selected, (Tarbuck *et al.*, 1985 and M.T. Belmar-Campero, Thesis, 1987).

The initial conditions employed of 1vvm air flow rate and 500rpm stirrer speed, as described by M.T. Belmar-Campero (Thesis, 1987), proved to be unsatisfactory for this study since oxygen depletion occurred rapidly and fell close to 0% oxygen concentration for between 5 -10 hours during the growth phase affecting nutrient consumption. It also documented that antibiotic production has a critical oxygen level of between 1 - 10%, (Scott *et al.*,1988 and Yegneswaran *et al.*, 1991). Therefore, in an attempt to prevent the fluctuations in dissolved oxygen level, (DO), a number of fermentations were carried out at different agitation speeds and air flow rates, to determine the effect on DO level, as described by Rollins *et al.*, (1988).

Significant foaming problems were encountered at higher agitation rates, above 750rpm which could only be controlled by reducing the stirrer speed, reducing the airflow and/or adding antifoam, as supplied by SmithKline Beecham. A similar problem occurred when the air flow rate was increased above 1.5 vvm. Therefore, the conditions selected for the fermentation were at stirrer speed of 750rpm and air flow rate of 1vvm. Although these conditions did not prevent the DO falling to approximately 10%, it was only at this level for 2 -4 hours as opposed to almost 10 hours given the initial parameters. In conclusion, the fermentation parameters selected were a temperature of 26°C, pH of 6.8 and a stirrer speed of 750rpm at an air flow rate of 1 vvm.

The on-line gas analysis data from fermentation using each medium is shown in figures 15.1 to 15.3. The results indicate significant variation in both the rates of oxygen uptake and carbon dioxide evolution between the different media which initially suggests that the growth rate of the organism will be dependent on the type of medium, and hence the carbon source available, used during fermentation.

Figure 15.1 shows the comparison of the oxygen uptake rate with using the three different media. On complex medium, after a lag phase of 5 hours, the OUR increases rapidly, over a period of 10 hours, signifying the organism has entered the initial growth phase. The OUR appears to slow between 15 - 16 hours but then increases again reaching maximum uptake rate of 25 mmol/l/h at 20 hours. This would suggest that the organism has started a second phase of growth, indicative of a switch in carbon source. This is supported by an observed change in the RQ value, (figure 15.3), which switches from 0.85 - 0.90, between 5 - 15, to 0.8 - 0.85, between 15 - 25 hours.

There is a plateau in the oxygen uptake rate between 25 - 30 hours which coincides with a fall in the dissolved oxygen level to below 10% thus preventing oxygen uptake. This reduced OUR is accompanied with a second change in RQ value to between 0.75 - 0.8 which remains relatively constant until the end of the fermentation. The oxygen uptake rate falls gradually which indicates the organism is in the stationary growth phase. A similar plot is produced for the carbon dioxide evolution rate, (figure 15.2), which demonstrates the same trends with respect to lag and growth phases.

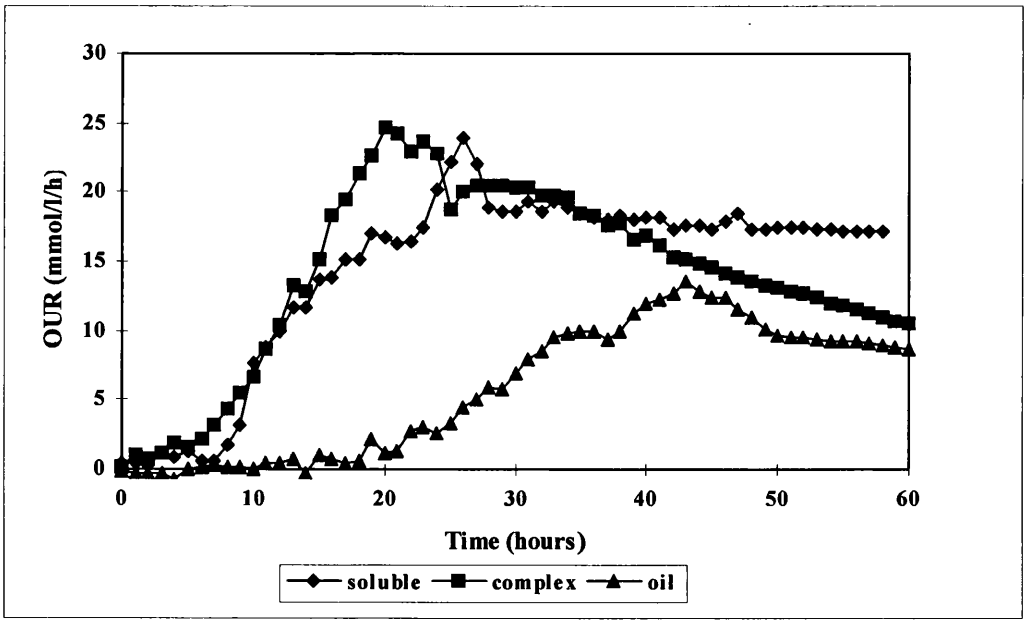


FIGURE 15.1 Comparison of OUR during *Streptomyces clavuligerus* fermentation using complex, soluble and oil growth media

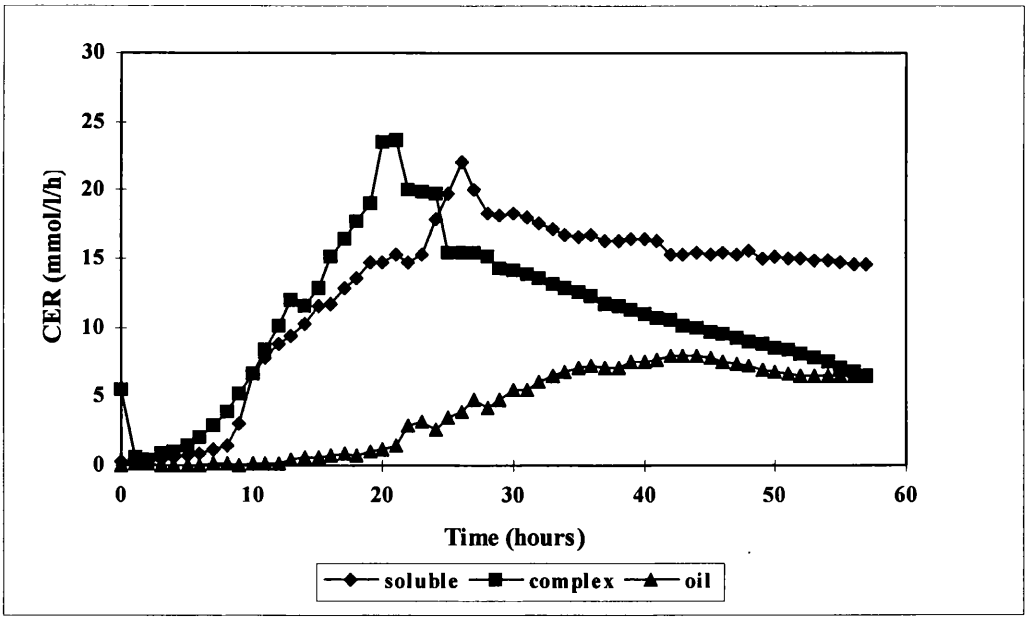


FIGURE 15.2 Comparison of CER during *Streptomyces clavuligerus* fermentation using complex, soluble and oil growth media

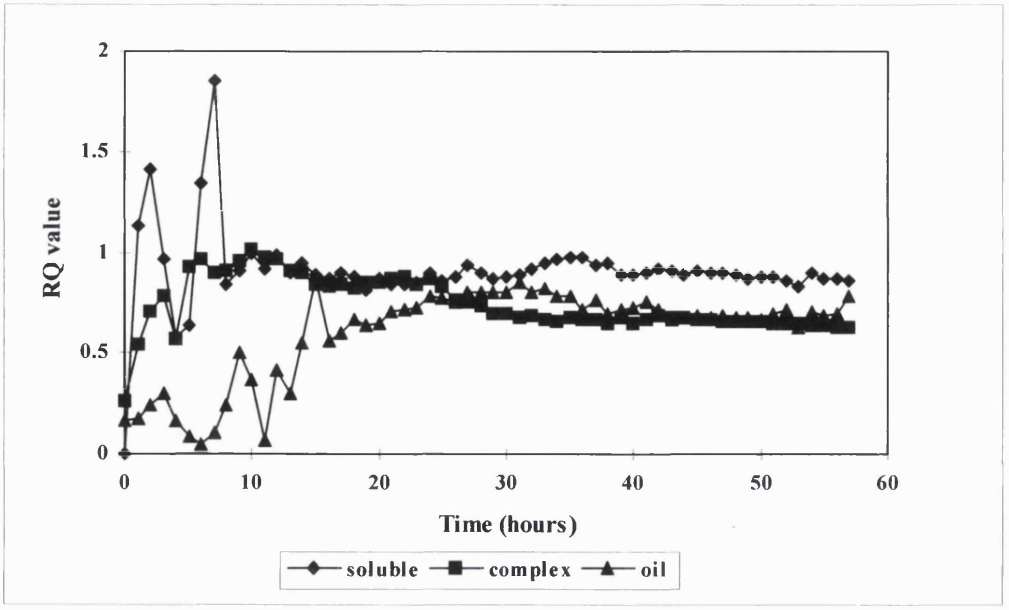


FIGURE 15.3 Comparison of RQ during *Streptomyces clavuligerus* fermentation using complex, soluble and oil growth media

The use of the soluble medium during fermentation resulted in a similar trend in the oxygen uptake rate and the carbon dioxide evolution rate as the use of the complex medium, which can be observed in figures 15.1 & 15.2. The plot demonstrates a similar lag phase, although slightly longer at 8 hours, followed by a rapid increase in oxygen uptake equivalent to the growth of the organism. The rate slows for a period of 3 hours but is followed by an increased uptake reaching a peak at 28 hours of 24 mmol/l/h. This is reflected, once again, in a change in RQ from 0.85 - 0.9 to 0.8 - 0.85, (figure 15.3). After an initial decline, from 30 hours the OUR and the RQ remain relatively constant.

Figures 15.1 to 15.3 show that the use of oil as a sole carbon source during fermentation results in a slower growth rate of the organism which would have implications on the time period of the fermentation. The lag phase on this medium is extended to 20 hours, from 5 hours on complex medium, and is followed by a steady growth phase reaching a maximum oxygen uptake at 45 hours of 12.5 mmol/l/h. The rate of oxygen uptake does slow for a period of 2 - 3 hours at 35 hours which coincides with the fall in dissolved oxygen below the critical level of 10%. During the fermentation the RQ remained relatively constant although there was a slight decrease after 45 hours. This would be expected since there is only one carbon source available to the organism.

The metabolic activity of the organism was also monitored, via biochemical assay, during fermentation. The rate of oil utilisation and level of lipase activity were determined using the assays previously developed, (chapters 12.3 & 13), as was the biomass concentration. The relative variation in pH value was monitored on line. The results of the analyses are represented in figures 15.4 to 6.

Figure 15.4 illustrates the pH fluctuation during the course of the fermentation. The pH was only controlled to prevent acidic conditions and therefore any alkaline deviations were not corrected. The first observation is that the pH profiles for both the complex and the soluble media show a peak in pH at the same point where the OUR and CER plots demonstrate the end of the first growth phase of the fermentation and switch in RQ, or substrate. With complex medium the pH increases from a starting value of pH 6.97 reaching a peak at 15 hours of pH 7.28. During the second growth phase, 15-20 hours, the pH falls rapidly to pH 7.14 and after 20 hours remains relatively constant. The pH profile of the soluble medium shows a similar trend, reaching a peak at 20 hours of pH 7.5 which then declines reaching a steady value of pH 7.14, the same as the complex medium fermentation, after 30 hours.

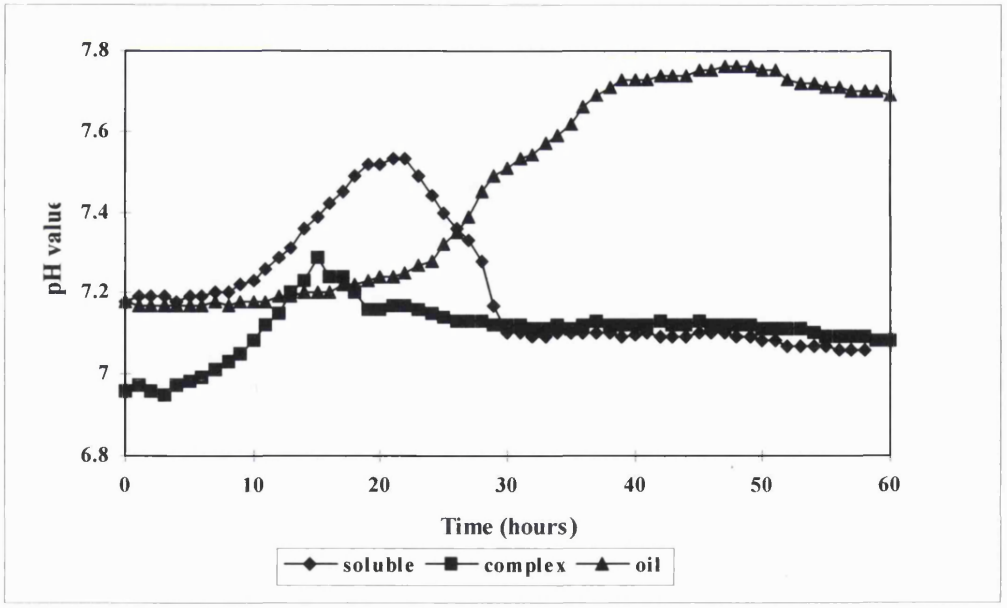


FIGURE 15.4 Comparison of pH profiles during *Streptomyces clavuligerus* fermentation using complex, soluble and oil growth media

The pH profile of the fermentation using the oil medium varies from the other profiles although the pH at the start of the fermentation is a similar value to that of the soluble medium fermentation. There is no significant increase in pH during the lag phase but pH starts to increase rapidly as the organism enters the growth phase, 25-40 hours, reaching an initial peak at 40 hours of pH 7.75. The plot then plateaus which corresponds to the fall in dissolved oxygen levels and cessation of growth. However, once the dissolved oxygen levels have recovered and growth is initiated, the pH increases again reaching a maximum of pH 7.78 at 45 hours. After 45 hours there is only a slight reduction in pH to 7.73 and then the pH remains relatively constant.

It is unlikely that the increase in pH is related directly to the growth of the organism since the hydrolysis of oil results in the production of fatty acids which would reduce the pH, a feature which can be observed in the later stages of the complex and soluble media fermentations. Therefore, it would appear that the variation in pH is the result of some other reaction occurring in the fermentation media. One possibility is that the respective nitrogen sources, soya flour and peptone, are being broken down or deaminated resulting in the release of ammonia causing an increase in pH. This would also suggest that oil is not utilised preferentially in either the complex or soluble media fermentations and was supported by analysis of both lipid concentration and lipase activity (figures 15.5 and 15.6).

Figure 15.5 shows that during both the complex and soluble media fermentations oil is not utilised in the early stages of growth and the cell appears not to begin to metabolise the oil until 20 and 25 hours, respectively. During this period, maximum lipase activity of 0.03 U/mg biomass can be detected irrespective of the medium used (figure 15.6). However, no lipase activity is detected prior to oil utilisation which the suggests the lipase is only produced when necessary i.e. it is induced as opposed to constitutive. It is also possible that the lipase is being repressed since it may not be energetically favourable for the organism to utilise the oil during the initial stages of the fermentation.

Oil utilisation during soluble medium fermentation appears to slow after 35 hours with negligible amounts of oil being used after 40 hours. This results in a residual oil concentration of 12.5 g/l. The oil utilisation period in the complex medium fermentation is longer and a greater amount of oil is metabolised but again oil uptake slows after 50

hours leaving a residual oil level of 9.0 g/l. No lipase activity can be detected in either fermentation after 40 - 45 hours which would suggest that there is either some form of lipase inactivation or a physical limitation to oil uptake.

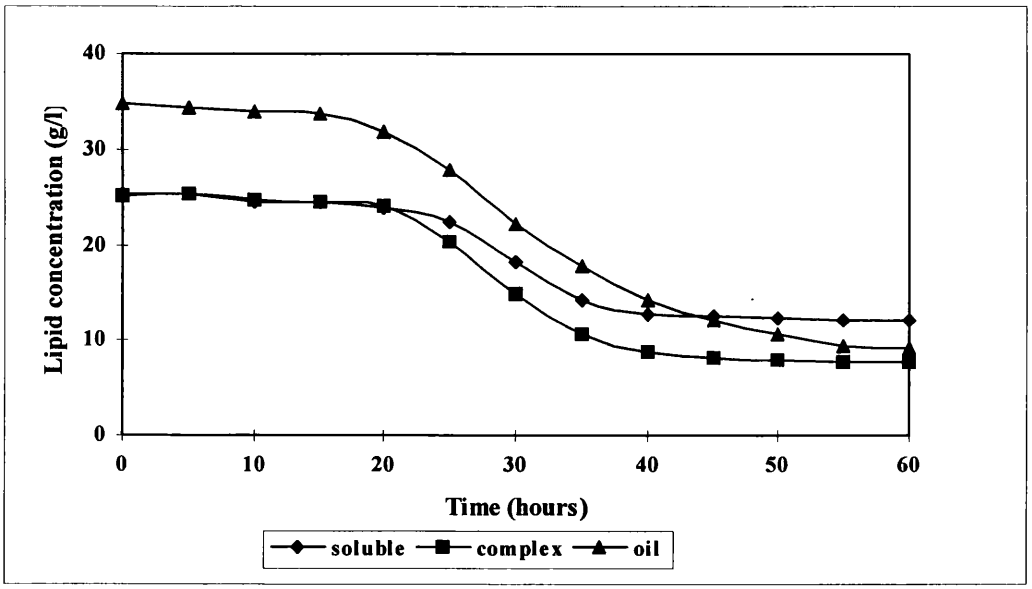


FIGURE 15.5 Comparison of lipid utilisation profiles during *Streptomyces clavuligerus* fermentation using complex, soluble and oil growth media

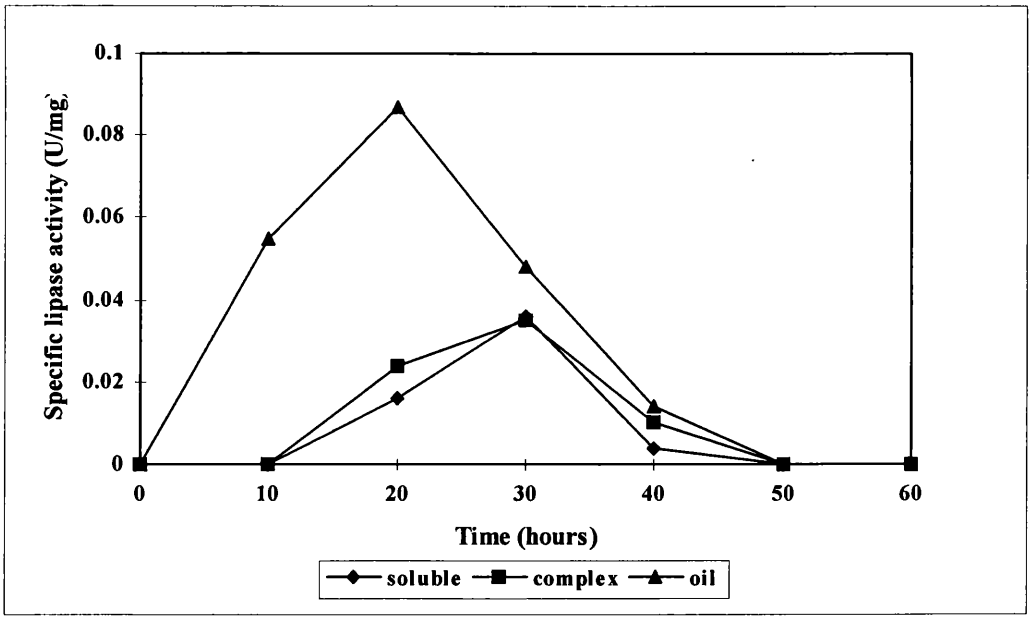


FIGURE 15.6 Comparison of lipase activity during *Streptomyces clavuligerus* fermentation using complex, soluble and oil growth media

During fermentation using the oil medium, the oil utilisation profile illustrates that the oil is used at a steady rate between 20 - 45 hours, following an initial lag period of 20 hours, which reiterates the OUR and CER profiles discussed previously. The rate of oil uptake decreases after 45 hours with a residual oil level of approximately 10g/l. Maximum lipase activity, of 0.087U/mg biomass, is detected prior to oil utilisation which again implies that the lipase may be induced (Tan & Gill, 1987). However, no lipase activity can be detected after 45 hours even though oil is present in the system and therefore, this again suggests that lipase has been inactivated/inhibited or there is a physical limitation to oil uptake.

The morphology of *Streptomyces clavuligerus* is also important when considering oil utilisation given the results from assay development which suggests that the lipase is cell associated. The filamentous nature of *S.clavuligerus* will also impact on the rheology of fermentation broth and hence oil dispersion. The variation in main hyphal length during fermentation on each of the media is represented in figure 15.7. This measurement does not include the length of the branching hyphae which would be the he total hyphal length.

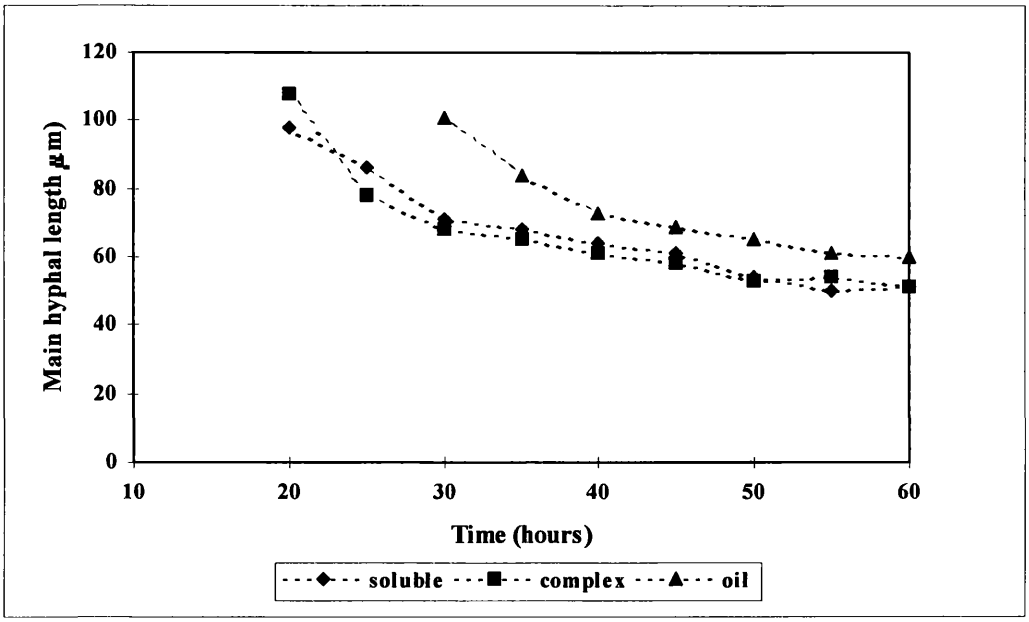


FIGURE 15.7 Comparison of main hyphal length during *Streptomyces clavuligerus* fermentation using complex, soluble and oil growth media

From these results, (figure 15.7), it becomes evident that there appears to be very little variation in hyphal length between the three types of media. The hyphae were very long and highly branched with a high degree of entanglement. During the early stages of growth determination of hyphal length was difficult but by 20 hours, with complex and soluble media, and 30 hours, with oil medium, hyphal measurements could be made. In complex and soluble media fermentations fragmentation of the hyphae occurs between 20 - 30 hours and branching frequency decreased. Other fragments of hyphae were visible with an approximate size of 5 - 10μm based on comparison with the main hyphal length. During the oil medium fermentation hyphal lengths could not be measured until 25 hours and fragmentation occurred between 30 - 40 hours due to the slower rate of growth. However, growth did appear to be more dispersed.

Fragmentation occurs prior to oil utilisation, except in oil medium fermentation and therefore, may play a role with respect to efficient oil uptake. Oil utilisation is dependent on cell-substrate association, as well as efficient oil dispersion and fragmentation would increase the specific interfacial area for lipase activation thus influencing oil uptake (Ohta *et al.*, 1995). This would be dependent on the mixing conditions within the fermenter and its relative effect on broth rheology and oil dispersion. It should also be noted that lipase activity was detected between 20 - 30 hours which would support this theory. However, lipase activity may have increased due directly to fragmentation. If the lipase is cell associated, the cell bound lipase will limit the secretion of any further enzymes, such as extracellular lipase, but fragmentation will allow their release into the fermentation broth inducing oil uptake (Jacobsen *et al.*,1989). This seems unlikely since lipase activity was not detected in broth supernatant during assay. It is also possible that fragmentation has increased oil utilisation by reducing viscosity of the fermentation thus improving mixing and hence oil dispersion. This would result in increased cell-oil association and therefore, improve oil uptake.

Chapter 5.1 Summary of initial fermentation studies

It can be concluded that oil utilisation is dependent on the medium used during fermentation and the organism will use an alternative carbon source, such as dextrin, in preference to the oil. Secondly, the lipase required for oil utilisation appears to be induced and is not readily detectable throughout the course of the fermentation. The highest activity level was measured during fermentation using the oil medium and suggests that the level of oil present will determine the amount of lipase produced as well as the time of expression. However, the lipase is either not induced or is inactivated during the later stages of the study thus reducing oil utilisation.

One of the most significant observations from these results is the fact that oil is only utilised for a very short period of time and therefore, in all the fermentations, a residual amount of oil is present at the end of the fermentation irrespective of the initial oil concentration. This has been reported in a number of different fermentations (Park *et al.*, 1994; Choi *et al.*, 1996 and Stahmann *et al.*, 1997)). This could be due to an inability of the organism to metabolise certain components of the oil since it may have undergone partial hydrolysis during the course of the fermentation. However, studies carried out by Stowell, (1987) demonstrated that the residual oil present at the end of an antibiotic fermentation had the same composition of the input oil.

There could also be a physical limitation of oil mass transfer such as reduced oil- cell association due to mixing and oil dispersion problems as discussed previously. It may be that the oil is in an inaccessible form for the organism to use such as bound to the soya flour particles, (chapter 11.2), or the aqueous phase has limited accessibility to the interface due to the broth rheology.

In order to determine the factors affecting oil utilisation, lipase expression and the problem of residual oil, further fermentations were carried using the three types of media with reduced levels of oil. The fermentation broths were monitored for additional parameters such as dextrin, ammonia, phosphate and protein levels as well as metabolic activity with respect to β-oxidation and clavulanic acid titre. The results of these studies are illustrated in the next chapter.

Chapter 15.2 *Streptomyces clavuligerus* fermentation using reduced oil media

During this section of the study, fermentations were carried out using the same media as previously described except for a reduction in the oil concentration. From the initial fermentations it was observed that a residual level of oil was present at the end of the fermentation of approximately 5-10g/l. Therefore, the initial oil concentration in the various media was reduced by 10g/l to 15 g/l in the complex and soluble media and 25g/l in the oil medium, respectively. It was anticipated that this would help to reduce the residual oil level. Further analysis of the broth samples was also done in order to obtain the nutrient depletion profiles so changes in the metabolism of the organism, which appear to be occurring during the fermentation, could be investigated.

The results of off gas analysis from the fermentations are shown in figures 15.8 to 15.10 for each of the respective media. The main observation is that although the plots exhibit similar trends in growth to those previously observed, with the use of the reduced oil media, the fermentation exhibits a longer lag period irrespective of the type of medium. The extended lag period delays the start of growth but does not appear to affect the length of the respective growth phases and hence, the growth rate of the organism.

On complex medium the lag phase increased from 5 hours to 8 - 10 hours which is followed by the first phase of growth until 18 - 20 hours when the rate of oxygen uptake slows and the organism switches carbon source as indicated by a change in the RQ value (figure 15.10). This is accompanied with the significant increase in pH as observed previously, from pH 7.28 to pH 7.69 (figure 15.11). The second growth phase continues until 30 - 35 hours, reaching a maximum oxygen uptake of 20 mmol/l/h, during which time the pH falls to pH 7.32. From 35 - 40 hours the OUR slows, the pH value steadies and the RQ falls 0.75 indicating that oil is being utilised. Again the CER plot demonstrates similar trends (figure 15.9).

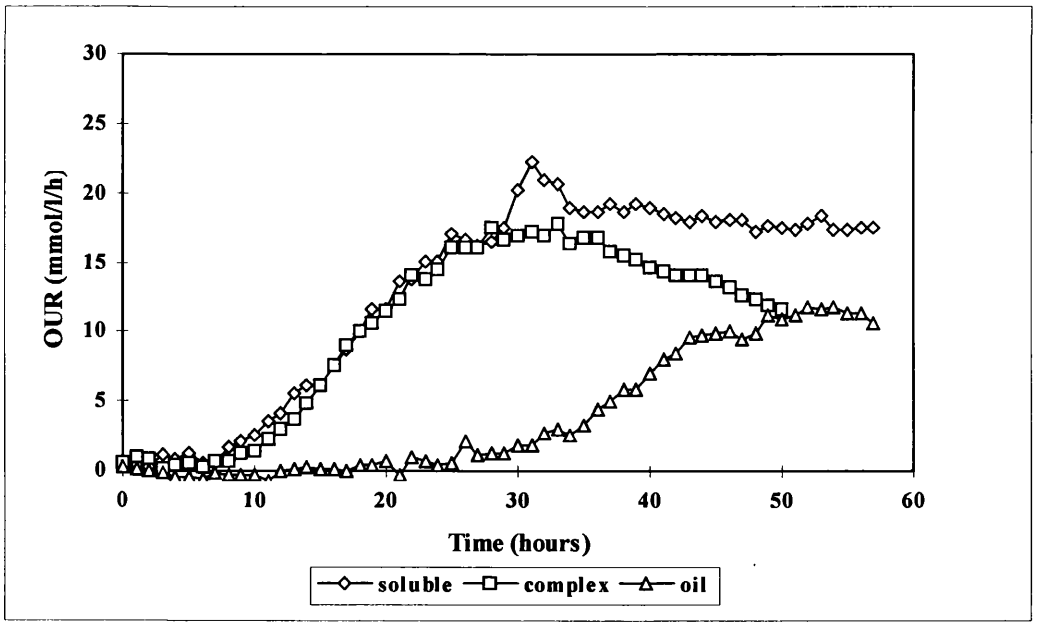


FIGURE 15.8 Comparison of OUR during *Streptomyces clavuligerus* fermentation using reduced oil complex, soluble and oil growth media

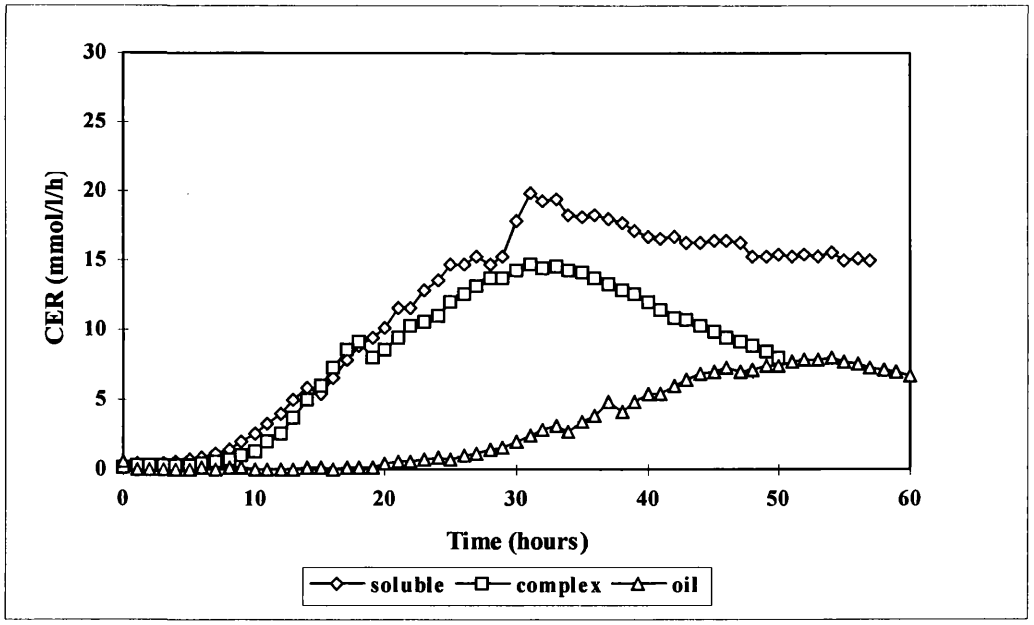


FIGURE 15.9 Comparison of CER during *Streptomyces clavuligerus* fermentation using reduced oil complex, soluble and oil growth media

The plot resulting from the use of the reduced oil soluble medium shows a minimal increase in the lag phase of approximately 1 - 2 hours which could attributed to batch variation (figure, 15.8). The OUR increases rapidly after the lag phase until 23 hours when the rate slows for 2 hours, equivalent to the peak in pH, (figure 15.11) and then the organism enters its second growth phase reaching a maximum oxygen uptake of 23 mmol/l/h at 32 hours. The rate of uptake decreases slowly over the next 30 hours during which time the pH falls rapidly and oil metabolism begins.

The most significant increase in lag phase was observed with the oil medium which resulted in a further 10 hours fermentation before growth began. This resulted in a total lag phase of 30 hours (figure 15.8). The delay in growth could be due to lipase induction. The role of oils as lipase inducers has been studied by Shimada *et al.*, (1992) who found that the presence of triacylglycerols will induce lipase in *Geotrichum candidum* although fatty acids have been shown to be better inducers of lipase activity (del Rio *et al.*, 1990; Obradors *et al.*, 1993 and Gordillo *et al.*, 1995). If the presence of oil is required for the lipase to be produced/expressed, reducing the initial concentration of oil would slow the rate enzyme induction which suggests that there may be minimum oil concentration required for lipase induction (Lee & Rhee, 1993).

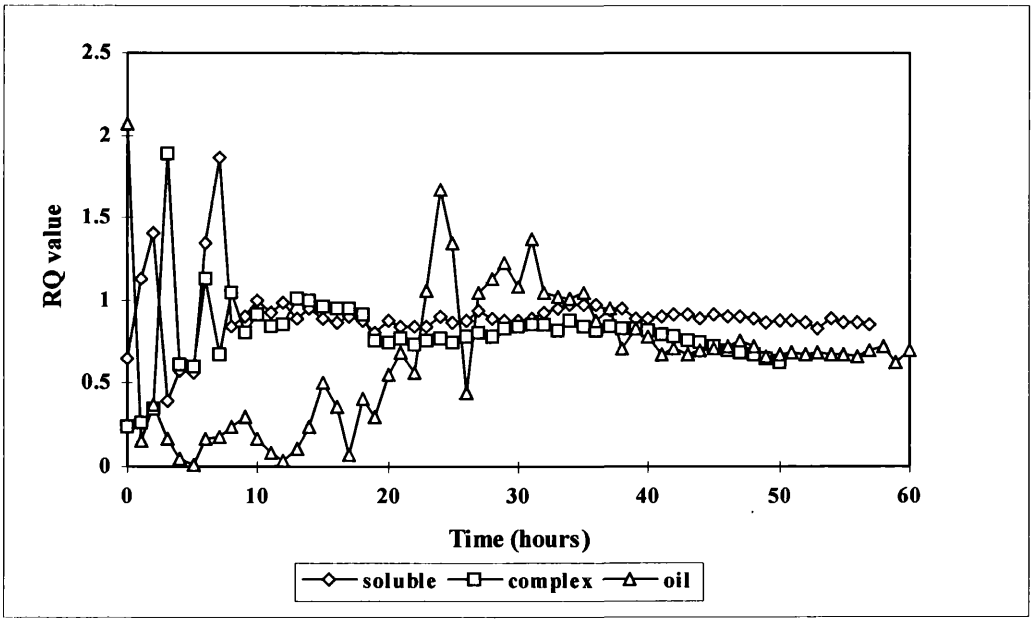


FIGURE 15.10 Comparison of RQ during *Streptomyces clavuligerus* fermentation using reduced oil complex, soluble and oil growth media

The use of the reduced oil media during fermentation is creating an increased lag phase. As mentioned previously, this could be due to lipase induction or some other factor. As discussed in Chapter 7.5.1, the presence of oil in the fermenter environment has been shown to improve the oxygen transfer capacity of the system. Rols and Goma, (1989) showed that the presence of 19% soya bean oil enabled a 1.85-fold increase of the K_La value in an *Aerobacter aerogenes* fermentation, by acting as an oxygen vector (Rols *et al.*, 1990). Therefore, reducing the oil level may have slowed the rate of oxygen transfer in the fermenter, when compared to non-reduced oil media fermentation, resulting in a lower microbial growth rate.

However, there does appear to be a variation in how long the lag phase is increased dependent on the medium used. The least increase was observed with the use of the soluble medium whilst both the complex and oil media exhibited significantly longer lag phases. When considering the media, the only difference is the presence of soya flour which suggests that this may also have an influence on cell growth and metabolism. The soluble medium has both a soluble nitrogen source, peptone and an additional soluble carbon source, starch, which are more accessible and will therefore have a lesser effect on growth. Soya flour, however, is insoluble and provides a far more complex nutrient source. It is possible that the presence of oil aids soya flour utilisation or breakdown by some form of interaction and this interaction may be of mutual benefit to both the oil and the soya flour. For example, soya flour can act as an emulsifier and hence would aid oil dispersion.

Soya flour provides both an important carbon and nitrogen source, (Zhou *et al.*, 1992) and the utilisation of the soya flour, during the initial period of growth can be observed by increased levels of ammonia due to deamination, (figure 15.12), which is reflected in an increase in pH, (figure 15.11) and a decrease in soluble protein levels (figure 15.13). It can be seen that there is a rapid increase in ammonia level from 10 - 20 hours with complex medium which corresponds to the first phase of growth described earlier. This suggests that the soya flour is being broken down and the carbon component used by the organism preferentially. A similar trend can be observed with growth on the oil medium. Oil is utilised at a later stage in both fermentations.

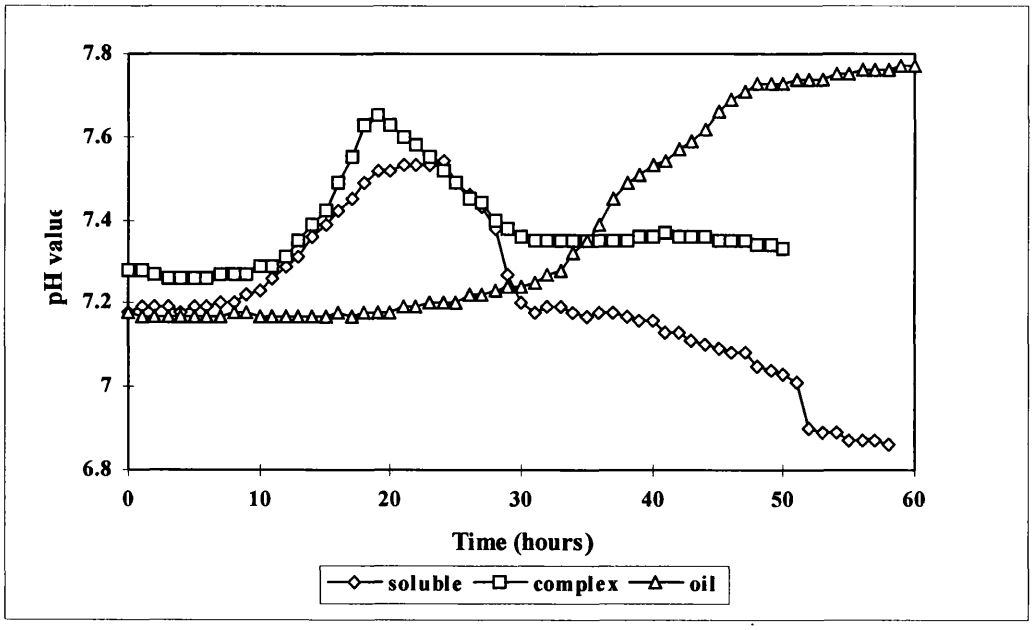


FIGURE 15.11 Comparison of pH during *Streptomyces clavuligerus* fermentation using reduced oil complex, soluble and oil growth media

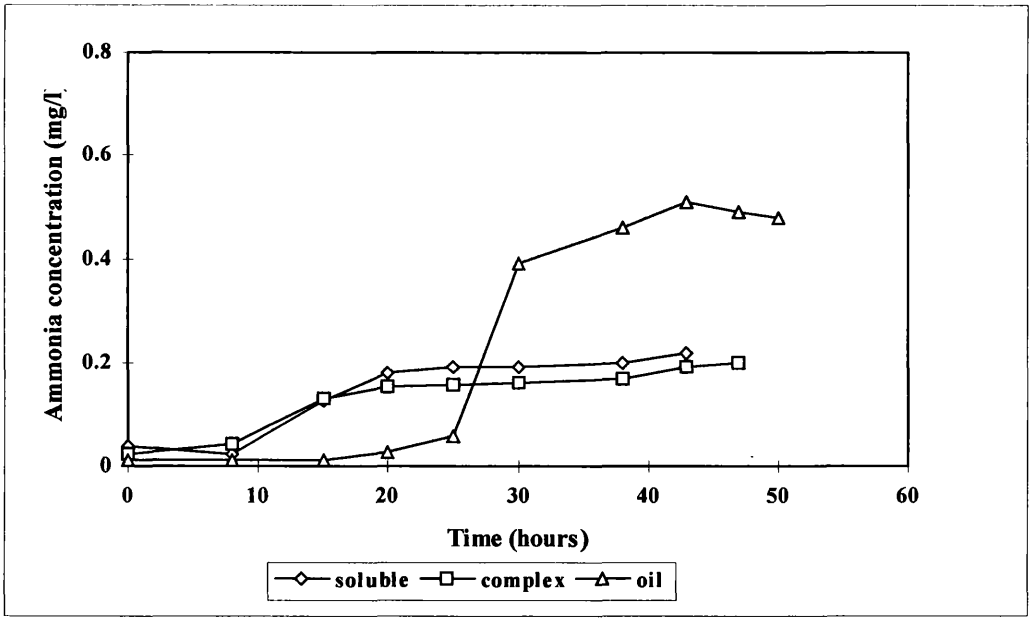


FIGURE 15.12 Comparison of ammonia level during *Streptomyces clavuligerus* fermentation using reduced oil complex, soluble and oil growth media

The use of the soluble medium, with reduced oil concentration, resulted in two phases of growth prior to oil utilisation. The first phase of growth could be due to either utilisation of the dextrin, starch or peptone. Starch is an important carbon source since it is hydrolysed slowly creating carbon-limiting conditions thus increasing antibiotic synthesis (Lebrihi *et al.*, 1988). However, the results suggest that the peptone is being used initially since there is an increase in the pH, (figure 15.11) and ammonia level, (figure 15.12), which would result from the breakdown of this nutrient source. This is supported by the levels of soluble protein which decrease rapidly during the first 25 hours of growth, (figure 15.13). It is also possible that other carbon sources are being utilised by the organism because the oil cannot provide sufficient energy for the metabolic demands of the cell during the initial growth phase. Hence, the oil cannot be used.

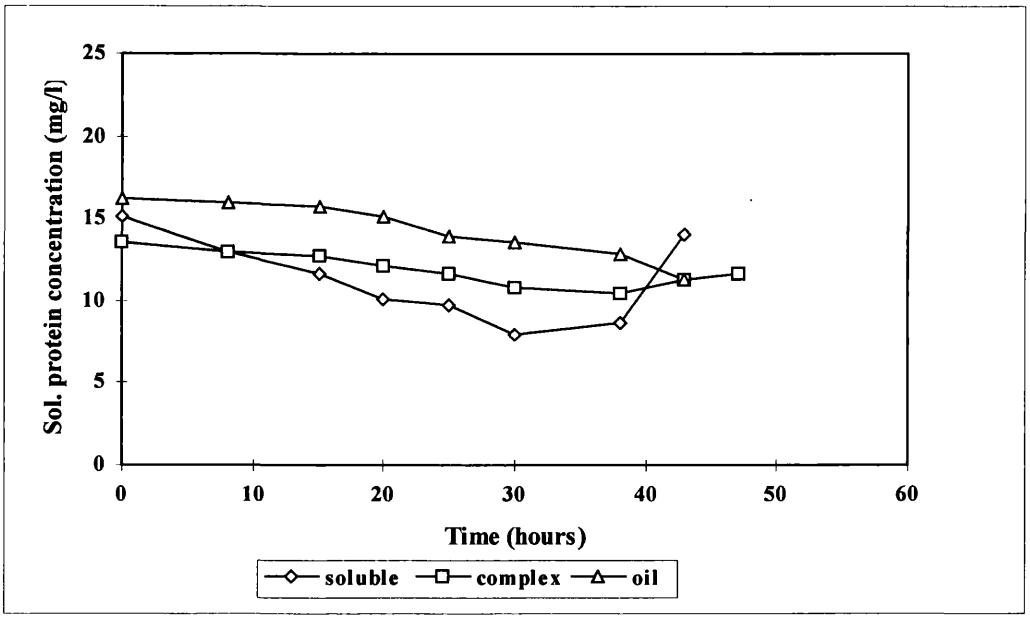


FIGURE 15.13 Comparison of soluble protein level during *Streptomyces clavuligerus* fermentation using reduced oil complex, soluble and oil growth media

The sequence of the use of carbon/nutrient sources by the organism can be further clarified by figure 15.14 which illustrates the rate of dextrin utilisation and figure 15.15 showing variation in the oil concentration through the course of the fermentations. During growth on the complex medium, dextrin levels only start to decrease after approximately 15 hours and dextrin is utilised until 30 hours at which point metabolism switches to oil. It can be seen that very little oil is actually utilised. A similar trend is

exhibited by the growth on the soluble medium. The plot for growth on the oil medium shows that the oil is only metabolised after 35 -40 hours which confirms the results observed previously.

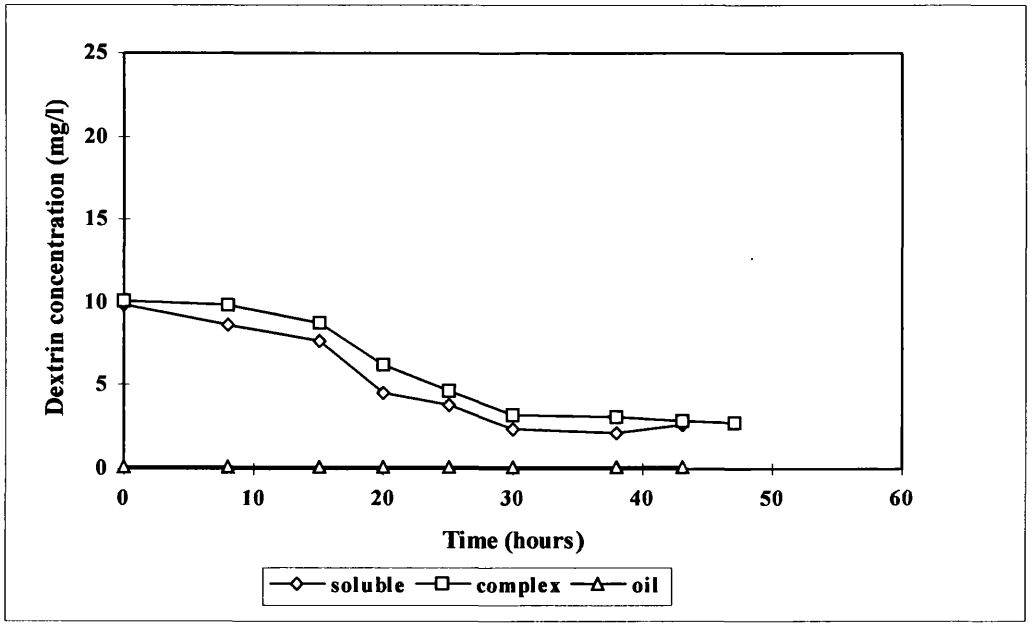


FIGURE 15.4 Comparison of dextrin level during *Streptomyces clavuligerus* fermentation using reduced oil complex, soluble and oil growth media

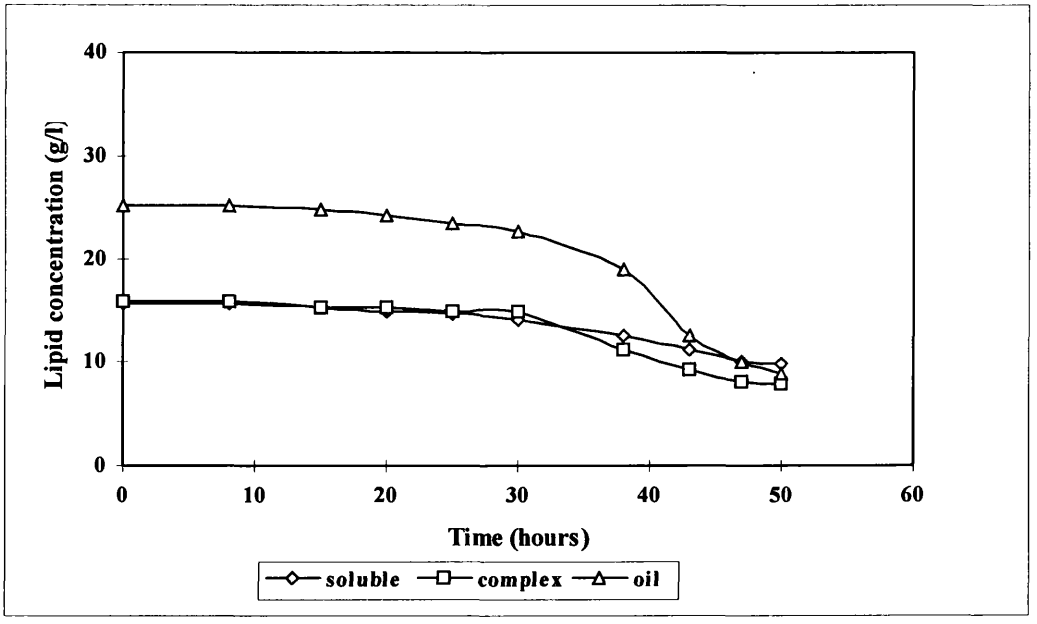


FIGURE 15.15 Comparison of lipid level during *Streptomyces clavuligerus* fermentation using reduced oil complex, soluble and oil growth media

Therefore, during growth on the complex medium the organism preferentially utilises the carbon present in the soya flour and then the dextrin before finally utilising the oil even though there is still dextrin present. This implies that it is of some advantage for the organism to utilise the oil whether it is energetically favourable or to stimulate secondary metabolism. During the growth on the soluble medium and the oil medium the organism expresses the similar preferences.

In order to utilise the oil the organism produces a lipase and the level of activity detected for each fermentation is shown in figure 15.16. This demonstrates that the lipase produced can be detected prior to oil utilisation irrespective of the medium used which again suggests that the lipase is induced. Maximum lipase activity was detected at 35 - 40 hours with complex, oil and soluble media fermentations but the greatest level of activity was exhibited in the oil medium which has a higher initial oil level. The activity level falls rapidly once oil metabolism has started. The level in the soluble medium is undetectable after 42 hours and less than 0.02U/mg specific activity can be detected in complex medium fermentation after 50 hours fermentation.

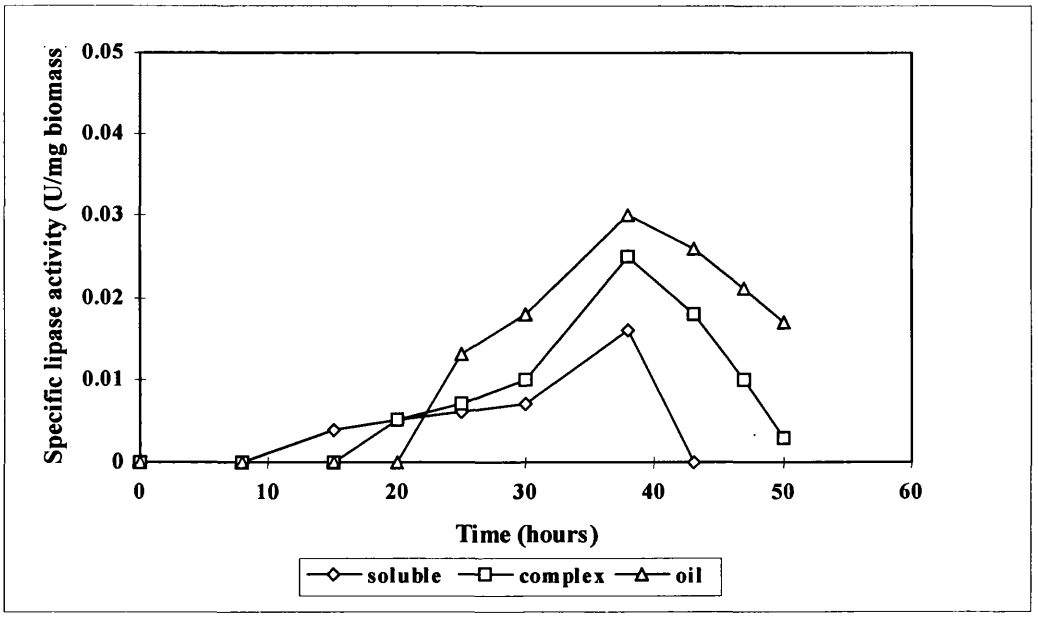


FIGURE 15.16 Comparison of lipase activity detected during *Streptomyces clavuligerus* fermentation using reduced oil complex, soluble and oil growth media

This implies that the level of activity is dependent on the concentration of oil within the system (Gordillo *et al.*, 1995). Although the opposite effect has been observed with *Ashbya gossypi* when grown on soy bean oil. It was shown that by reducing the oil concentration the lipase activity was increased (Stahmann *et al.*, 1997). It is also possible that, since the lipase appears to be cell associated, the induction of lipase activity requires cell - substrate association to be established and therefore, reducing the level of oil will decrease this association resulting in slower induction of the lipase (Tan and Gill, 1985). Large *et al.*, (1999) demonstrated that the activity of the cell bound lipase from *S. clavuligerus* is only activated in the presence of oil and will increase with increasing oil concentration.

The level of lipase activity decreases rapidly once oil is being metabolised and is undetectable by 50 hours even though there is oil present in the fermentation broth, (figure 15.15). This suggests that the lipase may be inactivated in some way thus limiting oil utilisation, for example by shear inactivation (Lee & Choo, 1988 and Stahmann *et al.*,1997). The metabolic activity of the organism may also be a limiting factor. Figure 15.17 shows the β-oxidation activity and hence the metabolic activity of the organism to oil through the course of each fermentation.

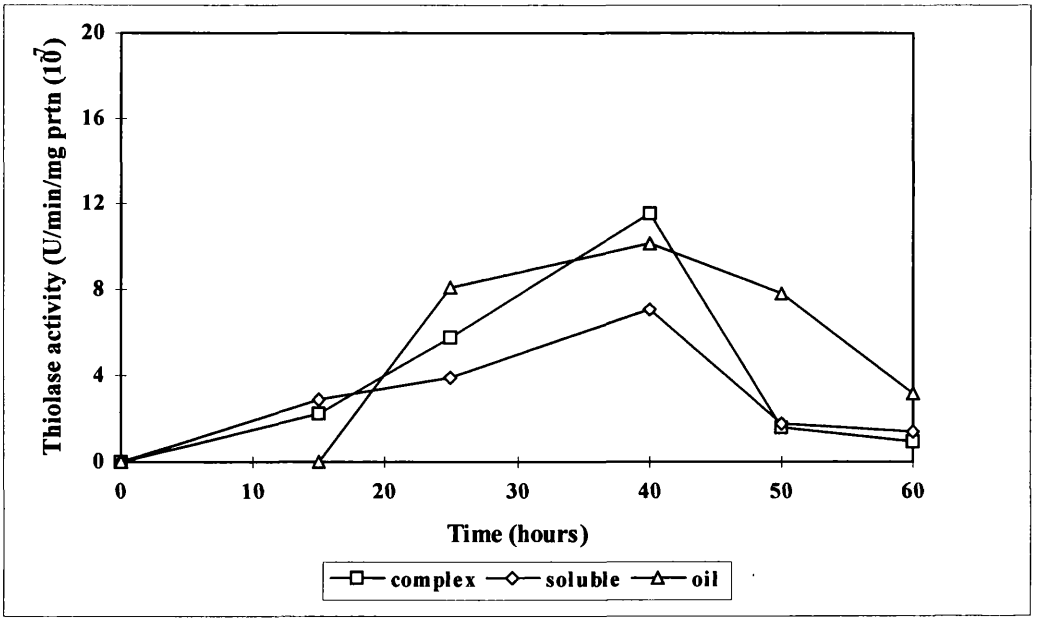


FIGURE 15.17 Comparison of β-oxidation activity during *Streptomyces clavuligerus* fermentation using reduced oil complex, soluble and oil growth media

The level of β-oxidation activity is shown to vary with the type of medium used during fermentation (figure 15.17). The plot indicates that when grown on an oil substrate, the organism demonstrates a greater metabolic activity toward oil although this does decrease rapidly after 50 hours. With the complex and soluble media fermentations, the organism is metabolically active for a shorter period and again the metabolic activity ceases after 50 hours even though oil is present which corresponds to the decrease in lipase activity. It is probable that the reduced level of β-oxidation activity is due to lower lipase activity thus limiting oil utilisation since oil is still present and in a utilisable form. The activity falls because it is not required, i.e. responding to the environment.

The lipase may be inactivated or it is also possible that lipase production is regulated/controlled on a cellular level. Lipase synthesis has been reported to be growth regulated (Lee & Rhee, 1993 and Kim *et al.*, 1996). After lipase has been induced, the growth rate slows due to the substrate and once the cells start to enter stationary phase lipase synthesis stops due to energy limitations (Gordillo *et al.*, 1995). Thus oil metabolism is limited. It is also possible that the lipase has been has been inactivated by the products of oil utilisation. Although fatty acids, such as oleic acid, linoleic acid and linolenic acid, have been shown to induce lipase activity, (Suzuki *et al.*, 1988 and Montesiros *et al.*, 1996), if they accumulate they will exhibit a negative feedback effect on lipase synthesis, (Obrados *et al.*,1993 and Choi *et al.*, 1996), which will be toxic to the cell. Therefore, the presence of fatty acids will inhibit the further hydrolysis of oil and oil metabolism will be growth related. In practice, this is unlikely in this situation since the fatty acids are transported directly into the cell and cannot be detected in the medium.

The use of oil during *S.clavuligerus* fermentation has been shown to improve antibiotic titre, (Park *et al.*, 1994 and Lee & Ho, 1996) and with the correct medium formulation of slow release nutrients, high rates of antibiotic biosynthesis can be sustained by avoiding carbon catabolite repression (Jain *et al.*, 1992 and Park *et al.*, 1994). Therefore, the level of clavulanic acid in the fermentation was measured during the study. The results are shown in figure 15.18. The actual antibiotic titre was relatively low irrespective of the media used although there was a difference in titre between the complex medium and the soluble and oil media fermentations.

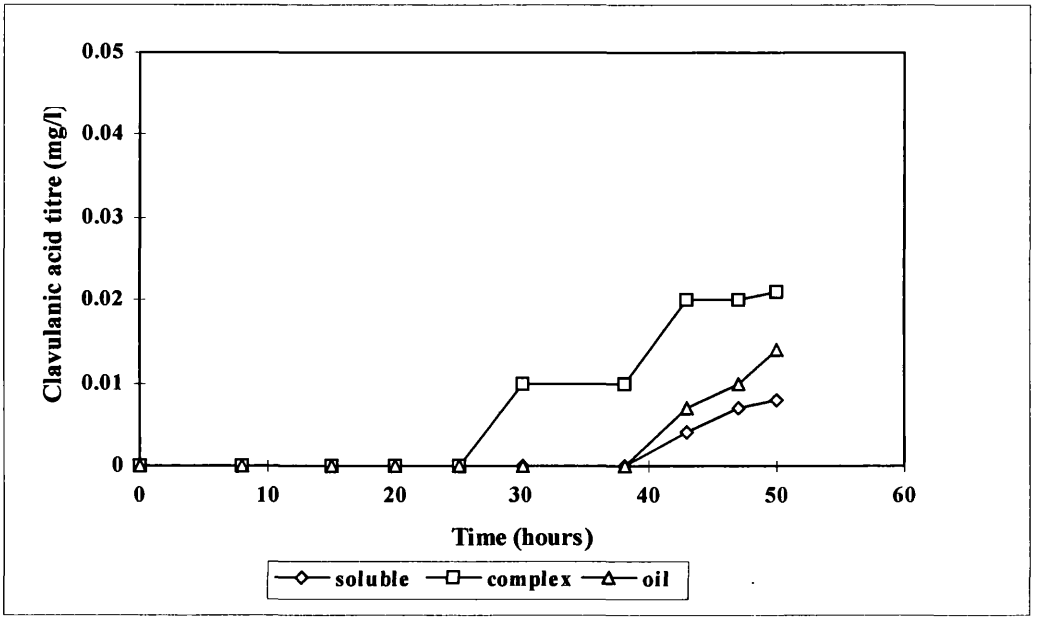


FIGURE 15.18 Comparison of clavulanic acid titre during *Streptomyces clavuligerus* fermentation using reduced oil complex, soluble and oil growth media

During fermentation with complex medium the clavulanic acid starts to be produced at 30 hours and the titre gradually increases as the fermentation proceeds and metabolism switches to oil. Clavulanic acid is not produced until 40 hours with both soluble and oil media. The level exhibited in the oil medium is approximately 50 % lower than that in the complex medium which could be due growth limitations. It should be noted that at 50 hours, clavulanic acid titre appears to still be increasing with the oil medium but stabilising in the complex and soluble media fermentations. The lowest level of antibiotic is observed when the soluble medium was used despite the presence of starch which has been reported to increase antibiotic synthesis (Lebrihi *et al.*, 1988). This suggests that the presence of oil does indeed stimulate antibiotic, clavulanic acid, production in *Streptomyces clavuligerus*.

The level of antibiotic produced is limited by a number of factors such as carbon source, nitrogen source and phosphate level (Hu *et al.*, 1984; Aharonowitz & Demain, 1979 and Fang & Demain, 1995). Phosphate has been shown to be of key importance and therefore, levels of phosphate were measured throughout the fermentations (figure 15.19).

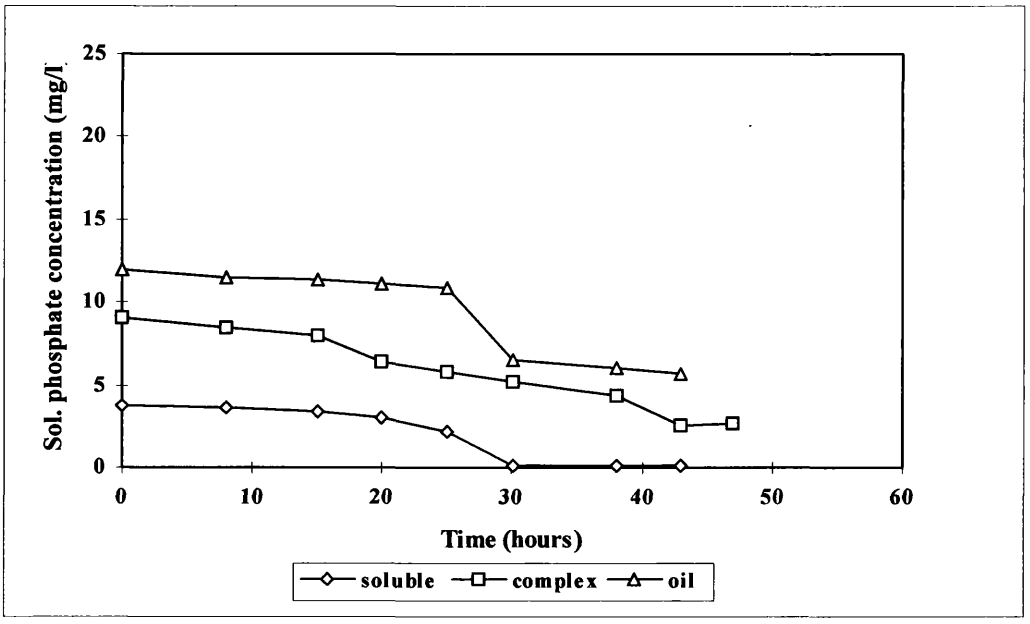


FIGURE 15.19 Comparison of phosphate level during *Streptomyces clavuligerus* fermentation using reduced oil complex, soluble and oil growth media

The main observation is that the phosphate is depleted by 30 hours in the soluble medium fermentation. This may account for the low antibiotic titres since at low aeration and non optimal growth conditions phosphate is required for antibiotic biosynthesis and will stimulate β-lactam antibiotic production (Fang & Demain, 1995). However, at higher concentrations phosphate will be limiting and thus antibiotic synthesis will be inhibited which explain the low clavulanic acid titres (Aharonowitz & Demain, 1977).

In summary, the results from this section of the study confirm the initial findings. The composition of the fermentation medium will have a significant effect on the rate of oil utilisation and has been seen that even in an oil medium, *Streptomyces clavuligerus* will utilise a more complex carbon source in the form of soya flour in preference to oil. This was also demonstrated when the organism is grown on complex and soluble media. The addition of oil did however increase the clavulanic antibiotic titre. The level of oil present in the fermentation medium will also determine the rate of growth of the organism as well as the rate of oil utilisation.

The level of lipase activity is also influenced by the composition of the medium in particular the concentration of oil present. In general terms the higher the oil concentration the greater the level of lipase activity and the sooner the lipase is produced. This was most significant when the oil concentration was reduced in the fermentation. This suggests that the lipase is induced. The reason for this may be that lipase induction is dependent on oil - cell association and at lower oil concentration the less oil is dispersed and hence less oil is physically available to the cell.

Finally, once again there is a residual level of oil present at the end of the fermentation and it is evident that oil metabolism has ceased. The lipase is no longer active which could be for a number of physical reasons. The lipase could have been inactivated due to changes in the morphology of the organism such as increased fragmentation reducing the affinity of the cell for oil (Ohta *et al.*, 1995). It is also possible that the lipase has been inactivated due to shear (Lee & Choo, 1989) or by a reduced level of dissolved oxygen, below a critical level, in the broth (Becker *et al.*, 1997). It should be noted also that Khan *et al.*, (1992) showed that proteins can be denatured by the air/liquid interface. The lipase may also be affected at the cellular level by a feedback mechanism such as fatty acid build up(Tan & Ho, 1991), or by the presence of the oil being self limiting (Stahmann *et al.*, 1997). These factors were investigated in the next part of the study.

Chapter 15.3 Factors affecting oil utilisation in *Streptomyces clavuligerus* fermentation

From the results of the previous studies it has become apparent that a number of parameters determine the efficient uptake of oil during *Streptomyces clavuligerus* fermentation. Therefore, this final section of the study investigated the effect of a number of variables such as oil concentration, aeration and agitation on the oil utilisation rate during fermentation on complex medium.

Chapter 15.3.1 Effect of oil concentration

It has been proposed that the concentration of oil present in the fermentation medium is a key factor in efficient oil uptake. It has been reported that there may be a critical concentration of oil required for lipase expression as mentioned previously or that the presence of higher concentrations of oil will prevent lipase activity. It was also suggested that the presence of oil at high concentrations would actually have deleterious effect due to mass transfer problems associated with oil dispersion. Therefore, a series of fermentations were carried out in which the initial oil concentration was varied between 25g/l - 10g/l. The results obtained are shown in figures15.20 and 15.21.

The lipase activity profiles exhibited a similar trend irrespective of the oil concentration although it will affect the rate of expression of lipase activity and the rate at which the oil is used. The start of oil metabolism occurred later at the lower initial oil concentrations and there was a delay in the expression of the lipase activity. At higher concentrations the lipase was detected earlier and in greater amounts and oil was metabolised at a faster rate. At 25g/l oil concentration the maximum lipase activity was detected at 30 hours prior to oil utilisation which began at 30 - 35 hours whereas at a lower oil concentration of 10g/l, lipase was not expressed until 45 hours and the oil did not start to be metabolised until 45 - 50 hours. The results also suggest that there may be a dual or biphasic lipase which is demonstrated clearly with the lipase profile at 25g/l of oil. This has been observed in other micro-organisms, (Heidrich *et al.*, 1991), but this can only be confirmed by further isolation and purification.

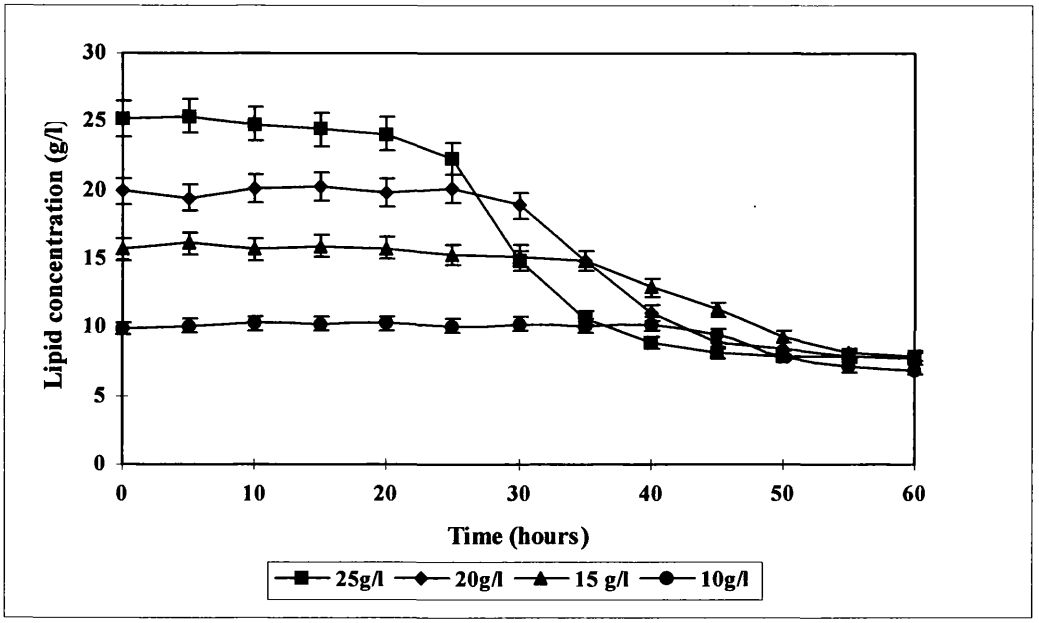


FIGURE 15.20 Effect of oil concentration on oil utilisation rate during *Streptomyces clavuligerus* fermentation using complex medium

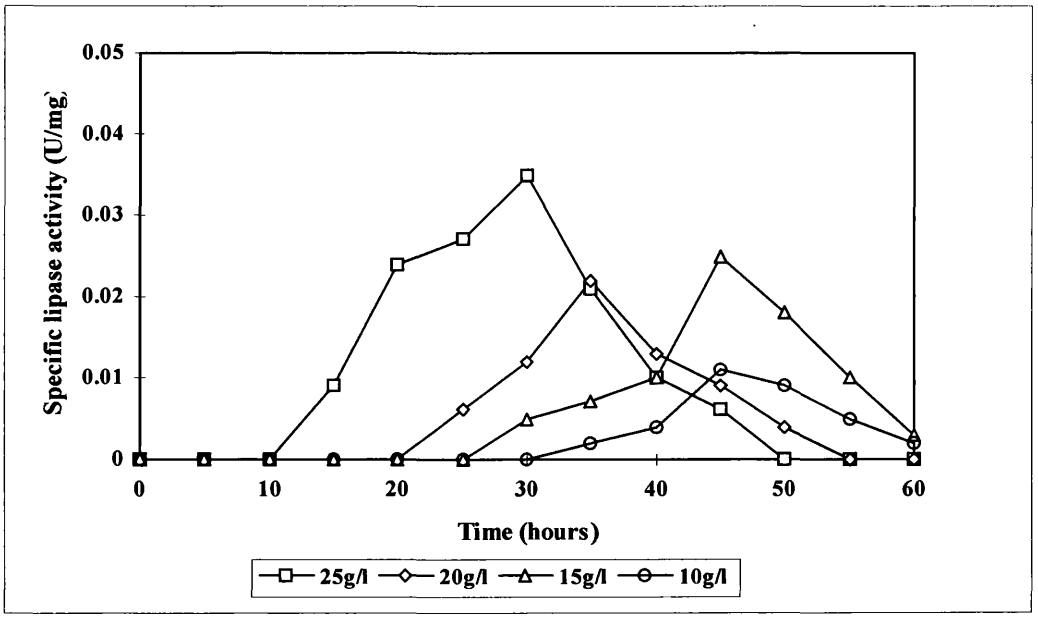


FIGURE 15.21 Effect of oil concentration on lipase activity during *Streptomyces clavuligerus* fermentation using complex medium

This results do indicate that there may be a critical oil level in order for lipase induction or as proposed earlier, at lower oil concentrations oil-cell association would be reduced. It has also been found that the delay in metabolism at lower oil concentrations could be due to the actual size of the oil droplets. Bakhuis & Bos, (1969) reported that at lower oil concentrations the size of the oil droplets dispersed in the fermentation broth would be below the region of 20-25μm. They found that minimum growth rate occurred and concluded that due to their size, the droplets were to rigid to allow good contact with the biomass thus resulting in lower growth rates. In this system this would have minimal effect since previous size distribution studies have shown that the oil droplets, when dispersed in water, have an average diameter greater than this even at high stirrer speeds,(chapter 11.2). However, Ohta *et al.*, (1995) reported that it was not the size of the oil which was crucial but the effect of the other medium components on the oil droplet size such as emulsification which would reduce droplet size thus increase cell-oil association.

The results of these fermentations demonstrated that the concentration of oil will determine the efficiency of oil utilisation. The study also showed that there was still a residual level of oil irrespective of the initial oil concentration in the medium. A standard lipase solution of 1U/ml volumetric activity, made from purified lipase from *Candida cylindracea* as supplied by Sigma, was added into the fermentation broth during the later stages of the fermentation. Of the 1U/ml added, only 0.25U/ml was detectable 30 seconds after injection and no activity was detected 90 seconds after injection. Similar results were observed by Stahmann *et al.*, (1997) when studying *Ashbya gossypii*. This does suggest that the limitation to oil metabolism is due to lipase inactivation. Therefore, the next section of the study observed the effect of physical conditions within the fermenter on oil metabolism such as agitation and dissolved oxygen concentration.

Chapter 15.3.2 Effect of dissolved oxygen concentration

From previous fermentations it was seen that maximum oil utilisation and lipase activity was observed at 40-50% dissolved oxygen concentration but once the level of dissolved oxygen fell below this level, oil metabolism slowed significantly. During normal operation, the dissolved oxygen concentration was not controlled due to the high viscosity of the medium. It is documented that antibiotic production has a critical oxygen level (Scott *et al.*, 1988 and Yegneswaran *et al.*, 1991) and therefore, it is possible that there is a critical level of dissolved oxygen for lipase production and/or oil utilisation.

The fermentation was run in the normal way using complex medium but when the dissolved oxygen level fell below 40% during the oil utilisation period the DOT was maintained at 50% by varying the air flow rate whilst maintaining a constant stirrer speed. Samples were taken at regular intervals and analysed for lipid concentration and lipase activity. The results are shown in figures 15.22 and 15.23.

This demonstrated that if the oxygen level was controlled the lipase activity profile varied little from an uncontrolled fermentation. However, after 40 hours there was a slight increase in activity when the oxygen level was controlled but this soon within10 - 15 hours. There was no significant change in the rate at which the oil was utilised although oil was utilised for approximately an additional 10 hours but a slow rate. There was no significant difference in the residual oil level. It is possible that the increased aeration has resulted in the deactivation of the lipase by contact with the air bubbles present in the system (Falk *et al.*,1991 and Michaels *et al.*, 1995). It is also likely that the system is limited due to viscous nature of the fermentation broth and therefore the demands on the air flow exceeded the capacity of the fermenter. Hence, the dissolved oxygen level will start to fall again.

These results show that maintaining the oxygen concentration in the fermenter has a limited effect on oil metabolism and the viscous nature of the broth rheology will limit its effect even further.

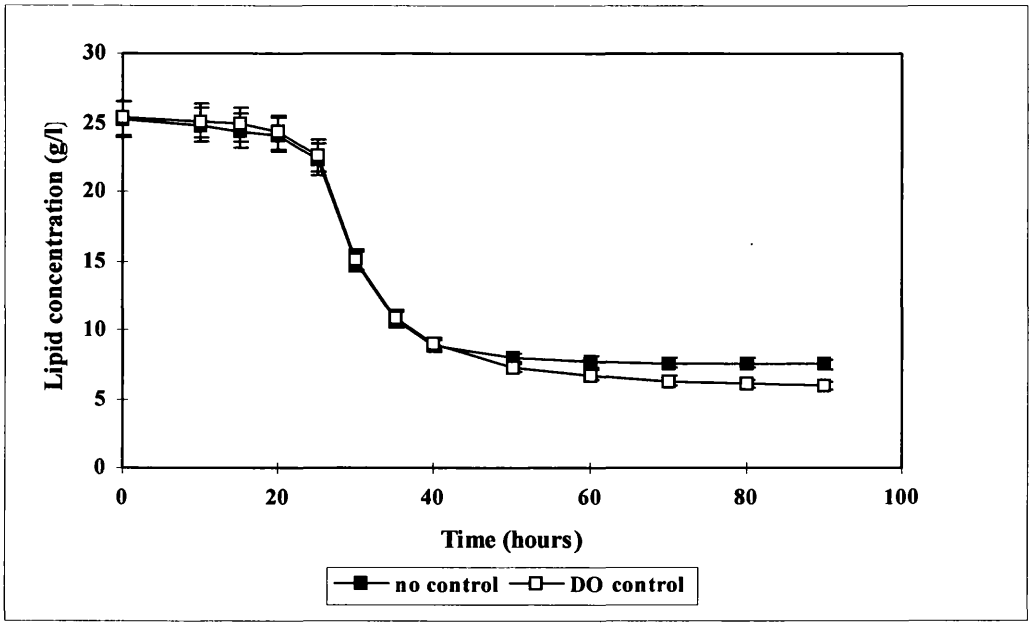


FIGURE 15.22 Effect of controlling DO level on oil utilisation rate during *Streptomyces clavuligerus* fermentation using complex medium

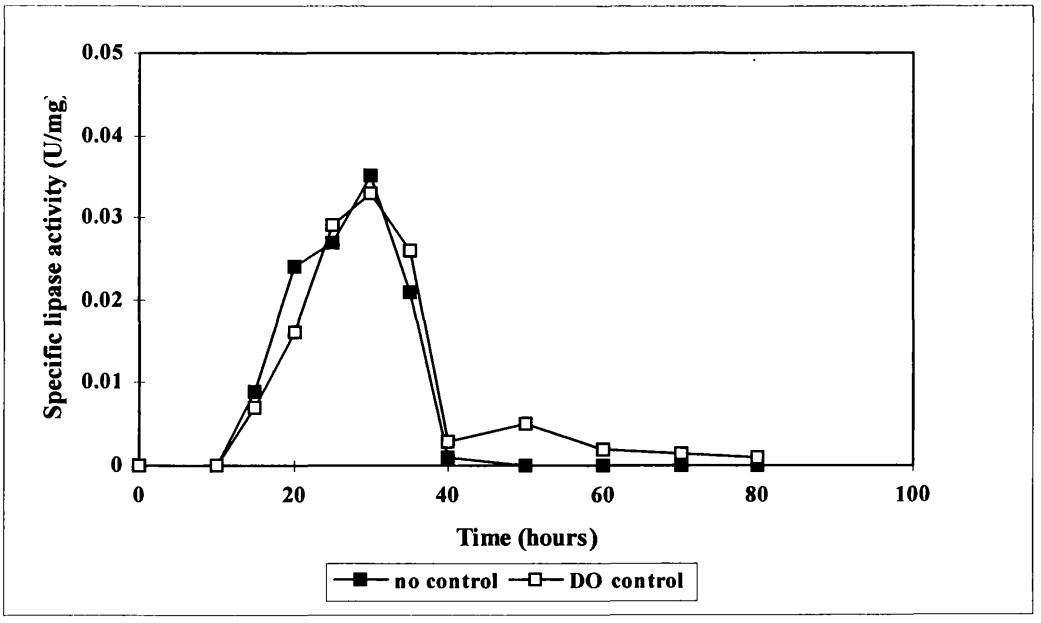


FIGURE 15.23 Effect of controlling DO level on lipase activity during *Streptomyces clavuligerus* fermentation using complex medium

Chapter 15.3.3 Effect of agitation

The rate of hydrolysis of an oil is a direct function of the interfacial area available for lipase activation and therefore good mixing and hence oil dispersion is essential in lipase catalysed systems. However, it has been reported that shear forces produced by mixing may inactivate enzymes. Lee & Choo, (1989) found that the lipase from *Candida cylindracea* lost activity as a function of shearing time and shear rate due to direct deformation of the lipase molecule. Therefore, the effect of increasing agitation rate was investigated during *S.clavuligerus* fermentation using complex medium. Whilst maintaining a constant air flow rate, the impeller speed was varied in order to maintain a dissolved oxygen level of 40 - 50%. At this concentration, oil uptake and lipase activity were at their maximum. Samples were taken and analysed for lipid content and lipase activity and the results are shown in figures 15.24 and 15.25.

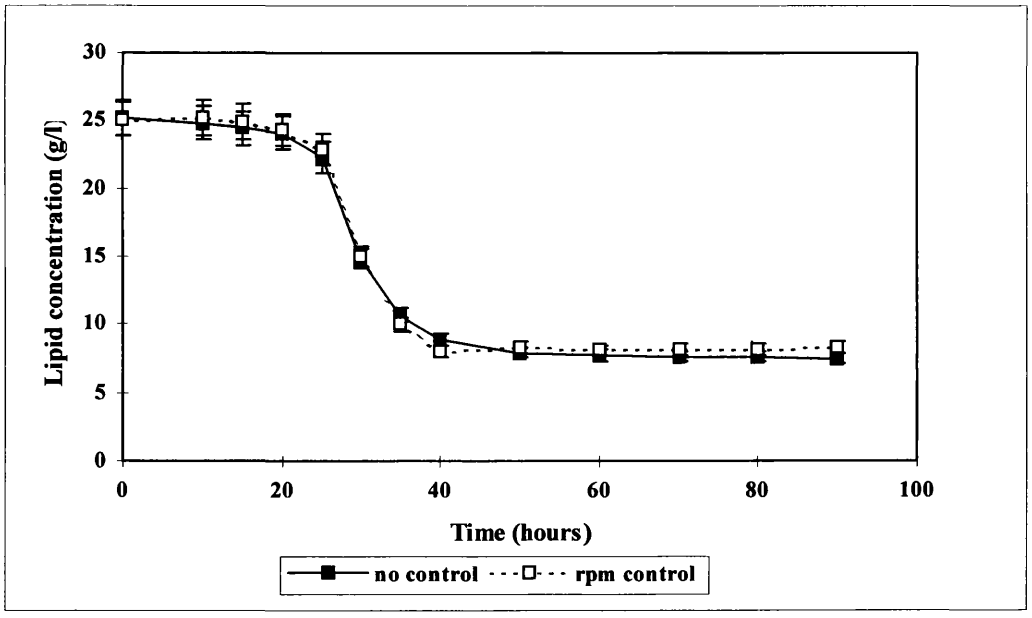


FIGURE 15.24 Effect of controlling stirrer speed on oil utilisation during *Streptomyces clavuligerus* fermentation using complex medium

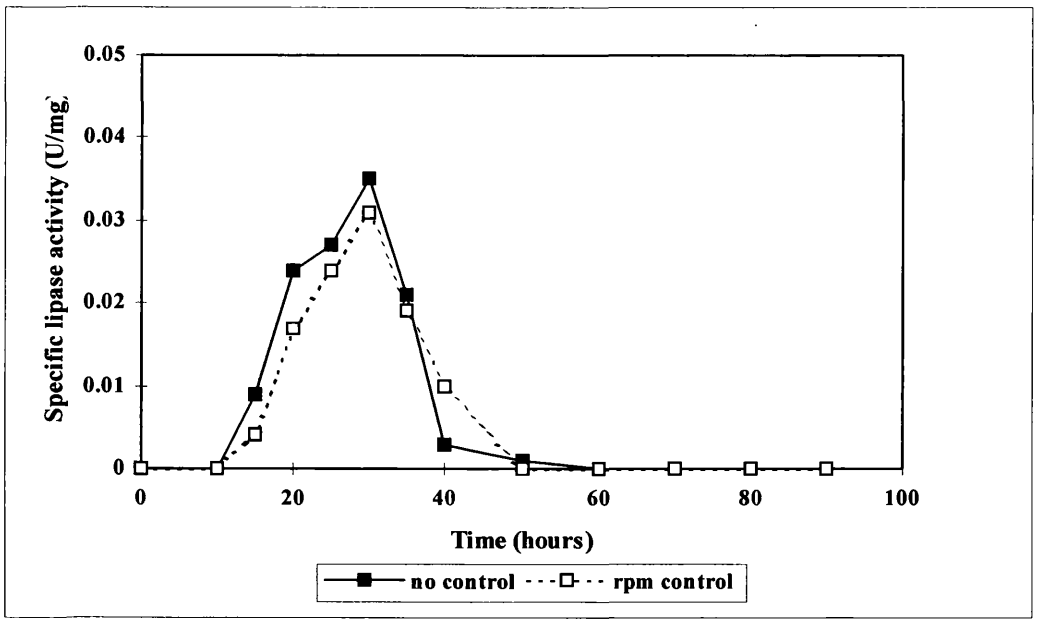


FIGURE 15.25 Effect of controlling stirrer speed on lipase activity during *Streptomyces clavuligerus* fermentation using complex medium

The plots demonstrate that there is very little change in the rate of oil utilisation although a lower level of lipase was expressed. This suggests that the lipase could have been inactivated due to a shearing effect or the increased stirrer speed has resulted in greater dispersion of the oil and hence smaller oil droplets. This would affect oil metabolism in the manner suggested by Bakhuis & Bos (1969) as previously explained. It is also possible that the increased stirrer speed has caused further fragmentation of the hyphae which, if the lipase is cell associated as suggested by earlier studies, has resulted in disassociation of the cell and lipase or physical damage to the enzyme.

To summarise, from these studies it becomes clear that the composition of the fermentation medium is crucial to the success of the fermentation with respect to antibiotic titre, metabolic activity and oil uptake. For example the study has shown that the use of a complex medium containing both oil and sugar resulted in the best productivity in relation to yield. The results also show that the initial concentration of the oil in the medium will determine the time of lipase expression and the level of activity. All these factors will be discussed in chapter 19.

Chapter 16.1 Emulsion stability

Chapter 16.1.1 Surfactant effects on emulsion stability

When two immiscible fluids are mixed in the presence of a surfactant an emulsion is formed and a direct emulsion results from oil dispersion in an aqueous continuous phase. Oil and water do not readily mix in the absence of surfactants and the addition of only a very small amount of surfactant can cause water and oil to form an isotropic phase or microemulsion where the oil and water regions are separated by layers of surfactant molecules. Two types of microemulsion exist: water in oil and oil in water. Thermodynamically stable mixtures of oil, water and surfactant are discriminated according to their major component, water or oil and the structure formed depends crucially on the water to oil ratio (Eicke *et al.*, 1996).

Surfactants are both hydrophilic and lipophilic which has promoted their use in a wide range of industries (Wasan *et al.*, 1988). A surfactant molecule consists of a hydrophobic tail that prefers to be surrounded by the oil environment and a hydrophilic tail interacting preferentially with the water molecules. Therefore, the surfactant molecules position themselves at the interface between the oil and water. The interaction between the water and the surfactant head significantly reduces the water-oil interfacial energy, by several orders of magnitude, thereby increasing their mutual solubility (Larson *et al.*, 1985 and Jin *et al.*, 1997). Experimental evidence suggests that under conditions of complete coverage by an anionic surface active agent such as SDS, the interfacial tension decreases, typically by a factor of 10 (Skelland *et al.*, 1987).

Surfactants, such as SDS, adsorb spontaneously onto the oil/water interface thus reducing the interfacial free energy and hence surface tension, σ. When no other forces are present in the system, the interfacial surface tension is the predominant factor in determining the dispersion characteristics but the addition of a surfactant will create an interfacial tension gradient by altering the electrostatic forces on the oil droplet. Since coalescence relies on collision of two oil droplets, changing the surface charge of the droplets will have a direct effect on droplet size. The surfactant provides an "electrical barrier" around the oil droplet which helps to stabilise the dispersion by slowing coalescence.

The efficiency of the surfactant molecules are dependent on not only their molecular length but the strength of the energy of interaction between the water and the head of the surfactant molecule. Although surfactant concentrations as low as 0.1% w/v have been shown to stabilise emulsions, (Shiomori *et al.*, 1995), increasing the concentration of the surfactant will also enhance the solubility of the oil in the water. Coalescence may also be further delayed by increasing the surfactant concentration. Therefore, in liquid-liquid dispersions the presence of surfactants can ultimately result in smaller droplets, (Eckbert *et al*, 1985), and in this study influence the size of the oil droplets formed. This will result in an over-estimation of the overall interfacial surface area present and in this case available for lipase activity. Therefore, the addition of SDS during this study will affect the oil droplet distribution although its effect will be minimal.

Chapter 16.1.2 Physical effects on emulsion stability

The viscosity of the dispersed phase will affect dispersion such that Selker *et al.*, (1965) observed that as the viscosity of the continuous phase increases, its tendency to disperse also increases. This may be an important issue in this study, where the rheology changes constantly during fermentation and becomes increasingly more viscous. However, this appears to have little effect on the actual droplet size. Only minor variations in droplet diameter of 10-20% were observed when cyclohexanol was dispersed in water which have significantly different viscosities.

Later studies by Stamatoudis & Tavlarides, (1987) proposed a model which related viscosity and coalescence and hence dispersion stability. During coalescence one drop collides with another resulting in a film of the continuous phase being trapped between the two droplets and this film has to drain before coalescence can occur. It was proposed that the coalescence efficiency depends on the time the two colliding droplets remain in contact as well as the time taken for the continuous phase to drain. Therefore, if the viscosity of the continuous phase increases, the film drainage rate will decrease thus slowing the coalescence rate and improving stability.

However, Vermuelen *et al.*, (1955) concluded that it was density which was more significant. The density difference between the two phases has a significant effect on the stability of the dispersion. A large difference in density between the continuous phase and the dispersed phase makes homogenous dispersion more difficult to achieve requiring more stirring power, (Treybal, 1958) and hence the rate of coalescence will be affected. Further work by McClarey *et al.*, (1978) found this particularly true at low agitation speeds. The agitation rate is an important factor to consider with respect to stability since at the higher impeller speeds and hence higher shear rates, a shear stabilised emulsion is formed whereas low shear rates have very little effect on the emulsion formation. This will be discussed in detail in the following section.

Chapter 16.2 Oil droplet dispersion

Two-liquid phase dispersion is a key step in many operations in the chemical, biochemical, food and pharmaceutical industries and is of key importance in this study involving fermentation in which a dispersed oil phase is the carbon source. The size and size distribution of the dispersed phase droplets will determine not only the stability of the dispersion and the efficiency of contact but will also aid the utilisation of oil since lipase activation requires the presence of the oil/water interface. Therefore, the lipase activity may be critically affected by the interfacial area available which is directly related to the oil droplet size. The extent of the effect will be determined by the rate limiting step in the lipase reaction.

On mixing in a mechanically agitated vessel the dispersed phase forms droplets which are subject to complex flow fields. The droplet size will be dependent on the interactions at the interface between the droplets and the continuous phase. Hinze, (1955) stated that droplet breakage occurs when the fluid dynamic forces which deform the droplet exceed the interfacial tension forces opposing deformation. The critical condition at which droplet breakage occurs is normally expressed as a ratio of the two forces, defined as:

$$\tau / (\sigma / d_{max}) \qquad \textbf{(16.1)}$$

where σ is the interfacial tension and τ is the fluid dynamic stress. The value of τ is dependent on whether the continuous phase field of flow is laminar or turbulent. The correlation assumes constant Newtonian behaviour of the continuous phase which is unaffected by the dispersed phase concentration and size distribution (Davies, 1985 and Davies, 1987). Later studies have demonstrated that at relatively high dispersed phase concentration these assumptions cannot be fully justified (Kumar *et al.*, 1991 and Boye & Shamlou, 1994). The apparent change in rheology of the dispersion will influence the flow conditions in the vessel and hence the mechanism of droplet breakage.

When two immiscible liquids are mixed in a stirred tank, energy is transferred to the mixture by the impeller which serves to suspend the dispersed phase thus creating turbulence in the fluid. This dispersion of one phase into another such as oil into water, takes place in three steps. Initially at the start of agitation, large droplets of the dispersed phase are present and then these large droplets are subjected to deformation depending on rotational directions resulting in further droplet break-up. These droplets are then further broken down until a dynamic equilibrium is established. It is generally accepted that this final breakage stage is due to local shearing action or by dynamic pressure fluctuation caused by localised turbulence and can be explained Kolmogoroff's theory of local isotropic turbulence.

It is assumed that there are turbulent flow conditions in the vessel. A turbulent flow field is regarded as collection of eddies or velocity fluctuations characterised by their fluctuation frequency, or length scale, and magnitude. The largest, primary eddies have the scale of the main flow and are unstable and disintegrate into smaller eddies which are unstable and disintegrate further and so on. Kinetic energy flows through this cascade from large to small until energy is dissipated as heat. As energy is transferred through the cascade the directional character of the primary eddies decays. This results in a wide droplet size distribution due to the spectra energies and eddy sizes.

Kolmogoroff's theory states that the small eddies are statistically independent of the primary eddies and are spatially uniform, locally isotropic. The smallest eddies capable of dissipating energy have length scale, λ, where

$$\lambda = \frac{[\mu^{3/4}]}{[\rho^{1/2}]} \times \frac{P}{V} \qquad \textbf{(16.2)}$$

According to Kolmogoroff's theory, therefore, the power input per unit volume, (P/V), is the key parameter in determining scales of eddies obtained and the intensity of turbulent velocity fluctuations of length scales comparable to drop sizes. This is an idealised situation and considers only the physical mechanism of energy input and as such does not consider other factors, for example interfacial tension. It states that turbulence is controlled by energy consumption; viscosity and to a lesser extent density. This explains why droplet size decreases with increasing power input as observed in the oil/water dispersions (figures 11.5 and 11.6).

For fully turbulent flow conditions, the fluid induced stresses tending to deform the droplet can either be shear or normal depending on the direction of the fluctuating eddies influencing the droplet. The ratio of the droplet diameter to the microscale of turbulence can be defined as:

$$\lambda = (\nu^3/\varepsilon)^{1/4} \qquad \textbf{(16.3)}$$

where ε is the energy consumption rate and ν is the kinematic viscosity. For droplets larger than λ, droplet deformation is assumed to occur by the normal, inertial stresses which originate from pressure fluctuations around the droplet and are dependent on the continuous phase density and local velocity fluctuations (Shamlou, 1994). Droplets smaller than λ reside within an eddy and breakage occurs by viscous stresses in the eddy which are the product of the shear rate and continuous phase viscosity (Boye *et al.*, 1996). Therefore, the oil droplets are subject to different deformation forces dependent on their size and will vary while a dynamic equilibrium is established.

In an agitated system, energy input is therefore necessary to equilibrate a stable drop size distribution. Agitation is the positive force that creates new interfacial areas while coalescence is a negative force which reduces surface area and forms larger droplets. The collision frequency and coalescence frequency of agitated droplets are dependent on the level of agitation, the phase ratio of the dispersed phase and the physical properties of the system whilst the rate of droplet break up is a function of the cohesive forces

which hold the droplet together such as surface tension and droplet viscosity, (Chapter 11.2) and the dispersive forces that tend to break up the droplets such as the turbulent eddies outside the droplets or the shear stress. These forces act in tandem resulting in a stable equilibrium.

Previous studies have shown that both positive and negative step changes in agitation rates have a direct impact on the droplet size distribution given time to attain a steady state. A positive step such as increasing the agitation rate results in the formation of an increased number of smaller droplets. However, increasing the number of droplets results in an increased collision frequency between the droplets and hence coalescence occurs. A local equilibrium is established between breakage and coalescence resulting in an overall decrease in the Sauter mean diameter (figure 11.5). Conversely, a negative step in agitation rate leads to larger droplet sizes (Chatzi *et al.*, 1991).

Chapter 16.2.1 Oil droplet size and interfacial area

The dispersion of one fluid into another produces large interfacial areas. This is important with respect to efficient heat and mass transfer and in the case of oil and water dispersions, providing a site for lipase activation as well as allowing cell-oil association with respect to fermentation. The utilisation of oil is limited by both its' solubility and the degree to which it is dispersed in the aqueous phase and therefore, the oil droplet size is important with respect to efficient oil uptake.

The oil droplet size distribution studies centred on the use of oil concentrations similar to those present in the fermentation medium adopted in later studies. From the size distribution studies the Sauter mean diameter, D_{32}, was obtained which can be used to calculate the interfacial area per unit volume, A, according to equation 16.4, where ϕ is the phase ratio of oil.

$$A = \frac{6\phi}{D_{32}} \qquad \textbf{(16.4)}$$

Using this equation the surface area of each oil droplet could be calculated which in turn could be used to find the total area available for lipase activation (Appendix B). Calculation of the interfacial area, based on a 2.5% v/v oil content at 350rpm with a Sauter mean diameter of 15μm, found that the interfacial area available for lipase activation was 2×10^5 cm^2/l. Assuming the diameter of the lipase molecule is between 50 – 90 Angstrongs, with circular adhesion, the estimated concentration of lipase required to saturate the available interface would be 10 -15mg/l. These results combined with those from initial shake flask studies would therefore indicate that the interfacial area is not a limiting factor of lipolytic activity in this environment. However, this is in an ideal situation of oil/water dispersion and assumes complete dispersion of the oil and does not take into account any other droplet/particle interaction which may occur in the fermenter environment. The surface area will also vary with agitation rate due to droplets of larger/smaller diameter being formed at lower/higher agitation rates thus reducing/increasing interfacial area.

Chapter 16.3 Effect of medium composition on oil droplet size distribution

The presence of any other compound in the oil/water dispersion will influence the oil droplet size distribution by oil/particle interaction and therefore, during fermentation the dispersion of the oil will be affected by various medium components. The addition of dextrin appeared to have very little effect on droplet size due to relatively high solubility whilst the addition of soya flour, a major medium component, causes a significant change in the oil droplet size distribution (figures 11.9 and 11.10).

It can be seen that the addition of soya flour causes a significant shift in the distribution resulting in the formation larger droplets/particles. The size of the soya flour particles when dispersed in water was relatively constant at a modal size of 60μm but the oil droplet size varied significantly when dispersed with soya flour when compared to oil/water only dispersions. There is an overall increase in droplet/particle size which that the soya flour is interacting with the oil in some manner.

There are a number of possible interactions occurring. Soya flour is added to the fermentation medium as a nitrogen source and has a high protein content. Various macromolecules, such as proteins, can be used as surfactants since they will bind to the surface of oil and form a stable emulsion. This will aid oil dispersion in the fermentation broth and hence, will influence oil utilisation (Ohta *et al.*, 1995 and Large *et al.*, 1999). Therefore, the soya flour has the potential to influence the interfacial tension gradient between the oil and water due to its surface active properties.

The presence of the soya flour may be counteracting the effect of the added surfactant, SDS, by changing the surface charge of the oil droplets resulting in an increased rate of coalescence. Previous studies have also shown that if the surfactant concentration in a dispersion is gradually increased, after an initial period of droplet size decrease, the droplet size will begin to increase again due to an increased rate coalescence resulting from an increased collision frequency caused by the presence of a larger number of droplets (Shiomori *et al.*, 1995). This would ultimately affect the size of the oil droplets in the fermenter environment because although SDS is not present, an antifoam is added, thus there may be a similar effect resulting in a reduced interfacial area for lipase activation.

However, the tendency to form larger droplets could also be explained more simply by a physical interaction between the soya flour and the oil droplets which appears more probable. There are two options. Firstly, the free soya flour particles could be coated by the oil which would suggest that the soya flour particles are surrounded by an oil film. This will in turn depend on the cohesive forces between the soya flour and the oil and/or the affinity of the oil for the soya flour. It should be noted that this would however, would make the soya flour inaccessible to the cell and hence, would not be readily metabolised. The second theory is that the soya flour is binding to the surface of the oil droplet as observed with the use of colloidal solids, such as mustard, as surfactants. The solid particles "sit" on the surface of the oil droplet and this would result in an increased particle size. Microscopic examination could help to clarify this further.

In either situation problems will be created in the fermenter environment. The physical interaction between the oil and soya flour would result in the presence of the oil in an inert and unusable form and hence would make both the oil and the soya flour inaccessible to the cell. This would reduce the free oil concentration in the fermentation medium, reducing oil/cell interaction which may in turn reduce both lipolytic activity as well as oil uptake (Mimura *et al.,* 1971 and Choi *et al.*, 1996). Therefore, these initial findings suggest that the composition of the fermentation medium will have a profound effect on the mixing characteristics and the dispersion of the oil within the fermenter environment as well as affecting efficient oil utilisation.

Chapter 17 Lipase determination

From the studies to determine a suitable lipase assay, it was seen that the type of assay actually used had a significant effect on the final result with respect to determining lipolytic activity. Of the three assays investigated, two proved to be successful, the agar plate assay and the titrimetric assay, both of which were easily adapted to suit this study and relatively simple to perform. The p-nitrophenyl palmitate assay was found to be unsuitable for this study.

Chapter 17.1 p-Nitrophenyl palmitate, (pNPP), assay

The pNPP assay had a number of limitations, the first of which is the incubation temperature of the samples, 37°C. This temperature was shown during optimisation studies not to be the optimum for the lipase from *Streptomyces clavuligerus* and therefore, would demonstrate a lower lipase activity during the assay. The assay also suffered from the fact that samples with low activity levels had to be incubated for longer which is not desirable when the stability of the lipase is not known. This may have been overcome, to a certain extent, by continuous on line assay of the p-NP palmitate lipase activity, (Janssen *et al.*, 1994), but this was not possible during this study.

The final consideration with respect to this assay is the specificity of the lipase toward the substrate. Rapp & Backhaus, (1992) demonstrated that a wide range of bacteria and fungi when screened for lipase activity, demonstrated very little activity toward pNPP. The rates of hydrolysis of pNPP of almost all the microorganisms tested were only in the range of 0.1 to 6% of the activity toward both triolein and tributyrin. It was concluded that pNPP is a very poor substrate for many microbial lipases due to its structure but could be used for the assay of certain esterases (Janssen *et al.*, 1994).

Chapter 17.2 Agar plate method

There were problems associated with this assay. Firstly, the type of oil used had a significant effect on the results as shown in figure 12.1. It was found that rape seed oil was unsuitable for use with this assay although the organism had been growing on a rape seed oil carbon substrate. This was thought to be due to problems emulsifying the oil into the medium since it remained as large globules and hence, was not evenly distributed throughout the medium. The mixture of triolein/Tween resulted in better emulsification than the rape seed oil and hence a more uniform distribution of the oil. However, at low lipase concentrations there were negligible clearance zones which made it difficult to make an accurate measurement of the final zone and hence lipase activity.

Olive oil proved quite successful with respect to detection of lipase activity. The zones of clearance were clearly visible and the oil was more readily emulsified into the medium. However, again there were large oil droplets distributed throughout the medium and hence it would appear that there was incomplete emulsification of the oil. This would cause inaccuracies in lipase activity determination. Tributyrin appeared to be the most suitable oil for this assay since complete emulsification of the oil was achieved and distinct clearance zones were formed which were easy to measure. It was concluded that this method could be used but may prove unreliable since a margin of error can be introduced by inaccurate measuring of the clearance zones. Therefore, it was suggested that this assay could be used for qualitative purposes since it gave a good indication of the actual presence of lipase or as a tool for screening (Rapp & Backhaus, 1992).

Chapter 17.3 Titrimetric assay

The third assay investigated was the titrimetric assay which was found to give an overall more accurate measurement of the lipase activity present. Similar results were observed with this assay with respect to the type of oil used as the agar plate method of analysis. Very poor activity was observed when both rape seed oil and the mixture of triolein/Tween were used as the reaction substrate which was again due to problems associated with emulsification of the oils. The use of an olive oil substrate gave a good

indication of lipolytic activity although the value was low compared to the standard lipase solution used. Once again tributyrin proved the best substrate to use giving the most accurate measurement of lipolytic activity (figures 12.3 and 12.4).

The rate of oil hydrolysis will be affected in this assay by a number of factors, such as the composition of the oil and the specificity of the lipase, which in turn will be strain dependent. The most significant aspect of the oil composition is the fatty acid content which will have a significant effect on the rate of hydrolysis of the triglycerides. The nature of the fatty acids affect the structure and the physico-chemical properties of triacylglycerols (Brockerhoff & Jensen, 1974). The degree of unsaturation, the position of the double bonds and their conformation are factors that determine the viscosity, the melting point and ultimately the ability of the triacylglycerol to be emulsified. Lipase-catalysed reactions are strongly influenced by the presence of an interface, (Malcata *et al.*, 1992) and thus the quality of emulsion, making it difficult to interpret the relative rates of hydrolysis in terms of specificity.

The actual chain length of the composite fatty acids is also a major rate determining factor. In general, the shorter the fatty acid chain lengths, the faster the rate of hydrolysis. For example, studies have shown that the fastest rate of hydrolysis occurs with tripropionin (6-carbon atoms) and tributyrin (4-carbon atoms) with pancreatic lipase due to esterase attack (Schϕnheyder & Volqvartz, 1944 and Wills, 1961). The rate of hydrolysis can be further affected by the actual experimental conditions (Entressangles *et al.*, 1961). However, if the substrates have the same number of carbon atoms and degree of unsaturation, the lipases will exhibit positional specificity (Macrae, 1983 and Sonnet *et al.*, 1993). It should also be noted that some microorganisms show no positional specificity such as *Ashbya gossypii* (Stahmann *et al.*, 1997).

This was demonstrated by Boutur *et al.*, (1995) during a study of the lipase from *Candida deformans* which showed a greater activity toward triacylglycerols than aliphatic esters. The esters of *cis* Δ-9 unsaturated fatty acids with 16 or 18 carbon atoms were generally hydrolysed at the highest rates. For the same number of carbons with the same degree of unsaturation, the presence of a Δ-6 or Δ-11 unsaturation was unfavourable compared to Δ-9. The *trans* conformation was also unfavourable to oil hydrolysis.

These studies imply that tributyrin, due to its composition and structure, may not be an ideal substrate since it is susceptible to hydrolysis by some esterases and would result in an over-estimation of the lipase activity. However, it has a number of advantages. For example, it can be more easily dispersed than the other oils tested and gives a good quantitation of lipolytic activity by automatic titration. The lipase activity measured when using tributyrin may be verified using another substrate, such as olive oil, which has been done periodically during analysis of fermentation samples.

For an insoluble substrate such as olive oil, which is a better substrate to use in terms of chain length, the observed lipase activity reflects the physicochemical properties of the substrate. This includes the surface area and charge as well as the concentration and activity of the enzyme. This is due to problems related to the emulsification of the substrate. Although some of the combinations used, such as olive oil, were as sensitive as tributyrin, none of them were as reproducible which is believed to be related to difficulties in obtaining stable and repeatable degrees of emulsification. More emulsifying agent or a surfactant could be added in order to improve dispersion but this could cause inaccuracies in measurement since they would affect the oil/water interface crucial for lipase activity.

The rate of hydrolysis of the substrate during this assay was also dependent on the temperature and pH at which the assay was carried out as shown by the results of the investigation (figures 12.3 and 12.4). The effect of pH on the rate of hydrolysis by lipase is a resultant of its effects not only on the enzyme itself, but also on the emulsified substrate and the properties of the substrate/aqueous interface. For example, Schϕnheyder & Volqvartz, (1946) demonstrated that the pH optimum for gastric lipase was 5.5-5.8 when tributyrin or tripropionin were used as substrates but 7.2-7.9 for the hydrolysis of trilaurin or tristearin.

In this study it was shown that the pH optimum for tributyrin and olive oil was between 7.0 and 7.2 dependent on temperature. The temperature optimum for the majority of lipases is between 30-40°C although exposure to higher temperatures can affect the stability of the enzyme and influence the capacity for enzymatic hydrolysis which is dependent on chain length of the fatty acids (Fodor, 1950). In the study it was found that the optimum was 30°C.

From this work it was concluded that lipolytic activity was best measured using a titrimetric assay which had been developed and optimised by varying operating conditions such as temperature, pH and sample preparation. It was found that the assay was best done with a tributyrin substrate at pH7.2 and 30°C and using a whole broth sample assayed directly after removal from the fermenter (Large *et al.*, 1999).

Chapter 17.4 Location of lipase

From the studies carried out, the preparation method of samples is of key importance in obtaining an accurate measure of lipase activity. The results show that the use of a whole broth sample taken directly from the fermentation was the best method of measuring lipase activity. However, the use of broth supernatant results in nearly a 100% loss in activity which indicates that the presence of cells are required for lipase activity. This is supported by the fact that the addition of the active cells to the inactive supernatant results in a recovery in lipase activity.

Similar results were obtained by Janssen *et al.*, (1994) when studying the lipase activity exhibited by a thermophilic *Bacillus spp.* Strain Wai 28A5. The level of lipase activity detected, using the pNPP assay, in the supernatant accounted for only 13% of the total lipase activity of the fermentation broth and the remainder was assumed to be cell associated. It was concluded that it was difficult to transport pNP-palmitate, or tripalmitin, into the cell and therefore, the enzyme is loosely cell associated or that the soluble activity is associated with subcellular particles lysed from the cells.

Studies carried out by Tsujisaken *et al.*, (1973) using *Geotrichum candidum* also found that lipase activity varied with assay sample. It was observed that the amount of lipase detected in culture filtrate was much less than that of the culture liquid with cells. However, this variation in activity was dependent on the growth phase of the organism with maximum cell bound lipase activity during the first 24 hours growth. Extracellular lipase increased during the later stages of growth, particularly during the stationary phase. Therefore, the age of the culture and hence the morphology of the organism will affect lipase production and possibly location.

These observations suggest that the lipase enzyme is in some way cell associated. This theory was reinforced by further studies in which the broth samples were treated in such a way as to disrupt the cell membrane such as sonication and the addition of Triton X-100. This resulted in almost a total loss of detectable lipase activity in samples containing cells. However, supernatant samples which were treated in the same way resulted in a slight increase in lipase activity which was thought to be due to the release of intracellular enzymes caused by the physical breakage of the cell or changes in cell membrane permeability.

Early studies by Reese & Maguire, (1969) reported similar results when studying the lipase from the fungi *Trichoderma reesei.* It was observed an increase in extracellular lipase secretion on the addition of Tween 80, a surface active agent and Triton X-100. However, later studies by Panda *et al.*, (1987) demonstrated that this increase was not correlated to the location of the enzyme or the membrane composition but the presence of Tween 80 exerting a stabilising effect of the enzymes already present.

If the enzyme produced by *Streptomyces clavuligerus* is cell associated, this may actually have a deleterious effect on oil utilisation as demonstrated by Jacobsen *et al.*, (1989). Studies using *Geotrichum candidum* showed that the presence of a cell bound lipase will inhibit the secretion of further lipase into the growth medium and thereby reduces extracellular lipase. This in turn limits the organisms' ability to utilise oil since lipase activity will be limited by not only cell bound lipase but also the morphology of the organism. Therefore, if the lipase is cell-associated in this study, the morphology of *Streptomyces clavuligerus* becomes an important consideration with respect to efficient oil uptake.

Chapter 18 Lipid analysis

Chapter 18.1 Thin layer chromatography

One of the simplest and most effective methods for analysing oil in the fermentation broth proved to be TLC, (Thin Layer Chromatography). The main advantages of TLC are all components can be visualised; the volume of solvent used is small and hence it is economic and the process requires little instrumentation. This technique also gave a good indication of the presence of oil and oil breakdown components within the fermentation sample. Therefore, results on a qualitative basis could be obtained relatively simply using the minimum of equipment.

The separated lipid classes could be visualised and the resulting chromatograms kept as permanent record or samples could be recovered for further analysis. This method does not quantify the results although the intensity of the iodine stain was indicative of the amount of triglyceride present. The use of iodine stains the lipids brown/black but care must be taken since iodine will bind irreversibly to polyunsaturated fatty acids.

As mentioned previously, (chapter 13.1), lipid mixtures are separated by differential migration which gives each lipid component a characteristic mobility known as its Rf value. This can vary due to the adsorbent used, temperature, solvent etc. The adsorbent used is variable. In this study, silica gel was used which is manufactured to contain pores of a definite diameter such as silica gel 60, where the 60 denotes the pore size. At a given humidity the amount of water absorbed by the silica gel increases as pore size decreases. The water content of the silica gel determines the polarity of the adsorbent and hence its activity and chromatographic properties. For good separations the water content must be carefully controlled. Therefore, the silica gel on the TLC plates is normally activated by heating the plates before use at temperatures above 100°C to remove the water.

Other adsorbents can be used such as Kieselguhr which is a diatomaceous compound based on a silicaceous material. However, since it is of fossil origin and non-synthetic its properties are more variable than silica gel and is more suited to reversed phase TLC. Aluminium oxide has also been used as an adsorbent for TLC of lipids but has considerably less capacity than silica gel plus the oxide is of basic pH and can therefore, cause hydrolysis of esters. Thus, silica gel is the most suitable adsorbent for this method.

In its normal form, silica gel is a polar adsorbent and consequently polar lipids are more tightly adsorbed than non-polar lipids due to polar-polar interactions. In TLC separation of lipids using standard silica gel, the most non-polar lipids therefore migrate at the fastest rates, (high Rf values) and the polar lipids at the slowest rates, (low Rf values). By increasing the polarity of the developing system the Rf values can be increased. The choice of a suitable solvent system is critical in the separation of lipid classes. It should be noted that complex polar lipids will remain at the origin irrespective of the solvent system used (Touchstone, 1995).

In the TLC studies carried out, the lipids involved were simple neutral lipids and as such the most commonly used solvents are hexane, diethyl ether and acetic acid, (Henderson & Tocher, 1987), although other non-polar solvents may also be used. In this case the hexane was replaced by petroleum ether which may affect the Rf values of the more polar lipids although non-polar lipids will not readily be affected. The actual Rf values obtained will vary depending on equipment as well as environmental conditions such as humidity as mentioned previously.

From the studies, the use of petroleum ether was suitable for the separation of the components present in the fermentation samples although the resolution of the lipid classes could be variable. However, if the proportion of diethyl ether is increased whilst the proportion of petroleum ether is decreased, the more polar lipids will migrate further from the origin and be defined more clearly. This may cause a loss of resolution of the least polar classes towards the top of the plate. Therefore, the choice of solvent(s) will be dependent on the lipids to be separated.

It can also be observed that there were significant variations in the Rf values of the triglycerides especially with respect to tributyrin which cannot be totally explained by differences in polarity (table 13.1). This is because although lipid classes are given in a single Rf value, some classes such as triacylglycerols are better represented by a range of values since in natural samples they frequently exhibit more than one band. This is due to their partial resolution into molecular species on the basis of fatty acid composition (Henderson & Tocher, 1989). The degree of spreading depends on the solvent system used. The separation of individual lipid classes on the basis of fatty acid composition can be achieved by reversed phase TLC using modified silica gel coated with silver nitrate. The silver ions form complexes with the double bonds of the fatty acid components.

There is some breakdown of the oil in its natural state since there are components which can be visualised with Rf values of approximately 0.2-0.3. These resolved spots correspond with the diacylglycerols and also the monoacylglycerols. These can be difficult to resolve clearly using this solvent system but may be observed by using a solvent system consisting of benzene/propan-2-ol/water. However, there will be different levels of migration depending on the specificity of the diacylglycerols. For example a 1,3-diacylglycerol will have a Rf value of approximately 0.45 whilst a 1,2-diacylglycerol will have an Rf value of approximately 0.54 which is due differences in the structural chemistry causing variation in the polarity of the components.

From running a TLC plate of standard fatty acids using the same solvent system it could be seen that again the resolution was not as clear as for the triglycerides and the resulting spots were harder to visualise even with bromophenol blue. Other stains can be used such as spraying the plates with a solution of 2',7'-dichlorofluorescein followed by 1% aluminium chloride in ethanol and 1% ferric chloride and then warming to 100°C after each spray. The fatty acids will give a rose-violet colour. However, the use of bromophenol blue will be sufficient in this study since only an approximate Rf value is required for fatty acids as an indication of the oil breakdown.

This use of TLC is a good qualitative method of lipid analysis and it can be used quantitatively by the use various methods such as scanning densitometry, elution of the separated components and weighing or by measuring the absorbance of the eluted samples. However, these tend to be inaccurate and as such a biochemical assay was investigated.

Chapter 18.2 Biochemical lipid assay

The biochemical lipid assay was shown to be more accurate on a quantitative basis than the TLC method and gave a comprehensive overview of the lipid utilisation profile. However, besides normal experimental error introduced by measurement of reactants, sample preparation, sample contamination and the like, the assay is subject to additional error sources. For example, the assay measures the total lipid present in the sample and hence will not just measure the added oil carbon source but will be sensitive to any lipid from the biomass and/or cellular protein present. This will result in an over-estimation of the lipid/oil present in the sample although this would be minimal with respect to nutrient value.

Further errors may be introduced into this assay due to the characteristics of the fermentation samples. In order to obtain a representative sample the oil needs to be uniformly dispersed when the initial sample is removed from the fermenter environment and this will depend on position within the fermenter where the samples were taken. For example, in the area of the impeller the oil will be dispersed more uniformly due to increased turbulence when compared to areas at the sides of the vessel which will exhibit more laminar flow. Therefore, if samples were taken from this region of the vessel the results of the assay may be more erratic and less reproducible. This dispersion limitation will also apply to time with respect to the stage of the fermentation since in the latter stages of the fermentation it would follow that the oil will be readily dispersed due to increased mixing time. However, this may be counteracted by the non-Newtonian nature of the fermentation broth.

Another consideration is the actual location of the oil with the system as a whole. Results from the oil droplet sizing experiments have indicated that the dispersion of the oil will also be affected by the composition of medium used. For example, there may be some physical interaction between various medium components, such as soya flour and the oil. If the soya flour is binding to the surface of the oil droplet, the oil is effectively hidden and may result in the protection of the oil during sample preparation resulting in reduction in the amount of lipid measured in the sample. This theory, however, does not seem very probable.

Given the errors associated with this method, at least triplicates of the samples had to be assayed for every sample, preferably from different areas of the fermenter vessel. From these measurements an average lipid concentration could be determined. This method was used in conjunction with TLC during initial studies.

Chapter 19 *Streptomyces clavuligerus* fermentation

As microbial cells grow, they break down complex carbon and energy sources through the action of catabolic enzymes. The end products of catabolism form the primary intermediates such as amino acids, nucleotides, vitamins, carbohydrates and fatty acids which are then used for cell maintenance or other biological function. These metabolic pathways and products often supply the precursors for secondary metabolism and therefore, the factors that influence primary metabolism will also have an indirect affect on secondary metabolism. The control mechanism for primary metabolism include substrate feedback, repression and inhibition, catabolite repression and inhibition and energy, (ATP), regulation. The interactions between primary and secondary metabolism must certainly involve these regulatory processes.

Improvement in productivity of microbial metabolites can be carried out by improving the microbial strain genetically or manipulating the nutritional or physical factors of the fermentation such as temperature, pH, oxygen concentration, stirrer speed and medium composition. Medium composition plays a vital role in the efficiency and economics of the industrial process since it influences growth and metabolism but also can result in higher antibiotic titres and consequently affect process economics. All these factors were considered in this study and therefore, will be discussed in the following sections.

Chapter 19.1 Effect of spore inoculum

One area not mentioned in the previous results section concerns the effect of the inoculum used for the initial seed culture for the fermentation. This can be one of the major factors with respect to batch to batch deviations and hence, reproducibility. Smith, (1985) found that different spore generations of *Penicillium chrysogenum* had a profound effect on biomass and hence antibiotic titre. Similar batch to batch variation has been reported by Tarbuck *et al.*, (1985) with respect to clavulanic acid production in *Streptomyces clavuligerus* whilst Tucker & Thomas, (1992) showed that the level of the spore inoculum would determine the morphology of the organism. For the purpose of *Penicillium chrysogenum* fermentation as the inoculum levels rose towards 5×10^5 spores/ml there was a sharp transition from pelleted to dispersed forms, but above this

level there appeared to be little additional effect. Therefore, the inoculum will affect not only the rate of growth but also the morphology of the organism and in turn the antibiotic titre.

Medium composition, with respect to carbon, nitrogen and phosphate levels has been shown to have a significant effect on spore preparation, (Nabais *et al.*, 1995 and Karandikar *et al.*, 1996) and therefore, standardisation and control of the preparation of the inocula is of key important in reducing this batch variation. All spore stocks were initially supplied by SmithKline Beecham, (Worthing) and the spores used for the each inoculum were taken from the same batch to reduce any variation and the size of the inoculum maintained. Their viability was checked as reported in the earlier methods section (chapter 10.3.2).

The spore inoculum is used to inoculate the seed culture used for the fermentation. The seed culture is important since it will introduce fermentation batch variation. A number of fermentations were carried out with seed cultures at different stages of growth. This was determined on the basis of time as well as lipase activity measurements and oil utilisation profiles. It is necessary to inoculate the fermenter with actively growing cells to reduce the lag phase as well as the number of cell generations and enhance growth. The results from this study indicated that a seed culture 48 hours old should be used.

Chapter 19.2 Effect of medium selection

Optimal design of culture media is of importance due to interactions between secondary metabolites and growth metabolism and therefore, product synthesis or secretion is influenced by growth limiting nutrient concentrations. The effect of medium composition on growth and antibiotic titre in *Streptomyces clavuligerus* fermentations has been widely studied (Lebrihi *et al.*, 1988; Bascaran *et al.*, 1990 and Ives & Bushell, 1997). With the correct medium formulation, the slow release of nutrients, as experienced with complex media used in this study, can be an advantage for sustaining high rates of biosynthesis both primary and secondary metabolites (Jain *et al.*, 1992). Computer modelling has now even been undertaken to design an optimal medium for activity and production with respect clavulanic acid (Sircar *et al.*, 1998).

As shown by the results obtained, (chapter 15), it becomes clear that the medium is of great importance with respect to the carbon, nitrogen and phosphate sources since all will have a significant effect on the outcome of the fermentation. This in turn will also affect the lipase activity and hence oil utilisation. The effect of these will be discussed in the following sections.

Chapter 19.2.1 Effect of carbon source

Streptomyces clavuligerus is not capable of utilising simple sugars such as glucose as previously mentioned. Dextrin is the preferred substrate since the slow release of glucose from dextrin hydrolysis creates a carbon limiting condition which prevents repression of antibiotic synthesis (Park *et al.*, 1994). Dextrin is used as the main carbon source in both the complex and oil media but in the oil medium additional oil is substituted for the dextrin component. Glycerol can also be used and is added to the seed medium. This carbon source promotes growth of the *Streptomyces clavuligerus* but has a negative feedback effect on antibiotic synthesis, (Lebrihi *et al.*, 1988), as well as inhibiting lipase activity (Stahmann *et al.*, 1997). The results from these fermentation studies show that the organism will preferentially use the dextrin before the oil (chapters 14 & 15). This could be for a number of reasons such as the availability of the substrate, rape seed oil, which is limited by the degree to which it is dispersed in the fermentation medium (chapter 16.3).

The other possibility for the delay in oil metabolism is due to carbon regulation of primary metabolism. When a favoured carbon source, which in this case is dextrin, is present the cell is inhibited from producing the enzymes capable of catabolising the second carbon compound which is the rape seed oil. This in turn may affect secondary metabolism since the intermediates and/or products of the secondary carbon catabolism, could serve as precursors for the biosynthesis of the secondary metabolites (Hu & Demain, 1979). This would explain why the cells grown without any oil exhibit lower antibiotic titres.

The presence of soya flour in the complex and oil media provides an additional carbon source which can be readily catabolised by the organism. Although the actual composition of the carbon component is not known, it is probable that the soya flour consists of a 5:1 ratio of carbon to protein, based on manufacturers information. The soya flour is substantially broken down during the sterilisation process and is utilised initially, due to its' availability. This is indicated by the fermentation protein profiles, (figure 15.13) and the change in pH during the first growth phase (figures 15.4 and 15.11).

The soluble medium exhibits a lower growth rate and lower antibiotic titre than the complex medium which could be due to both the carbon and nitrogen sources used (chapter 15). In the soluble medium the dextrin is supplemented with rape seed oil and starch both of which are known to support growth of *Streptomyces clavuligerus*. However, there are reports of the suppression of cephem antibiotic synthesis by the presence of glycerol and starch, (Hu & Demain, 1979 and Hu *et al.*, 1983), which is indicated by the results in this study. Although, later studies showed that starch actually enhanced antibiotic synthesis due to the slow and continuous hydrolysis of starch creating carbon limiting conditions (Lebrihi *et al.*, 1988). The results show low clavulanic acid production when the soluble medium is used during fermentation (figure 15.18).

This effect is reported to be a generalised carbon catabolite effect in that the formation of an enzyme during glycerol/starch utilisation inactivates one or more of the cephem synthetases. Hu *et al.*, (1983) suggested that the organism had to be actively growing and hence protein synthesis had to occur for this suppressive effect to be observed, which is a view supported by Miambres *et al.*, (1992). However, the organism appears to utilise starch plus the peptone initially, before the period of dextrin catabolism. This initial growth phase is slower than that with the use of the complex medium which could firstly, be due to the nature of the polypeptides present in the peptone which are strongly bonded and hence maintain their structure, (Petrovic *et al.*, 1990) and also the slow hydrolysis of the starch.

Chapter 19.2.2 Effect of nitrogen source

It is well documented that the source of nitrogen used in a fermentation will determine the pattern of growth, the formation of enzymes and intermediates for biosynthesis as well the actual end products. In a significant number of antibiotic fermentations the highest productivity can only be achieved under nitrogen limitation (Aharonowitz & Demain, 1979 and Fang & Demain, 1995). For the study two main nitrogen sources were used which were an insoluble soya flour in the case of the complex and oil media respectively and in the case of the soluble medium. The nitrogen source is important since the availability of precursors for antibiotic biosynthesis depend on nitrogen metabolism with respect to the protein and amino acid components.

The nitrogen source with respect to the protein used for the fermentation is also important since it has been shown that proteins can promote cell growth and antibiotic production under nitrogen limited conditions as well as inhibiting lipase activity. Nahas, (1988) showed that the use of soybean flour was the only nitrogen source which did not limit lipase activity in a fermentation of *Rhizopus oligosporus.*

This could help to explain why the broth from a soluble medium fermentation expressed the lowest level of lipase activity and the slowest rate of oil utilisation as compared to the complex and oil media fermentations (figures 15.1 - 15.6). This is further supported by Narasaki *et al.*, (1968) who found that lipase activity decreased when peptone was added to a *Pseudomonas species* fermentation medium although this did not reduce lipase activity by as much as the presence of ammonium sulphate (Petrovic *et al.*, 1990). However, Prabhakar & Raju, (1993) found that peptone was the best nitrogen source for growth and enzyme, glucose isomerase, production but was dependent on nitrogen limited conditions. Nitrogen limitation appears to be crucial in maintaining antibiotic biosynthesis.

The most important nitrogen source with respect to productivity is ammonium concentration. High ammonium levels are known to inhibit antibiotic production as well as in some instances inhibit growth (Aharonowitz & Demain, 1979). The ammonium effect is only expressed early in the growth phase and therefore, if the ammonium levels are high in the secondary metabolism phase, there is no suppression of either antibiotic

production or growth. This suggests that if the synthetic machinery needed for antibiotic synthesis is already present, the ammonia can have no inhibitive effect.

Ammonia levels increased during fermentation in the first growth phase but fell rapidly during the second phase of growth (figure 15.12). If *Streptomyces clavuligerus* is utilising the soya flour present in the fermentation medium and hence causing the release of ammonia into the fermentation medium, the antibiotic production ability of the organism will, in turn, be inhibited.

This also implies that the effect of ammonia cannot be attributed to a specific inhibition of an antibiotic forming enzyme. The enzymes which supply the cells with nitrogen are regulated by ammonium ion concentration which acts directly on the enzymes involved in the catabolism of nitrogenous compounds or the mechanisms of nitrogen assimilation. High ammonia levels were seen in all the fermentations at an early stage in the growth phase which would account for the low or negligible clavulanic acid titres. Therefore, the biosynthetic machinery for antibiotic synthesis is regulated by nitrogen metabolism and is established during the trophophase.

Similar results were obtained by Bascaran *et al.*, (1990) who found that protease production in *Streptomyces clavuligerus* was under ammonia control. It was also demonstrated that the synthesis of protease and antibiotic is suppressed during rapid growth with complex nitrogen sources. It was concluded that the regulatory mechanism for protease synthesis is related to growth in much the same way as the production of secondary metabolites (Martin & Demain, 1980). This would appear to contradict the results of this study which showed that the highest antibiotic titre was produced when a complex nitrogen source of soya flour was utilised although low level of antibiotic were actually expressed. Therefore, careful selection of the nitrogen source is essential for a successful fermentation and antibiotic biosynthesis is favoured in conditions of low ammonia and/or the absence of a rapidly utilised carbon source (Aharonowitz & Demain, 1979 and Fang & Demain, 1995).

Chapter 19.2.3 Effect of phosphate

From the results of this study the effect of phosphate is not altogether clear. It is known that variation in the levels of phosphate will affect the growth and antibiotic producing ability of *Streptomyces clavuligerus* (Aharonowitz & Demain, 1977) and is growth limiting (Ives & Bushell, 1997). It has been found that a concentration of 1 mmol phosphate, (PO_4), will inhibit growth and antibiotic production whilst a concentration between 10-20 mmol will have very little effect. However, levels of phosphate above this value were shown to have a deleterious effect on both growth and antibiotic production. Bosnaj *et al.*, (1985) also reported faster growth and increased biomass concentration in *Streptomyces rimosus* on addition of phosphate up to a concentration of 20mmol and above this level growth is inhibited due to adverse effects on spore germination.

During the soluble medium fermentation it can be seen that there was phosphate limitation at the end of the fermentation where the level of phosphate fell rapidly to 0mmol (figure 15.19). This would therefore result in reduced antibiotic synthesis and hence a lower antibiotic titre. During the complex medium fermentation the phosphate levels were between 10-20 mmol which could account for higher antibiotic titre observed since this is the required phosphate concentration for antibiotic synthesis (figure 15.19). The oil medium fermentation shows rapid utilisation of the phosphate with falling levels as the fermentation progresses. It would appear that if the fermentation had progressed to full term there would have been a phosphate limitation resulting in a reduced antibiotic titre.

Phosphate is crucial in maintaining a stable pH which is required for growth as well as antibiotic production. The buffering capacity of the medium was improved by the use of organic buffers, such as MOPS. These have been proved to be the better buffers for this type of fermentation with respect to antibiotic production and stabilisation of the pH (Aharonowitz & Demain, 1977). Inorganic buffers, such as potassium phosphate, were shown to support good growth but their rapid utilisation resulted in acid production and insufficient buffering capacity. This caused a declining pH and cell lysis. The buffering capacity is of increased importance during a fermentation using oil in the medium since the breakdown of the oil results in the production of fatty acids (chapter 5.1). These will

reduce the pH as well as being toxic to the cells. MOPs buffer was used in this study and was shown to have a good buffering capacity although in the later stages of the soluble medium fermentation there was a rapid decline in pH, (figure 15.11), due to the metabolic activity of the organism.

Chapter 19.3 Effect of medium composition on lipase activity

Streptomyces clavuligerus produces a lipase in order to utilise an oil carbon source. The lipase appears to be induced as opposed to being constitutive. A constitutive enzyme is an enzyme which is synthesised under all growth conditions and does not require a specific substrate to induce its activity. However, an induced enzyme such as lipase, is formed in the presence of, or response to, an inducing agent. In the case of lipase this may be oils, (Tan & Gill, 1987 and Pokorny *et al.*, 1994), and / or fatty acids (Iwai *et al.*, 1973; Suzuki *et al.*, 1988 and Montesiros *et al.*, 1996). Therefore, the composition of the fermentation medium is of key importance with respect to lipase expression as is the manner in which the organism is grown (Rivera-Munoz *et al.*, 1991).

Chapter 19.3.1 Effect of carbon source on lipase activity

The presence of a suitable carbon source is important for not only growth and biomass development but also lipase production. Although other compounds such as starch, (Lebrihi *et al.*, 1988) and glycerol, (Christakapopoulos *et al.*, 1992), have been shown to increase or stimulate lipase activity. In general oils or oil compounds are required for lipase induction (Large *et al.*, 1999b). It has been proposed that this variation is caused by differences in the nutrients available for lipase synthesis (Alford *et al.*, 1963).

A range of oils have been shown to induce lipase activity and their efficiency depends to a certain extent on their fatty acid content. Both oleic acid and linoleic acids have been shown to stimulate lipase production, (del Rio *et al*, 1990; Gilbert *et al.*, 1991; Obradors *et al*, 1993 and Choi *et al.*, 1996), whilst longer chain fatty acids have been reported to repress lipase production (Chander *et al.*, 1990). The use of oleic acid has also been shown to increase cephamycin C production in *Cephalosporium acremonium* (Paul *et*

al., 1997). The selected oil, rape seed oil, has a high oleic and linoleic acid content and therefore, the chosen oil would not have been a limiting factor with respect to lipase activity and hence oil utilisation in this study.

However, the composition of the oil, and hence the efficiency of oil utilisation, can also have a negative effect on lipase activity. Fermentation showed that the level of lipase activity decreases rapidly once oil is being metabolised and is undetectable by 50 hours even though there is oil present in the fermentation broth (figure 15.15). This suggests that the lipase may be inactivated in some way thus limiting oil utilisation. For example by shear inactivation or by gas-liquid inactivation. Since the lipase is cell associated, inactivation of this type will be dependent on morphology and hence broth rheology.

It is also possible that the lipase has been inactivated by the products of oil metabolism. Although fatty acids such as oleic acid, linoleic acid and linolenic acid, have been shown to induce lipase activity, as mentioned previously, if they accumulate they will exhibit a negative feedback effect on lipase synthesis, (Obrados *et al.*,1993 and Choi *et al.*, 1996), and will be toxic to the cell. The presence of fatty acids will inhibit the further hydrolysis of oil. Therefore, oil metabolism will be related to the rate of uptake of the fatty acids into the cell and hence growth of the organism (Kim *et al.*, 1996).

The results obtained also indicate that the level of oil used in the fermentation medium will determine the level of lipase activity induced. The use of the oil medium, with the highest initial oil concentration of 35g/l, showed both the greatest volumetric and specific lipase activity during the study (figure 15.6). When the initial oil concentration was reduced later in the study to 25g/l, (chapter 15.2), the lipase activity detected fell (figure 15.16). Further studies showed that the level of lipase activity exhibited was dependent on the oil concentration present in the medium, (figure 15.21), which showed that by increasing the oil concentration the level of lipase activity increased (Large *et al.*, 1999b). However, maximum lipase activity was detected at the start of the exponential phase of growth irrespective of the medium used or the concentration of oil present.

This implies that the level of activity is dependent on the concentration of oil within the system, (Gordillo *et al.*, 1995), although the opposite effect has been observed with

Ashbya gossypi when grown on soy bean oil. It was shown that by reducing the oil concentration the lipase activity was increased (Stahmann *et al.*, 1997). It is also possible that since the lipase appears to be cell associated, the induction of lipase activity requires cell - substrate association to be established and therefore, reducing the level of oil will decrease this association resulting in slower induction of the lipase (Tan & Gill, 1985). This again will be determined by the affinity between the cell and the oil (chapter 12.5).

Chapter 19.3.2 Effect of nitrogen source on lipase activity

The fermentation using the soluble medium showed least lipase activity although the initial oil concentration was the same as that of the complex medium (figures 15.6 and 15.16). One explanation for this could be the nitrogen source used, peptone, which replaced soya flour and enhanced the solubility of the medium. Soya flour is important with respect to enhancing antibiotic production, (Nahas *et al.*, 1988 and Zhou *et al.*, 1992), as well as aiding oil emulsification.

Although an organic nitrogen source such as peptone, has been shown to have advantages over an inorganic nitrogen source, such as ammonium nitrate, (Pokorny *et al.*, 1994); it has also been shown to have a detrimental effect on enzyme production and oil utilisation (Bascaran *et al.*, 1990 and Wrenn *et al*, 1994). The presence of soluble protein in particular has been reported to significantly reduce lipase activity, (Narasaki *et al.*, 1968) and this is most significant with the use of peptone. However, Prabhakar & Raju, (1993) stated that during medium optimisation for the production of glucose isomerase in *Arthrobacter spp.*, peptone was the most suitable nitrogen source providing there were nitrogen limiting conditions.

Ammonium ions have also been demonstrated to have a more significant effect on lipase activity (Wrenn *et al.*, 1994 and Kim *et al.*, 1996). Priest, (1977) found that extracellular enzymes excreted from various species of *Bacilli* were influenced by the nitrogen source and that ammonium had a detrimental effect on enzyme production. Similar results were reported by Bascaran *et al.*, (1990) when studying the production of extracellular protease in *Streptomyces clavuligerus*. This is important in the later stages

of fermentation where it was demonstrated that ammonium concentration increased and lipase activity decreased (figure 15.12).

The soluble medium also has magnesium present in the form of magnesium sulphate and observations by Petrovic *et al.*, (1990) reported that the presence of magnesium ions will reduce lipase activity. This could further explain the lower lipase activity levels in the soluble medium fermentations. However, these observations are contradicted by results seen by Iwai *et al.*, (1973) who found that lipase production in *Geotrichium candidum* had a requirement for both iron and magnesium ions as well as being induced by the presence of both fatty acids such as oleic and linoleic acids and oil. Therefore, it would be appear that, like oil, the most suitable nitrogen is dependent on the microorganism.

Chapter 19.3.3 Effect of phosphate on lipase activity

The level of phosphate during the fermentation could also help to explain the lower lipase activity observed with the soluble medium. By 30 hours no soluble phosphate could be detected in the fermentation broth, (figure 15.19), whilst on both complex and oil media the phosphate level was approximately 5mg/l at 40 hours when lipase expression was at its maximum (figure 15.16). Phosphate level has been shown to regulate not only growth and secondary metabolite production, (Aharonowitz & Demain, 1977 and Fang & Demain, 1995), but also affect lipase expression.

When the levels of phosphate are very low in the fermentation broth, lipase synthesis has been shown to be inhibited (Alford *et al.*, 1963). It was reported that a phosphate optimum of 10mmol was required for maximum lipase synthesis in a *Pseudomonas species* fermentation but when the concentration exceeded 20mmol lipase synthesis was inhibited. A similar result was observed by Pokorny *et al.*, (1994) when studying lipase biosynthesis in *Aspergillus niger*. Shaker *et al.*, (1994) also found that cellulase production in *Aspergillus niger* could be regulated by phosphate levels using ammonium phosphate. Therefore, the level of phosphate present in the medium may also determine the level of lipase expressed and hence oil utilisation.

Chapter 19.4 Effect of aeration & agitation

Chapter 19.4.1 Effect on morphology, growth and antibiotic production

Most antibiotic fermentations involve mycelial species which result in highly viscous and often non-Newtonian broths. At high enough viscosities the circulation in agitated vessels becomes slow and stagnant zones may develop. Under such conditions the transport of oxygen is limited resulting in dissolved oxygen gradients throughout the fermenter and thus the microorganisms are subjected to fluctuating dissolved oxygen levels which has been shown to result in loss of productivity (Steel & Maxon, 1966). Therefore, antibiotic production is limited by the oxygen concentration.

The levels of clavulanic acid produced during this study were relatively low, (figure 15.18), with the use of the complex medium resulting in the highest antibiotic titre. However, when the dissolved oxygen concentration was maintained at a level of 50% the amount of clavulanic acid produced, when the complex medium was used, increased. The results are not shown graphically since it was only possible to take a final antibiotic titre measurement. Similar findings were reported by Rollins *et al.*,(1988 & 1990) who found that when the dissolved oxygen level was maintained at saturation levels, cephamycin C production increased in *Streptomyces clavuligerus*.

It has been proposed that the reason for this is due to the biosynthetic pathway for antibiotic manufacture. Yegneswaran *et al.*, (1988) showed that oxygen is a requirement for cephem biosynthesis for two reasons. The presence of a high oxygen level will depress the synthesis of hydrolytic enzymes which would result in a decline in antibiotic titre since there would be a loss of biosynthetic activity. The second reason suggests that at low levels of oxygen key biosynthetic enzymes are repressed or inhibited and the availability of antibiotic precursors becomes limited in a manner similar to that observed during carbon catabolite repression. Zhang *et al.*, (1987) proposed a different theory involving the level of amino acids present which are required for antibiotic synthesis. It was suggested that a low level of oxygen would denature the amino acids and hence reduce the amino acid pool.

Morphology appeared to be independent of the oxygen concentration as long as the dissolved oxygen level was maintained above a critical level of 5- 10 % (Yegneswaran *et al.*,1991 and Cui *et al.*, 1998). Park *et al.*, (1993) found that the oxygen level required to maintain morphology and itaconic acid was between 20 - 60% with *Aspergillus terreus* which implies the critical dissolved oxygen level is species dependent. As oxygen levels fall below this critical level and carbon dioxide levels increase, the filamentous nature of the organism changes resulting in irregular branching and shorter hyphae and even pellets (Smith & Ho, 1985). It is proposed that changes occur to the cell membrane which alters the transport of various substances which then affects morphology (Jones & Greenfield, 1982). This in turn will affect oil utilisation and lipase activity.

The effect of agitation on morphology is more significant although this may not have a direct effect on antibiotic production (Belmar-Beiny & Thomas, 1992). The morphology of *Streptomyces clavuligerus* varied very little once initial fragmentation had occurred, (figure 14.7) and the medium composition had very little effect on the nature of the broth. However, increasing the agitation rate increased fragmentation and branching frequency but had no effect on biomass. Similar results were observed by Belmar-Beiny & Thomas, (1991). Increasing agitation increases the effects of mechanical forces on the hyphae which results in a tendency to form pellets as opposed to mycelium (Makagiansar *et al.*, 1993). However, these changes in morphology were not accompanied by changes in productivity indicating that there is no direct link between morphology and clavulanic acid titre, a view supported by Belmar-Campero, (1987).

Chapter 19.4.2 Effects on oil utilisation

Growth of an organism on an oil substrate requires complete dispersion of the oil with a relatively small particle size to provide a high surface area for lipase activation as well as for cell attachment. Therefore, oil utilisation will be directly affected by the agitation rate and hence, power input (chapter 11.2). Due to the viscous nature of the substrate, at higher oil concentrations there will be less efficient mixing resulting in larger oil droplets and a hence reduced surface area. The cohesive forces such as surface tension, which hold the droplet together and provide resistance to breakup, will also increase as

oil concentration and viscosity increase. This may result in a longer lag period with respect oil utilisation, (figure 15.5), since the organism is not able to attach to the oil surface readily (Mimura *et al*, 1971).

Therefore, the availability of the substrate will affect the efficient uptake of the oil which in turn will be limited by the degree to which the oil is dispersed in the aqueous phase. In order to maintain a high growth efficiency it necessary to ensure uniform dispersion or emulsify the oil in some way and allow contact with the organism (Ohta *et al.*, 1995). It has also been shown that increasing the agitation rate may also increase oil utilisation due to increased fragmentation of the mycelium since utilisation rate is dependent on specific substrate-mycelia interfacial area (Choi *et al.*, 1996). However, increasing the agitation rate during this study to improve oil utilisation with respect to residual oil, had very little effect on the level of oil uptake. These findings were supported by studies by Large *et al.*, (1998).

In addition, the dissolved oxygen concentration will affect oil metabolism. To maintain a culture in stationary phase with oil instead of a sugar carbon source involves a high OUR since three times the amount of oxygen is required for oil oxidation compared to glucose. It has been shown that low oxygen concentrations result in depletion of OUR and antibiotic synthesis, (Bader *et al.*, 1984) and hence it would appear that the oxygen transfer rate can determine the rate limiting factor for oil metabolism.

Increased aeration in this study resulted in no significant improvement in oil utilisation, (figures 15.22 and 15.24), although maintaining the dissolved oxygen tension at 50% saturation using variable air flow rate may be beneficial due to extending fermentation time. This effect would be limited due to the nature of the fermentation broth. It should also be noted that in this situation the oil had a deleterious effect on oxygen transfer unlike previous studies which suggested that RSO could be used as an oxygen vector and hence improve the oxygen transfer rate (Rols & Goma, 1989).

Chapter 19.4.3 Effect on lipase activity

Both aeration and agitation have been shown to affect lipase activity during this study. During normal operation, the dissolved oxygen concentration was not controlled due to the high viscosity of the medium but maximum oil utilisation and lipase activity was observed at 40-50% dissolved oxygen concentration. When maintained at this level, neither expression of lipase activity or the period of oil metabolism increased (figures 15.22 and 15.23). At lower levels of dissolved oxygen fell below this level, oil metabolism reduced significantly as did lipase activity. Therefore, it is possible that there is a critical level of dissolved oxygen for lipase production / synthesis.

The dissolved oxygen concentration during several fermentations has been reported to be of importance with respect to lipase activity. Kim *et al.*, (1996) found that in order to achieve maximum lipase activity during *Pseudomonas fluorescens* fermentation the dissolved oxygen level was maintained above 20% whilst Choi *et al.*, (1996) observed maximum lipase activity when the dissolved oxygen level was maintained between 3 - 5ppm. However, lipase activity did decrease after a further 10 - 20 hours (figure 15.23). It is possible that the increased aeration has resulted in the deactivation of the lipase by contact with the air bubbles present in the system (Falk *et al.*,1991; Khan *et al.*, 1992 and Michaels *et al.*, 1995).

The effect of agitation on lipase activity has not been widely studied which is unusual considering the requirement of interfaces for lipase activation and the need for uniform dispersion of the oil. The agitation rate appears to have little effect on the rate of oil utilisation and lipase activity (figures 15.24 and 15.25). However, lipase activity decayed more rapidly at higher agitation rates which does suggest that the lipase is being inactivated in some way.

When, this was investigated more thoroughly, the first observation made was that there was an increase in lipase activity in fermenter cultures as compared to shake flasks (chapter 15). This does initially suggest that agitation and aeration is advantageous to lipase production which is supported by Jacobsen *et al.*, (1989). However, studies conducted by Tahoun *et al.*, (1982) found that the opposite was true and that shaking or aeration of liquid cultures resulted in a decrease in lipase activity. It was proposed that

this was due to either shear or oxidation effects resulting in inactivation of the lipase enzyme.

Lee *et al.*, (1989) conducted similar experiments using *Candida cylindricae* and found that the lipase activity declined as a function of shearing time and shear rate due to direct deformation of the lipase molecule. It was shown that the addition of antifoam, which was necessary at higher stirrer speeds to control foaming, also caused the denaturation of the enzyme due to changes in the oil/interface and thus lipase activity decreased. Later studies by Stahmann *et al.*, (1997) found that by lowering stirrer speed and removing baffles during *Ashbya gossypii* fermentation an increased lipase activity was observed due to reduced shearing effects.

The other possibility is that increasing the agitation rate will decrease lipase activity due to gas – liquid interfacial inactivation. Stahmann *et al.*, (1997) found that the input of an air bubble into a stirred gas/water system resulted in a decrease in lipase activity. To exclude chemical oxidation, the air was replaced by argon and the lipase was still inactivated. Similar results were observed by Falk *et al.*, (1991) who found that gassing with helium lead to the same decrease in lipase activity in *Staphylococcus carnosus* as gassing with air. This does suggest that the interface between the gas and water phases is the destructive element with respect to lipase activity. Further studies showed that by the addition of Pluronic^{R} F-68, a nonionic detergent, which coated the air bubbles in a stirred gas/water experiment resulted in full stability of lipase activity. This is also supported by Khan *et al.*, (1992) who demonstrated that proteins can be denatured/inactivated by an air/water interface. Therefore, it is possible that the lipase associated with *S. clavuligerus* is inactivated by the presence of air bubbles in the system.

A final consideration is temperature and heat. Heat will be produced as heat of agitation, as well as metabolic heat, which will be continually increasing during this experiment. However, providing the fermenter vessel is sufficiently controlled this is unlikely to have a significant effect although there may be some localised denaturation

Chapter 19.5 Factors affecting the use of oil during fermentation

Oils have been used in fermentation for many years. Pan *et al.*, (1959) first stated that oils could be used as a carbon source when it was reported that penicillin production increased when fed with oil as an antifoam. The efficient uptake of oil is dependent on a number of parameters; physical, (bio)chemical as well as metabolic. Firstly, the efficiency of the oil as a substrate is dependent on its composition as well as lipase specificity (chapter 6). Results from assay development, (*in vitro*), showed that the lipase produced by *Streptomyces clavuligerus* had low specificity toward triolein, olive oil but hydrolysed tributyrin rapidly (figures 12.1 and 12.7). This can be linked to the fatty acid composition of the oil as well as fatty acid chain length. This would suggest that the oil composition will be of significance with respect to efficient oil uptake.

The composition of the oil also affects the antibiotic titre. It has been observed that there is a correlation between the unsaturated fatty acid composition of the oil and a higher antibiotic production rate (Stowell, 1987). This is thought to be due to the fact that these fatty acids can be more easily incorporated into the cell and the antibiotic biosynthetic pathway. Khaoua *et al.*, (1992) reported that the presence of longer chain fatty acids, which result from oil degradation, are responsible for this increase. During *Streptomyces ambofaciens* fermentations, the presence of the fatty acids induced the synthesis of acyl CoA synthetase which is linked to primary metabolism and is responsible for the biosynthesis of necessary cell material. The fatty acids also induced acylkinases and acylphosphotranserases which are linked to spiramycin biosynthesis.

Similar results were observed by Huber *et al.*, (1985 & 1990) whilst studying *Streptomyces roseosporus* fermentation. It was suggested that low concentrations and/or poor fatty acid transport into the cell will result in poor lipase induction necessary for oil metabolism and hence reduced β-oxidation to acetyl Co-A, essential for growth and antibiotic production. However, as mentioned earlier, the correct fatty acids are required in order to stimulate lipase production and hence oil metabolism (Iwai *et al.*, 1973). Lipase production can be induced by the presence of both fatty acids such as oleic and linoleic acids which are present in RSO.

In addition to composition, the initial oil concentration will influence the rate at which the oil is utilised. The study has shown that the lipase appears to be induced and a higher level of activity can be observed when the oil concentration is higher (figures 15.20 & 15.21). These findings were confirmed in later studies by Large *et al.*, (1999). However, increasing the oil concentration further would create problems with respect to mixing and mass transfer by resulting in a more viscous broth as well as increasing the interfacial forces.

In order to utilise an insoluble carbon source, such as oil, the substrate has to be dispersed within the fermentation broth (chapter 19.4.2). Therefore, agitation can be a key factor with respect to oil dispersion and hence oil utilisation. As discussed previously, (chapter 19.4.2), there is an optimum degree of mixing necessary for mass transfer which will also subject the mycelia to a degree of mechanical stress (Choi *et al.*, 1996). Therefore, the rate of agitation best suited to oil dispersion may not be possible within the fermenter environment without causing damage to the mycelia as well as the lipase enzyme. Therefore, the agitation rate has to be optimised to dispersed oil whilst maintaining product formation.

Dispersion of the oil is necessary for the formation of the oil/water interface for lipolytic activity as well as to allow for cell-interaction and cell-substrate association (Tan & Gill, 1985). The contact relies on a degree of affinity between the oil and the cell and therefore, if no affinity exists, the organism will not be capable of growing on the oil. Raymond & Davis, (1960) found that during *Norcardia spp.* fermentation, the oil surrounded the cells which implies that there was a very strong oil/cell affinity. It is possible a similar situation is occurring in this study although image analysis could not distinguish between the oil and the organism due to the complex nature of the broth rheology.

A number of factors can affect the affinity between cells and oil such as, cell and oil concentrations; the ratio of cell concentration to oil concentration and agitation/aeration. The presence of surface active agents may also affect cell-oil affinity since they are capable of separating the cells from the oil droplets which inhibits growth due to cell/oil configuration rather than any physiological reason. This suggests the use of an antifoam may reduce oil metabolism.

Therefore, the medium composition becomes of prime importance since a number of components have surfactant properties (chapter 16.3). For example, some natural surface active agents such as peptone and soya flour, can favourably influence growth by improving emulsification of the oil (Ohta *et al.*, 1995). This effect did not appear to occur in the soluble medium where peptone was present, since the lowest oil metabolism was observed when this medium was used. However, it could be that this beneficial effect was counteracted by the deleterious effect of other medium components such as ammonia concentration. The results also appear to demonstrate that the soya flour is deaminated during sterilisation, (figures 15.11 and 15.12) and is then utilised as an alternative carbon source as well as providing a nitrogen source (figures15.10). Therefore, this would limit its emulsification effects on the oil.

Emulsification will enhance oil utilisation by reducing the interfacial tension and thus reducing the resistance of the oil droplets to the dispersive effects from the stirrer. Therefore, the forces maintaining the integrity of the droplet will be weakened and smaller droplets will be formed. This will increase the surface area for both cell association as well as lipase activity, thus increasing oil uptake.

The cells themselves can have emulsification properties which can be used favourably by varying the size of the inoculum. In general, a larger inoculum is necessary to emulsify oil and high levels of biomass would be required before there was sufficient emulsification of the oil for it to be utilised. This can be linked to the lipid present in the cell as phospholipid which has a strong surface affinity and shows emulsification properties (Munk *et al.*, 1969). Therefore, as the fermentation progresses, the presence of higher levels of biomass should encourage oil dispersion but the effect may be limited due to the nature of the broth.

Inoculum size can also influence cell-oil interaction which may link with the emulsification effect previously described. Eltola *et al.*, (1965) observed that no growth occurred when a small inoculum was added to a high oil concentration medium. He proposed that the microbial cells attach first to the surface of the oil droplets and prepare metabolically for forthcoming growth. The highest affinity can be seen with oil grown cells which again would explain the long lag phase experienced.

It becomes apparent that a number of factors will affect the efficient uptake of oil into the cell such as medium composition, oil composition, lipase specificity and aeration and agitation as well as the manner in which the organism is grown. This study also illustrates the complex relationship between primary and secondary metabolism in an antibiotic producing fermentation and shows the importance of medium selection and operating parameters.

Chapter 19.6 Residual oil

From the study it is apparent that there is a residual level of oil present at the end of the fermentation which has been demonstrated to be the same in composition as the oil at the start of the fermentation (Stowell, 1987). Therefore, oil utilisation is limited in some manner whether physical or metabolic. There could be a number of explanations for these observations which are discussed in this chapter.

One of the first considerations is the manner in which the organism is grown and its metabolic effects. During this study *S. clavuligerus* was grown in batch culture and hence oil utilisation can be limited not only by growth kinetics but the accumulation of toxic products of hydrolysis, such as fatty acids (chapter 19.3.1). The latter is of less significance since the fatty acids are taken up directly be the cell and would not accumulate in the fermentation broth. No fatty acids have been detected in the broth during lipid analysis with TLC. However, it has been demonstrated that oil metabolism may be improved by using a fed-batch or continuous process.

Becker *et al.*, (1991) found that the accumulation of oleic acid during a *Bacillus spp.* fermentation, resulted in the cessation of oil metabolism and hence a residual oil level of 30% of the initial oil concentration. By using a continuous system, there was a 50% increase in lipase activity which resulted in a two fold increase in the amount oil metabolised and a residual oil level of less than 5% of the initial oil concentration. Montesiros *et al.*, (1996) observed similar results by converting to a continuous fermentation for *Candida rugosa.* This is a physical solution to what appears to be a metabolic problem with respect to feedback inhibition of the lipase by fatty acid build

up. It assumes that the utilisation of oil is regulated metabolically and does not consider the physical aspect of oil substrates.

Another consideration is the lipase produced by the organism. In order to utilise the oil *S. clavuligerus* produces a lipase which has been shown to be cell associated. The level of lipase at the end of the fermentation is negligible, (figures 15.5 and 15.16) and may be inactivated in some way. To verify lipase inactivation was taking place during fermentation, a standard lipase solution was injected into the broth at the end of the fermentation and it was found that no activity could be detected 90 seconds after injection into the system (chapter 15.3.1). Therefore, this does suggest that the lipase is inactivated which could be due to shear stresses within the fermenter, (Lee *et al.*, 1989), or inactivation by the gas/liquid interface (Falk *et al.*, 1991; Khan *et al.*, 1992; Michaels *et al.*, 1995 and Stahmann *et al.*, 1997). Therefore, oil metabolism could be limited due to lipase inactivation. It should also be noted that the β-oxidation activity will reduce. This metabolic function will be switched off as a direct response to the reduced level of lipase activity and hence oil uptake i.e. an environmental response.

The low level of lipase activity at the end of the fermentation could be a result of the limitation to oil utilisation and not solely due inactivation. If the oil is prevented from forming the interface or contacting the cell, then the lipase will not be induced. From previous discussion, (chapter 16.2), the oil will form large droplets due to the shear forces exerted by agitation. There is efficient mixing and flow is sufficiently turbulent to disperse the oil. The droplets should then break down further due to deformation depending on rotational directions. They are further broken down due to local shearing action or dynamic pressure fluctuation caused by local turbulence, Kolmogoroffs theory.

As the fermentation progresses the broth becomes more viscous and hence the flow regime is less turbulent. Kolmogoroffs theory assumes the flow is Newtonian and therefore, could not be applied to the fermentation broth during the later stages of fermentation. The shear stresses exerted on the oil droplets may be insufficient to overcome the increased interfacial forces holding the droplets together due to these changes in rheology.

In this situation the oil would remain in large droplets and would be inaccessible to the cell. This would reduce the interfacial area which would in turn limit lipase activity. It has been shown that increasing the agitation rate can increase oil utilisation, (Ohta *et al.*, 1995 & Choi *et al.*, 1996), but the effect is limited. If the agitation rate is gradually increased, at some point it will have a deleterious effect on the mycelia. This will result in increased fragmentation and cell decay, thus reducing product formation. In this study increasing agitation had a negligible effect on oil uptake (chapter 15.3.3).

Due to the viscous nature of the broth the oil is not being sufficiently dispersed and as such the shear forces, to which the droplets are being subjected to, are having very little effect. Therefore, the cohesive forces which maintain droplet integrity become dominant and further droplet breakage does not occur. This could also lead to increased coalescence due to increased contact time between the droplets. By the addition of a surfactant, which would lower the interfacial forces and thus reduce resistance to deformation, oil utilisation has been seen to improve and reduce the level of residual oil (Large *et al.*, 1999b). The level of clavulanic acid also increased on the addition of a surfactant. This demonstrates that there is physical limitation to oil utilisation, the oil droplet size and hence oil dispersion which is enhance by the broth rheology.

Chapter 20 Conclusion

In summary, from the results of the study the following conclusions can be drawn;

•*Streptomyces clavuligerus* utilises rape seed oil during fermentation on a complex medium by the production of a lipase. The lipase can be detected using either a quantitative titrimetric assay or a qualitative agar plate method. The lipase exhibits substrate specificity as well as pH and temperature optima and it was found that optimum assay conditions for this study were a tributyrin substrate at pH 7.2 and 30°C. The lipase could not hydrolyse rape seed oil *in vitro* although RSO is used during fermentation, due to emulsification problems and therefore, the suitability of the substrate for the assay is determined by its composition.

•Further assay optimisation studies suggest that the lipase is cell associated. Lipase activity was detected in whole fermentation broth samples whilst no lipase activity was detected in the fermentation broth supernatant. Disruption of the cell membrane also resulted in loss of lipase activity.

•The level of lipase activity measured was dependent on a number of factors. The lipase was induced by the presence of oil and maximum activity is detected prior to oil metabolism. The level of activity of the lipase could be increased by increasing the initial oil concentration. The level of lipase activity was also affected by the composition of the fermentation medium, such as the nitrogen and phosphate sources.

•The lipase appears to be subject to inactivation by denaturation at the gas/liquid interface as well as susceptible to shear effects during fermentation which can reduce activity.

•In order to be utilised, oil needs to be dispersed in the fermenter and is achieved by agitation of the fermentation broth. The efficient dispersion of the oil is dependent on:

-the physical properties of the oil such as μ, ρ and σ

-the power input

-the rheological nature of the fermentation broth

Therefore, the agitation rate must be optimised to enhance oil utilisation whilst maintaining product formation.

•There is a residual level of oil present at the end of the fermentation irrespective of the initial oil concentration. Increasing the agitation rate did not enhance oil utilisation although emulsification of the oil can result in increased oil utilisation. This would suggest that oil uptake is limited by its dispersion in the fermentation broth.

Further recommendations:

The study has demonstrated that there is a physical limitation to oil uptake. It may be that this can be overcome by the addition of a surfactant which would enhance oil dispersion since increasing agitation had a limited effect. A further suggestion might be to perhaps use a fed batch process, feeding the oil as required or even feeding the composite fatty acids of the oil to observe the effect on antibiotic production. This would also prevent accumulation of any toxic by-products.

Chapter 21 References

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APPENDIX A Kinetic model for lipolysis of lipids

The actual pathway of lipolysis has been investigated by a number of researchers who have proposed a reversible enzyme adsorption to, or penetration into the interface. This step has been suggested to take place before the formation of the enzyme-substrate complex and hence is considered to be the rate limiting step. Verger *et al.,* (1973) have proposed a simple model to explain the kinetic action of a lipolytic enzyme and this illustrated in figure A1.

The model consists of two equilibria the first of which describes the reversible penetration of a water-soluble enzyme into an interface, (E=E*). This is followed with a second equilibrium in which one molecule of penetrated enzyme binds to single substrate molecule resulting in an enzyme-substrate complex, (E*S). Once this complex is formed it undergoes catalytic changes regenerating the enzyme in the form E* and liberating the product, P. This model is only valid if the products of reaction are water soluble, diffuse rapidly away and induce no change in the physicochemical properties of the interface with time.

Later studies indicated that the enzyme may be progressively inactivated at the interface and has been reported to occur in lipid monolayers at low surface pressures as well as at triglyceride-water interfaces (Brockerhoff, 1971). This inactivation was shown to be irreversible and resulted in the conversion of the penetrated enzyme E* into an inactive form E*i which competes with the formation of the productive complex E*S. Since these steps of penetration and inactivation occur consecutively, it is predicted that the kinetics of lipolysis will be controlled by an adsorption flux and hence will be the rate limiting step in the overall reaction.

It is thought that this reversible penetration of the enzyme into the interface converts the soluble enzyme E to a more catalytically efficient form E* and thus is considered distinct from other adsorption steps which may occur. This theory is supported by studies done by Wells, (1974) who investigated the kinetics of pure snake venom phospholipase A2 on a micellar substrate. However, conflicting evidence has been obtained from studies by Dervichian *et al.*, (1973). They showed at low surface pressure that irreversible binding of the enzyme takes place at the interface in the monolayer

state. Therefore, it follows that the reversibility of the enzyme depends on the state of the substrate and the type /form of the enzyme itself.

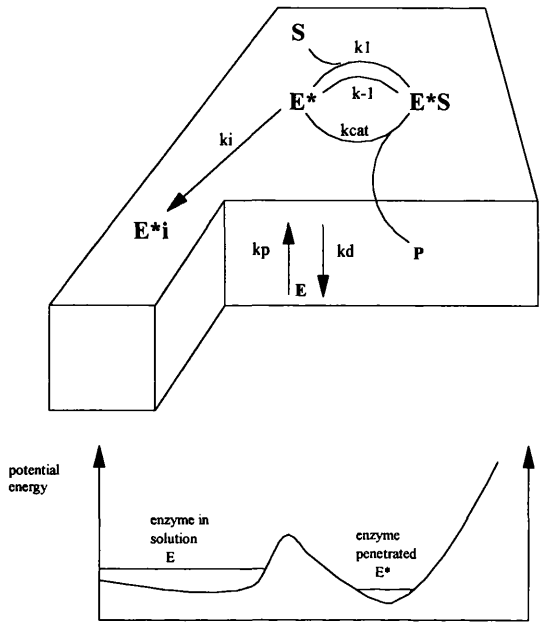

FIGURE A.1 Proposed model for a soluble enzyme at an interface

The model illustrated may be applied to any other enzyme system besides lipolytic enzymes in which theoretically the enzyme is soluble and the substrate is insoluble. For the purpose of kinetic studies the shape/type of interface present should not impose any restrictions and as such the substrate may be in micellar, emulsified or monolayer state.

Form this it was derived that the rate expression for enzyme velocity, at steady state, can be given as:

$$vb = vm \cdot I/V := \frac{kcat \cdot Eo \cdot S}{S + Km^*} \cdot \frac{S(I/V)}{S(I/V) + (kd/kp) \times (Km^*S/S + Km^*)} \quad \textbf{(A.1)}$$

where:

vb = bulk expression of the velocity (mols/cm^3)

vm = monolayer expression of the velocity (mols/cm^2)

I = total interfacial area (cm^2)

V = total volume (cm^3)

kcat = catalytic rate constant (s^{-1})

Eo = total quantity of enzyme (mol/cm^3)

S = substrate concentration (mol/cm^2)

Km* = kcat + k-1/k1 = interfacial Michaelis-Menton constant (mols/cm^2)

kp = penetration rate constant (cm^3/cm^2)

kd = desorption rate constant (s^{-1})

This implies that the enzyme velocity is dependent upon the enzyme concentration present as well as theoretically being linearly dependent on the substrate concentration. It hence, becomes evident that the kinetics of lipolysis are dependent on the surrounding environment.

APPENDIX B Interfacial area calculation in oil/water dispersions

Example: 2.5% oil in water mixture at stirrer speed of 350rpm

Assuming that the average droplet size of approximately 30μm and that the test rig has a total volume of 1.5l & the volume of RSO is 76.6ml

Therefore, the volume of a single droplet;

$$\mathbf{V} = 4/3\Pi (15 \times 10^{-6})^3 = \mathbf{1.41 \times 10^{-14}}$$

Calculating the number of droplets;

$$\mathbf{N} = \frac{7.66 \times 10^{-5}}{1.41 \times 10^{-14}} = \mathbf{5.42 \times 10^{9}}$$

and the surface area of one droplet;

$$\text{surface area} = \Pi . D^2 = \Pi (30 \times 10^{-6})^2 = \mathbf{2.83 \times 10^{-9}}$$

Therefore there is a total surface area of

$$5.42 \times 10^{9} \times 2.83 \times 10^{-9} = \mathbf{15.33 m^2}$$

Interfacial measurements show that the molecular diameter of a lipase (assuming circular adhesion) lies between 50 and 90Angstrongs

Calculating the number of lipase molecules required to saturate the surface:

At 50A

$$\mathbf{Area} = \Pi D^2/4 = \Pi(50 \times 10^{-10})^2 /4 = \mathbf{1.96 \times 10^{-17}\ m^2}$$

At 90A

A = 6.36 x 10^{-17} m^2

Number of lipase molecules required to saturate the area;

At 50A 15.33/1.96x10^{-17} = **7.82x10^{17}**

At 90A 15.33/6.36x10^{-17} = **2.41x10^{17}**

Number of moles of lipase required;

At 50A 7.82x10^{17}/ 6.023x10^{23}= **1.3x10^{-6}**

At 90A 2.41x10^{17}/ 6.023x10^{23}= **4.00x10^{-7}**

Taking the lower number of moles and assuming that the molecular weight of the lipase is 50,000g/mole, the saturation level can be calculated;

so 4.00x10^{-7} moles x 50 000 g/mole = 0.02g

The concentration of lipase in the test rig is 0.02g in 1.5l

Therefore, the concentration of lipase required to saturate the interface is

13.3 mg per litre