Geobacillus thermoleovorans:

Growth and Lipase Production

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MAGISTER SCIENTIAE

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Chapter 1
Literature Review

1.1. General Introduction

The first recorded studies of subsurface microbiology were those of Edson S. Bason, dating as far back as the 1920's, studying the microbiology of deep oil reservoirs. His work was later followed up by a colleague Frank E. Greer (Frederickson *et al*, 1996; Monastersky, 1997). However, it is now apparent that he was not studying a new form of microbial ecology, but rather evidence suggests that some microbes may have been trapped for 80 million years, and possibly as long as 160 million years. This has led some researchers to believe that this may be an explanation as to how bacteria survived in a hostile environment in early earth, when other life forms were struggling to survive (Monastersky, 1997).

In 1996, members of the Princeton University group isolated a bacterium able to reduce several heavy metals at high temperatures from a borehole in a South African gold mine at a depth of 3.2 km below surface (De Flaun *et al.,* 2004, personal communication and submitted). Samples collected from other mines confirmed that the Witwatersrand basin contains "extremophile" microbial populations that may have novel applications in biomining.

1.2. *Bacillus* GE-7

The new isolate; GE-7 (*Geobacillus thermoleovorans*) is a novel obligate thermophilic bacterium that grows in the temperature range of 45-70ºC and has a reported optimum of 65ºC. The organism was isolated from fissure water collected approximately 3.1km below ground surface in East Driefontein Goldmine situated in the Boonton Shales. The *in situ* rock temperature was measured at 45ºC and the fissure water's pH measured 8. It is an aerobic, rod-shaped, gram positive, spore forming bacteria which showed high lipase activity and a broad substrate specificity against triacylglycerides ranging from C4 to C18. This isolate was not only able to grow (specific growth rate of $2.5h^{-1}$) on olive oil as the sole carbon source, but also on a variety of other lipid substrates and even emulsifiers. (De Flaun *et al.,* 2004, personal communication and submitted).

Different strains of *Geobacillus thermoleovorans* have been reported to degrade a variety of bio-hazardous compounds ranging from naphthalene (Annweiler *et al*., 2000), cresol (Duffner *et al*., 1998) and even phenol (Feitkenhauer *et al.*, 2001).

1.3. Classification of Bacterial Strain

1.3.1. The Family *Bacillaceae*

The *Bacilli* is a large and diverse collection of aerobic to facultative anaerobic, rod-shaped, Gram positive or Gram variable, endospore-forming bacteria (Claus & Berkeley, 1986). This diverse group includes thermophilic, psychrophilic, acidophilic, alkalophilic, freshwater as well as halophilic bacteria that mostly grow either heterotrophically or autotrophically. Harwood (1992) reported that most Bacilli are generally accepted to be well suited for the production of industrial

enzymes due to their ability to secrete large amounts of protein directly into the culture medium.

The 16s rRNA gene sequences revealed a high heterogeneity within species (Ash *et al.* 1991) with ten clearly distinguishable phylogenetic groups (genera) (Bacillus Genetic Stock Centre, 2002).

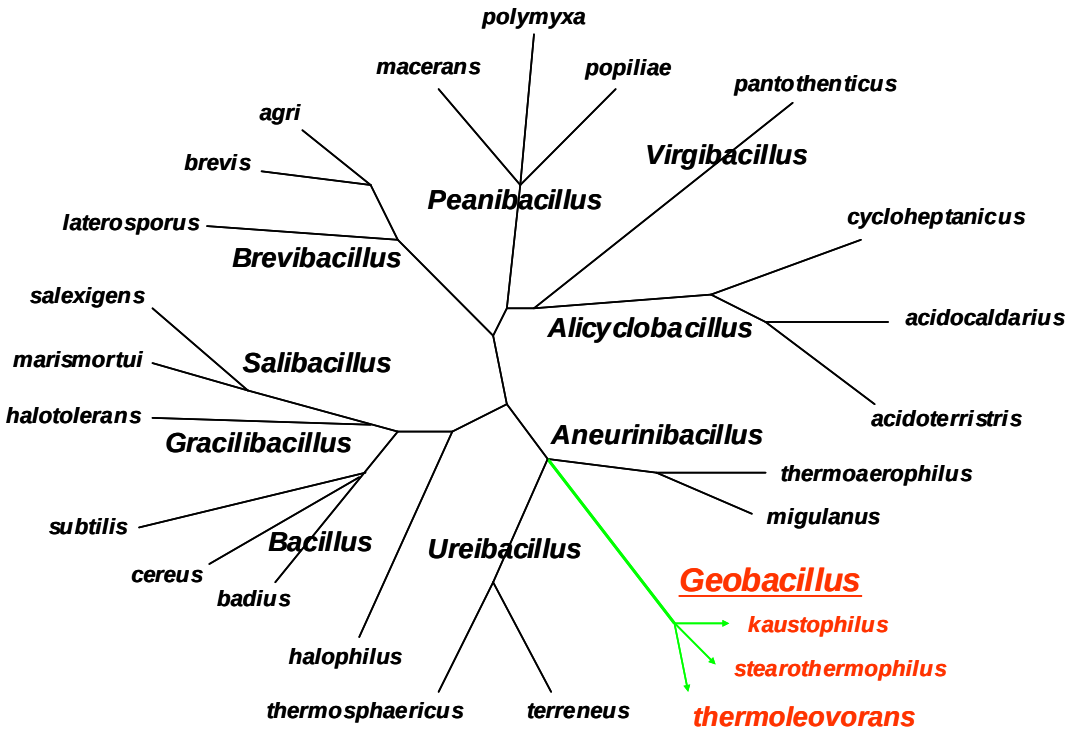

Figure 1.1: Unrooted phylogenetic tree of the bacilli based on 16S rDNA gene sequences showing most relevant species. Adapted from *Bacillus* Genetic Stock Centre Catalog (2002).

1.3.2. The Genus *Geobacillus*

Nazima *et al*., (2001) recently reported on the transfer of two new species *Geobacillus subterraneus* and *Geobacillus uzenensis* to a new Genus *Geobacillus*. This new Genus already contained the transferred *Geobacillus thermoleovorans*, *G. thermocatenulatus*, *G. kaustophilus*, *G.*

thermoglucosidasius and *G. thermodenitrificans* previously all part of the genus *Bacillus*.

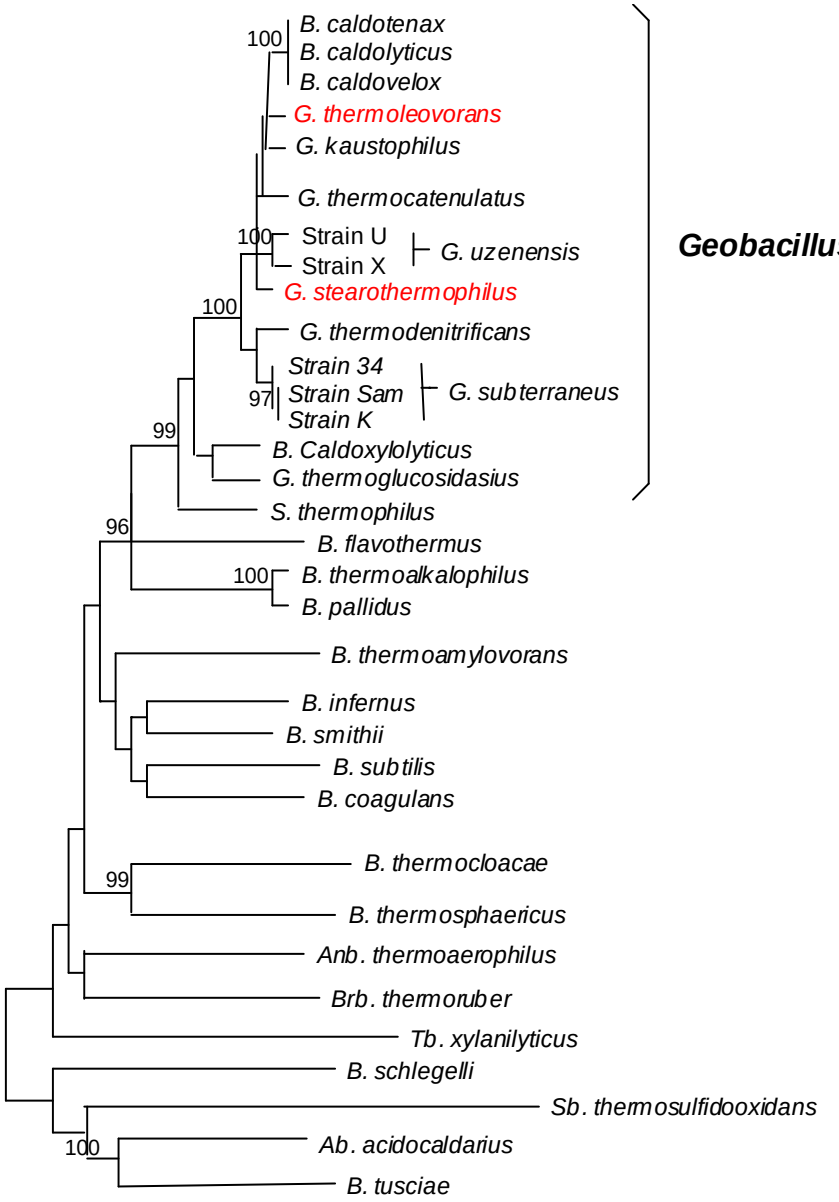

Figure 1.2: Phylogenetic tree showing position of most relevant *Geobacillus* sp. Indicated in red is *Geobacillus thermoleovorans* (studied organism) and *Geobacillus stearothermophilus* (Type strain). Adapted from Nazima *et al.* (2001).

This new Genus; *Geobacillus* will consequently contain the group of obligate thermophilic Bacilli with *Geobacillus stearothermophilus* (Strain DSM22^T) as the type species (Nazima *et al*., 2001).

1.4. Lipases

Lipases (Triacylglycerol ester hydrolases, EC 3.1.1.3) are ubiquitous enzymes that catalyze the breakdown of fats and oils with subsequent release of fatty acids, diacylglycerol, monoacylglycerol and glycerol (Papon *et al.*, 1988).

The increasing interest towards lipase research has occurred mainly due to the following reasons:

- Firstly the molecular basis of the enzyme's catalytic functions. Lipases, though water-soluble, catalyze reactions involving insoluble lipid substrates at the lipid-water interface. This phenomenon known as interfacial activation is due to this enzyme's unique structural characteristics (Alberghina *et al.*, 1998).
- Secondly the enzyme's medical relevance, especially in cases of atherosclerosis and hyperlipidemia (Farooqui *et al.*, 1987). Since products of lipolysis such as fatty acids play critical roles in signal transduction and cellular activation, its importance in regulation and metabolism cannot be ignored (Shinomura *et al*., 1991).
- The third reason was the discovery that lipases are also capable of catalyzing the reverse reactions, such as various esterification reactions and aminolysis in organic solvents. The equilibrium between the hydrolysis and the synthesis reactions is controlled by the water activity of the reaction mixture (Gandhi, 1997).

Furthermore the interest in lipase research is directed towards various biotechnological applications, for example the resolution of racemic mixtures, bioconversion of oils and the syntheses of pharmaceuticals and new surfactants. The use of lipases as catalysts for industrial reactions has the added bonus of lower waste treatment costs, reduced side reactions and milder reaction conditions.

The increasing interest was not only for lipases, but more specifically for lipases with higher stability, especially thermostability. The enzyme's catalytic function can be inhibited if the substrate (e.g. fat) has a melting point higher than that of room temperature, therefore an enzyme from a mesophilic organism will not be sufficient to catalyze reactions on such a heterogeneous substrate. With respect to the enzymatic processing of lipids and oil rich industrial effluents at high temperatures, thermostable bacterial lipases from thermophilic bacteria could be the key. (Schmidt-Dannert *et al*., 1994; Rua *et al*., 1997 and Kim *et al.*, 1994)

The number of studies on extremophilic microorganisms has grown exponentially in the last few years. Recent developments show that these organisms could possibly be a valuable source of biocatalysts. Although these compounds/enzymes seldom meet the exact requirements of industry the possibility of modification still exists (Madigan & Mars, 1997). Major advances in protein engineering have been reported on recently by Cavicchioli & Thomas (2000) identifying new pathways for the modification of enzymes (proteins). These enzymes, modified or natural possess extraordinary catalytic capacity as well as stability in harsh environments opening up new opportunities for biotechnological application (Madigan & Mars, 1997). Table 1.1 highlights extremozymes and their various applications, source and property exploited.

1.4.1. Bacterial Lipases

Bacterial lipases have recently been classified by Arpigny & Jaeger (1999) into eight families with the first family being the largest and consisting of six subfamilies with 22 members. Parmar *et al*. (1998) explained that the affinity towards long-chain fatty acid ester hydrolysis shows the presence of a true lipase in contrast to esterases that hydrolyze short chain fatty acyl esters.

Families I.1 and I.2 contain lipases from the genus *Pseudomonas* which are represented almost throughout each family. The lipases of family I.1 and I.2 are secreted via the type II secretion pathway (Outer membrane secretion) and show differences in regio-and enantio-selectivity despite a 40% amino acid sequence homology (Gilbert, 1993, Svendson *et al.,* 1995). Lipases belonging to the I.3 family utilize the Type I secretion pathway (ABC exporters).

Table 1.1 The variety of industrial applications of extremophilic enzymes.

Industrial application of extremophilic enzymes			
Extremophile	**Habitat**	**Enzyme**	**Representative application**
Thermophile	High Temperature	Amylases	Glucose, Fructose for Sweeteners
	Moderate thermophiles	Xylanases	Paper bleaching
	Thermophiles (65-85ºC)	Proteases	Baking, Brewing and detergents
	Hyperthermophiles (>85ºC)	DNA polymerases	Genetic engineering
Psychrophile	Low temperature	Proteases	Cheese maturation, Dairy production
		Dehydrogenases	Biosensors
		Amylases	Polymer degradation in Detergents
Acidophile	Low pH	Sulfur oxidation	Desulphurization of coal
		Chalcopyrite concentrate	Valuable metals recovery
Alkalophile	High pH	Cellulases	Polymer degradation in detergents
Halophile	High salt concentrations		Ion exchange resin degenerant disposal, producing poly (У-Glutamic acid) (PGA) and Poly (β-Hydroxy Butyric acid) (PHB)
Piezophile	High Pressure	Whole microorganism	Formation of gels and Starch granules
Metalophile	High Metal concentration	Whole microorganism	Ore-bioleaching, Bioremediation and Bio-mineralization
Radiophile	High Radiation levels	Whole microorganism	Bioremediation of Radionuclide contaminated sites

Nthangeni *et al*. (2001) found that the lipases of *Bacillus subtilis and B. pumilus* form a group of their own, subfamily I. 4 which contains the smallest enzymes known. *Bacillus licheniformis* should be added to this family on the basis of amino acid sequence similarity and biochemical process. Rua and co-workers (1997) grouped the lipase of *Bacillus thermocatenulatus* in the Subfamily I. 5.

Family II consists of a novel family of lipolytic enzymes reported by Upton & Buckley (1995) with seemingly unknown functions. The extracellular lipases of *Moraxella* sp. and *Streptomyces* sp. make up family III, with the cold adapted lipases grouped in family IV. Family VI lists esterases that have been partly identified from genome *sequences*.

1.4.2. Lipases as Catalysts

The biological function of lipases is to catalyze the hydrolysis of esters, especially long chain triacylglycerols, to yield free fatty acids, di-and mono-acylglycerols, and glycerol. These enzymes are however also capable of catalyzing the reverse reactions, achieving esterification, transesterification and (acidolysis, interesterification, alcoholysis), aminolysis, oximolysis and thiotransesterification in anhydrous organic solvents (Gupta, 1992; Klibanov, 1989), biphasic systems (Brink *et al*., 1988) and in micellar solution with chiral specificity (Martinek *et al*., 1986; Nagao *et al*., 1990).

Lipases generally exhibit low activity against water-soluble substrates with an increase in activity as soon as the substrate reaches its solubility limit. This is a result of interfacial activation caused by conformational changes in the enzyme. In the inactive state the substrate binding site and the active site are covered by peptide loops (“lids”). The active site is therefore not accessible to the substrate in this state. During activation the lid is able to pivot and expose the active site, increasing its own hydrophobicity thereby facilitating interaction between the

enzyme and its hydrophobic substrates (Cygler, 1997; Brzozowski, 1991). The involvement of a lid structure with interfacial activation was however questioned by Verger in 1997. Lipases from *Pseudomonas aeruginosa, Bacillus glumae, Candida*

Table 1.2. Recent bacterial lipase classification as adapted from Jaeger and Eagert (2002).

Family	Subfamily	Species	Family	Subfamily	Species
I	1	*Pseudomonas aeruginosa (Lip A)*	II		*Pseudomonas aeruginosa*
		Pseudomonas fluorescens (C9)			*Aeromonas hydrophilia*
		Vibrio cholerae			*Salmonella typhimurium*
		Pseudomonas aeruginosa (Lip C)			*Photorhabdus luminescens*
		Acinetobacter calcoaceticus			*Streptomyces scabies*
		Pseudomonas fragi	III		*Streptomyces exfoliateus*
		Pseudomonas wisconsinensis			*Streptomyces albus*
		Proteus vulgaris			*Moxarella* sp. *(Lip 1)*
	2	*Burkholderia glumae*			***(Psychrophile)***
		Chromobacterium viscosum	IV		*Moxarella sp. (Lip 2)*
		Burkholderia cepacia			*Archaeoglobus fulgidus*
		Pseudomonas luteola			***(Extreme Thermophile)***
	3	*Pseudomonas fluorescens*			*Alicyclobacillus acidocaldarius*
		Serratia marcescens			*Pseudomonas* sp.
	4	*Bacillus subtilus (Lip A)*			*Escherichia coli*
		Bacillus subtilus (Lip B)	V		*Moxarella* sp. *(Lip 3)*
		Bacillus pumilus			***(Psychrophile)***
		Bacillus licheniformis			*Psychrobacter immobilis*
	5	*Geobacillus stearothermophilus L1*			*Pseudomonas oleovorans*
		Geobacillus stearothermophilus P1			*Heamophilus influenza*
		Geobacillus thermocatenulatus			*Sulfolobus acidocaldarius*
		Geobacillus thermoleovorans			*Acetobacter pasteurianus*
	6	*Stapphylococcus aureus*	VI		*Pseudomonas fluorescens*
		Stapphylococcus heamolyticus			*Synechocystis* sp.
		Stapphylococcus epidermis			*Spirulina platensis*
		Stapphylococcus xylosus			*Rickettsia prowazki*
		Stapphylococcus warneri			*Clamydia trachomatis*
	7	*Propionibacterium acnes*			
		Streptomyces cinnamoneus			

antarctica and coypu pancreatic lipase did not demonstrate interfacial activation even though they all possess an amphiphilic lid covering the active site.

Lipases are only generally active over a small pH and temperature range; however Muderhwa *et al*. (1986) mentioned that the bacterial lipase of *Pseudomonas mephitica var. lipolytica* is stable over a wide pH range of 3.4-11.2. Madigan & Mars (1997) reported on lipases able to survive at high temperatures.

Generally however a number of enzymes belonging to extremophilic organisms have shown extraordinary capabilities. A few examples are psychrophilic enzymes that enhance yields of heat sensitive products, halophilic enzymes that are stable in high salt concentrations in low water activity media, and thermophilic enzymes that are highly resistant to proteases, detergents and chaotropic agents, which may also afford them resistance to the effects of organic solvents (Demirjian *et al*., 2001).

Thermophilic and alkaliphilic microorganisms have been investigated as possible sources of lipases that are stable in the extreme environments found in most industrial processes. *Bacillus* species such as *B. subtilis and B. pumilus* have been found to produce lipolytic enzymes under extreme alkaline conditions. Their enzymes were however thermo labile. In contrast the lipases from closely related species such as *B. thermocatenulatus, B. thermoleoverans and B. stearothermophilus* were thermo-tolerant, but only showed stability at milder pH values**.** The lipase of *Bacillus licheniformis* retained full lipase activity in the presence of a strong disulfide bond reducing agent. This confirmed the absence of disulfide bonds. This suggested that these proteins' tertiary structure facilitate conformational changes necessary for enzymatic activity when the water-soluble enzyme reacts with a hydrophobic lipid substrate (Kim *et al*., 1998; Rua *et al.*, 1997; Ward & Moo-Young, 1988).

Table 1.3 Relevant stabilities of extremophilic enzymes with possible future industrial application. Adapted from Demirjian *et al*. (2001).

Extremophilic enzyme stabilities		
Enzyme	**Organism**	**Stability**
Hyperthermophilic esterase	***Pyrococcus furiosus***	**Opt. T = 100ºC** $T_{1/2}$ **= 50min @ 126ºC**
Thermophilic esterase	***Bacillus licheniformis***	**Opt. T = 45ºC** $T_{1/2}$ **= 60min @ 64ºC**
Thermophilic esterase	***Bacillus acidocaldarius***	**Active @ 70ºC**
Thermophilic esterase	***Archeoglobus fulgidus***	**Active @ 70ºC**
Thermophilic lipase	***Bacillus stearothermophilus***	**Opt. T = 68ºC Stable 30min @ 55ºC**
Thermophilic lipase	***Bacillus thermocatenulatus***	**Opt. T = 60-70ºC**
Psychrophilic lipase	***Pseudomonas Sp. B11-1***	**Opt. T = 45ºC**
Hyperthermophilic pullulanase	***Thermococcus aggregans***	**Opt. T = 95ºC** $T_{1/2}$ **= 150min @ 100ºC**
Thermo-acidophilic α-amylase	***Alicyclobacillus acidocaldarius***	**Opt. T = 75ºC (pH 3)**
Halophilic B-galactosidase	***Haloferax alicantei***	**Active @ 4M NaCl**
Halophilic class I Fructose aldolase	***Haloarcula vallismortis***	**Opt. Act. @ 2.5M KCL**
Hyperthermophilic Fructose aldolase	***Stapphylococcus aureus***	**Opt. T = 37ºC Stable 100min @ 97ºC**
Thermophilic 2-keto-3-deoxygluconate aldolase	***Sulfolobus solfataricus***	$T_{1/2}$ **= 150min @ 100ºC**
Halophilic protease	***Halobacterium halobium***	**Opt. Act, @ 4M NaCl**
Hyperthermophilic alcohol dehydrogenase	***Pyrococcus furiosus***	$T_{1/2}$ **= 420min @ 95ºC**
Barophilic glutamate dehydrogenase	***Pyrococcus furiosus***	**36X more stable at 105ºC and 750atm**

Ward & Moo-Young (1988) discussed the primary structure of a protein and how this contributes to its thermostability as well as other environmental factors including cations, substrates, co-enzymes and modulators often increase thermostability.

Bacillus thermoleovorans is a thermophilic bacterial strain, which grows optimally at 65^0C and pH 6.0; these conditions however did not correlate with its maximal lipase activity which was found at 50^0C (Vileneuve, 2000). In 1997, Rua *et al*. reported on thermoalkalophilic bacteria, *Bacillus thermocatenulatus* that showed lipase activity at pH 8 and 9 with Tributyrin and Triolein as the inducers. The optimum temperatures for the two inducers however differed and were found to be 55 and 75^0C respectively.

1.5. Growth and Lipase Production in Bacteria

Papon and Talon (1988) reported that most bacterial lipases are produced during the exponential growth phase and that growth conditions greatly influence enzyme production. They found that maximum lipase production is generally obtained at optimum temperature and pH for growth.

Independent studies performed by Makhzoum *et al*. (1995) on *Pseudomonas* sp. and Papon & Talon (1988) on *Bronchothrix thermospacta* and *Lactobacilli* strains showed that glucose concentrations of 0.5-10 $g.l^{-1}$ stimulate growth but suppress lipase production. Results from the combined affects of temperature, pH, salt concentration and age of culture on lipase production by *Staphylococcus xylosus* showed that it was not significantly affected by temperature. Statistical analysis performed by Sorensen & Jacobsen (1996) did however confirm that lipase production was influenced by pH, salt concentrations and the age of the culture. They found that in general lipase production was high during stages of vigorous

growth, but high enzyme production was limited to a more narrow range of environmental conditions than growth.

Other organisms like the yeast *Candida rugosa* was recognized as a potent producer of extracellular lipase by Brockerhof & Jensen in 1974. It produced lipases in the presence of sterols and fats with cholesterol being the most effective inducer (Ota *et al*., 1968). This species also showed extracellular lipase activity and its production to be mostly induced by the addition of fatty acids to the culture broth (Lotti *et al*., 1998).

1.5.1. Enhancing Lipase Production

Literature suggests that every microorganism capable of the production and secretion of lipases requires a very distinct set of environmental conditions for optimum production. Lipase production has been shown to be directly affected by cultivation temperature, pH, agitation and oxygenation. Furthermore, nitrogen and carbon sources, their ratios, the inducer type and salt concentration all had a notable effect on production. These influences of culture conditions and other factors on lipase production have been studied extensively (Tan *et al*., 1984; Nesbit *et al*., 1993; Dharmsthiti *et al*., 1999).

Del Rio *et al.* (1990) and Montesinos *et al*. (1996) described the effect of carbon and nitrogen source, oxygenation and other parameters influencing the microbial process of lipase production. These authors focused mainly on factors such as carbon: nitrogen ratios, carbon and nitrogen sources, temperature, pH, concentrations of inducers and of course, different fermentation strategies e.g. submerged fermentation, solid-state fermentation, fed-batch and continuous culture in a Chemostat.

In the case of *Yarrowia lipolitica,* the addition of both lipid materials and soybean meal in the culture broth increased lipase production. The maximum production

was obtained after 72 hours. This maximum activity was correlated with an increase in dissolved oxygen concentration and pH. Destain and co-workers (1997) also saw these two parameters as an indirect sensor that signals the end of enzyme production.

Different inducers as well as different carbon sources have an effect on enzyme yield. As was the case for the yeast *Candida rugosa* where olive oil, the inducer of choice for lipase production induced production successfully but replacing glucose with maltose as a secondary carbon source further improved the lipase yield (Benjamin *et al*., 1996). Ferrer & Sola (1992) found that aeration rate showed a significant influence with minimum dissolved oxygen concentration encouraging enzyme production. Lipase yield also showed a proportional increase with the increase in concentration of olive oil up to 10%. Higher concentrations of the inducer did however not increase lipase yield. Although inclusion of olive oil at different concentrations had an effect on enzyme production, other factors cannot be ignored. The effect of carbon and nitrogen sources and ratios as well as the addition of macro and micro elements also achieved an increased yield (Benjamin *et al*., 1996). Media composition and other parameters all have an influence on the physiology of growth and this can affect both the synthesis and the secretion of lipase (Lotti *et al*., 1998).

In the first of two closely related articles the production of lipase by *Candida rugosa* was studied in solid state fermentation (SSF). Enzyme production by solid state fermentation was reported by Ramesh *et al*. (1990) and Madamwar *et al*. (1989) in two closely related articles. Both illustrated the use of SSF for the production of several industrial enzymes. Factors such as particle size and oil content of the rice bran substrate and the addition of urea and maltose had pronounced effects on lipase production. Malt extract was found to be the best organic nitrogen source, however the cost precludes its use for industrial purposes. The pronounced difference between growth and lipase production on organic as opposed to inorganic nitrogen sources has also prompted many

researchers to study this phenomenon more closely. In this SSF experiment the addition of a variety of extra carbon sources demonstrated that the growth limiting factor in SSF with rice bran as substrate was the available nitrogen rather than the carbon source (Venkato *et al*. 1992).

In the second article, different control strategies of fed-batch cultivation were explored. Gordillo *et al*. (1998) found that a constant low feeding rate proved to be optimal for lipase production. In this research it was demonstrated that with higher feeding/dilution rates there was a trend towards intracellular accumulation of lipase. In most cases Delmau *et al.* (2000) found that mixed carbon sources only improved biomass yield and did not improve on lipase yields obtained with lipid substrates as the sole carbon source.

The research performed by Nesbit & Gunasekaran (1993) involving *Nocardia asteroides* again highlighted the importance of the nitrogen source on the production of lipase. Cells grown in synthetic medium supported the highest growth and extracellular lipase production, with monosaccharide primary carbon sources being best for enzyme production. Various nitrogen sources were tested with glutamate supporting maximum growth and enzyme activity. Ammonium proved to be the least effective nitrogen source. The minimal growth and lipase production obtained with a very high C:N ratio explained why lipolytic activity is not induced when this organism undergoes starvation.

Sztajer and Maliszeska (1988) indicated that complex organic nitrogen sources such as soybean meal and peptone enhanced lipolytic activity followed by ammoniacyl nitrogen in *Bacillus circulans, B. licheniformis* and *Pseudomonas fluorescens.* They also showed that lipolytic activity was supported by different carbon sources in different organisms. Starch induced maximal lipolytic activity in *Bacillus circulans, Streptomyces sp.* and *Pseudomonas fluorescens,* with galactose and sucrose exerting an enhanced activity in *Bacillus sp.* Furthermore,

Sztajer & Maliszeska (1988) proved maltose to be the most effective lipolytic inducer in *Bacillus licheniformis.*

The fed-batch cultivation of *Pseudomonas fluorescens* for mass production of lipase showed that initiation and duration of lipase production was not only dependent on olive oil concentration but also cell concentration. As was the case in many other lipase production experiments the addition of excess olive oil was inhibitory to growth as well as lipase production. The authors were not able to explain the biochemical and genetic aspects of regulation, but did note that the limitation of olive oil and a semi-starved state was in most cases preferential to lipase production. This partially explained why many enzymes including lipases are excreted in the late exponential or early stationary phase where essential substrates become limiting (Suzuki *et al.*, 1988). It was also reported that the activity of *Pseudomonas aeruginosa* lipase increased in the presence of polysaccharides (Soberon-Chaves & Palmeros, 1994) and was decreased by the presence of long-chain fatty acids, specifically Oleic acid (Winkler & Stuckmann, 1979).

In the research of Becker *et al*. (1997) regarding the lipase producing thermophile *Bacillus sp.* IHI-91, olive oil was used as the sole carbon source. The continuous cultivation proved to be better than batch fermentations, improving lipase production by as much as 50%. Again the addition of excess olive oil had a detrimental effect on lipase production. This was clearly demonstrated by the decrease of lipase activity with an increase in dilution rate during chemostat growth.

Lipase production of *Pseudomonas aeruginosa* MB 5001 was enhanced 6.6 fold by the development of a fed-batch fermentation process by Chartrain *et al*. (1993). Again lipase activity was first detected during stages of decelerating growth. In this system however, the addition of oil was separate and in excess to that of the added culture medium. This oil feeding mechanism will probably not

be applicable to organisms, like those mentioned in the previous paragraphs, that Becker *et al.* (1997) and Suzuki *et al*. (1988)have demonstrated are repressed by excess oil. Once again the carbon: nitrogen ratio proved to be of great importance in enzyme production. Elevated ratios of between 1.66 and 4.13 gave a 3 fold increase in lipase production (Chartrain *et al*., 1993).

1.5.1.1. Effect of Carbon

1.5.1.1.1 Carbon Sources

Olive oil is the most widely used inducer for lipolytic activity. The presence of 1% olive oil has been reported to successfully induce lipase production in culture medium. The alkaline lipase producing *Pseudomonas fluorescens* (Lee *et al*., 1993) and *Penicillium expansum* (Sztajer *et al*., 1993), both showed lipolytic activity when grown on oil containing medium, but *Pseudomonas fluorescens* preferred the lipid substrate to be emulsified. In both cases, the addition of Tween 20 and Lubrol PX had a stabilizing effect on the produced lipase. The addition of Triton X-100 also enhanced the lipase production of *P. pseudoalcaligenes* by 50-fold when used in conjunction with olive oil as an inducer. In the bacterium *Pseudomonas aeroginosa* (strain KKA-5), lipase activity was successfully induced by castor oil addition at 2% (v/v).

Wang *et al*. (1995), found that most *Bacillus* strains, including thermophilic strains, produced highest levels of activity when vegetable oils (olive oil, soybean, sunflower, sesame, cotton seed, corn and peanut oil) were used as a carbon source, with maximal activity almost always obtained with olive oil as the inducer.

For the yeast *Rhodotorula glutinus,* both carbohydrate (fructose) and lipid sources induced lipase production. Further investigation however showed that a lipid inducer, in this case palm oil, gave 12-fold higher lipase yield

(Papaparaskevas *et al*., 1992). *Aspegillus niger* produced lipase in a lipid free media, but the introduction of lipids increased production. In the yeast *Candida rugosa,* Gordillo *et al*. (1995) established that the initial concentration of oleic acid influenced lipase production. Oleic acid is a major product formed during the hydrolysis of lipid inducers such as olive oil and Tween 80. Maximal production was found at 2 g/l oleic acid with inhibition occurring at higher concentrations. The constitutive extracellular lipase produced by *C. rugosa* was found by Lotti *et al*. (1998) to be induced by fatty acid (oleic acid) addition, with the constitutive lipase being produced (induced) when glucose was added as a carbon source.

Essamri *et al*. (1998) stated that inducer type and concentration not only influence lipase activity but also has a direct effect on biomass formation. The addition of various oils increased both the lipase production and cell growth of *Rhizopus oryzae* by up to 3-fold compared to oil-free media. Rapeseed and corn oil was most suitable for cell growth and lipase production, with 3% oil yielding maximal biomass, but maximal lipase production was obtained at 2% oil concentration.

1.5.1.1.2 Type of Inducer

Not only does different lipases have specific responses to different types of lipid substrates and even chain lengths but the type of lipid inducer also has an effect on the amount of lipase produced.

In two closely related articles reporting on the lipase production of two thermophilic *Bacillus* strains (*Bacillus* sp. THL027 and *Bacillus thermoleovorans* ID-1) it was showed that induction by a variety of different lipid substrates showed significant differences in the amounts of lipase activity obtained. For the *Bacillus* sp.THL027 strain the highest production was obtained with rice bran oil with minor lipase production also seen when using olive oil as the inducer (Dharmsthiti *et al*., 1999).

Furthermore, *Bacillus thermoleovorans* ID-1 was reported to have a very high growth rate on Tween 80 as a sole carbon source. The maximal lipase activity was found when Triolein was used as the inducer, with Tween 80 proving to be the least effective inducer. Tween 20 was the second most effective inducer, closely followed by soybean oil, mineral oil and olive oil (Lee *et al*., 1999)

1.5.1.1.3 Triacylglycerol (TAG) Accumulation

According to Alvarez *et al.* (2002), the principal function of bacterial TAG seems to be as a reserve energy source. Other possible functions include regulation of cellular membrane fluidity by excluding unusual fatty acids from membrane phospholipids, or acting as a sink for reducing equivalents.

Biosynthesis and intracellular accumulation of TAG has been reported for both Gram positive and Gram negative bacteria. In most bacteria, accumulation of TAG and other neutral lipids is usually stimulated by excess carbon source and limited nitrogen source. The accumulation occurs predominantly in the stationary growth phase. Under growth restricted conditions however, utilization of carbon source is able to continue in certain microorganisms principally for the biosynthesis of fatty acids that are subsequently accumulated intracellularly in the form of TAG (Olukoshi *et al*., 1994). In contrast Huisman and co-workers (1993) found that many bacterial strains block lipid metabolism, fatty acid biosynthesis and importantly β-oxidation due to the limitation of essential nutrients.

β-oxidation of fatty acids contained in TAG produces large amounts of reducing equivalents. Therefore the reason why most TAG accumulating bacteria are aerobic is most probably due to the fact that β-oxidation occurs only under aerobic respiratory conditions (Alvarez & Steinbuchel, 2002).

1.5.1.2. Effect of Nitrogen

It was reported by Sztajer & Maliszewska (1989) that in most cases organic nitrogen sources, specifically peptone, yielded highest lipase production. Other organic nitrogen sources such as corn steep liquor and soybean meal also had a stimulating effect on lipase production, but to a lesser extent than peptone. Inorganic nitrogen sources such as ammonium sulphate inhibited lipase synthesis. A few reported exceptions are *Rhodotorula glutunis*, where organic nitrogen such as yeast extract and tryptone favored growth, but ammonium phosphate had a positive effect on lipase production (Papaparaskevas *et al.* 1992). An increase in lipase production by *Aspergillus niger* was observed by Pokorny *et al*. (1994) as a result of supplementation of medium with ammonium nitrate. However the addition of both an organic and inorganic nitrogen source (ammonium sulphate and peptone) enhanced the production by *Ophiostoma piceae* (Gao *et al*., 1995). This was also seen in a *Bacillus* strain A 30-1 (ATCC 53841) by Wang and co-workers (1995) with the addition of yeast extract and ammonium chloride.

The use of amino acids and tryptone was found by Cordenons *et al*. (1996) to increased the lipase yield of *Acinetobacter calcoaceticus* by 2 and 3-fold respectively, compared to the use of ammonium, yeast extract or protease peptone. Further supplementation of the organic nitrogen source with ammonium again improved not only the yield, but the stability of the produced lipase.

1.5.1.3. Effect of Metal Ions, Dissolved Oxygen Tension and NaCl

A thermophilic bacterium, *Bacillus* sp. A30-1 isolated from a mineral-rich hot spring required a very complex lipase production medium that contained Ca^{2+}, Mg^{2+}, Na^{+}, Co^{2+}, Cu^{2+}, Fe^{2+}, K^{2+}, Mn^{2+}, and Zn^{2+} (Wang *et al*., 1995). For another thermophilic *Bacillus* sp. the addition of Mg^{2+}, Ca^{2+} and Fe^{2+} increased lipase

production several fold (Janssen *et al*., 1994). For *Pseudomonas pseudoalcaligenes* KKA-5 optimum lipase production occurred at Mg^{2+} concentrations of 0.8 M, and its exclusion resulted in a 50% decrease in lipolytic activity (Sharon *et al*., 1998). The addition of Ca^{2+} generally had little effect on lipase production except in the case of a thermophilic *Bacillus* sp., RS-12 where lipase production was found to be growth associated (Sidhu *et al.*, 1998).

Another interesting observation made by Chartrain *et al*. (1993) was the sensitivity of lipase production to dissolved oxygen tension. Lipase production during preliminary batch fermentations was increased when the cultivation was performed under oxygen limiting conditions. During the fed-batch cultivation of *Pseudomonas aeruginosa* it was found that cycling of oxygen tension achieved increased lipase production. It was found that biomass production occurred during the oxygen limited phase and lipase production during the phase with excess dissolved oxygen.

Salt tolerance in specifically thermophilic bacteria was first reported by Reeve (1994) to enhance the stability of thermostable enzymes even further. Work performed by Dharmsthiti & Luchai (1999) on a thermophilic *Bacillus* sp. THL027 showed a decrease in lipase activity over and below a NaCl concentration of 3%v/v.

1.6. Physicochemical Effects of High Temperatures

1.6.1. Bioavailability

In general an elevation in temperature is accompanied by a decrease in viscosity and an increase in diffusion coefficient of organic compounds (Antranikian, 1990). The solubility of these insoluble compounds will therefore increase with increasing temperature causing an increase in available substrate concentration,

and this will in turn lead to higher bioconversion rates (Solubility Data series, 1984).

1.6.2. ‘Mailard’ Products

Belitz & Grosch (1992) stated that Mailard products are only of real consequence if hyperthermophilic conditions are maintained. These dark-colored, insoluble precipitates form when sugars and amines are heated at neutral or alkaline pH. Otero *et al.* (1994) reported on the possibility that these products may be inhibitory to growth or lead to a deficiency in essential amino acids and/or available carbohydrates.

1.6.3. Oxygen Transfer Rate (OTR)

It is commonly accepted that the OTR will decrease at higher temperatures due to the decrease in oxygen’s solubility. However, the OTR depends not only on the solubility of oxygen but also on the volumetric mass transfer coefficient.

$$OTR = k_L a (c^* - c_L)$$

OTR - Oxygen transfer rate

$k_L a$ - Volumetric mass transfer coefficient for O_2 (h^{-1})

k_L - Liquid film coefficient of absorption ($m\ h^{-1}$)

a - Volumetric area of the gas/liquid interface ($m^2\ m^{-3}$)

c^*- Conc. of O_2 in liquid, in equilibrium with conc. in the gas phase ($mol\ m^{-3}$)

c_L - Dissolved O_2 conc. in the liquid phase ($mol\ m^{-3}$).

The diffusion coefficient for gasses in liquid increases with temperature, therefore the mass transfer resistance due to the boundary layer of the liquid is smaller at higher temperatures and this should in turn compensate for the decrease in oxygen solubility (Higbie, 1935).

1.6.4. Removal of Inhibitors

On the other hand, Sundquist *et al.* (1990) mentioned that the increase in liquid/gas mass transfer due to higher temperatures could possibly be useful in the removal of inhibitory products of growth. The removal of ethanol produced by thermophilic microorganisms during fermentation was reported by Sundquist (1990). The volatility of ethanol was increased further by the addition of salt, yeast extract or by applying a vacuum over the fermentor. In previous articles it was quoted that with hydrolysis of olive oil by lipases there is a subsequent release of oleic acid. This oleic acid, at high concentrations was found to be a direct inhibitor of lipase production (Gilbert *et al*., 1991). Unfortunately the only organic acid volatile enough to be removed as a vapor was shown to be formic acid.

1.6.5. Stripping of Volatile Components

Unfortunately due to the increased diffusion coefficient for gasses in liquid at high temperatures some volatile nutrients can be stripped out of the growth media under hyperthermophilic conditions. These nutrients may become deficient and can cause cessation in growth. An example of such a volatile nutrient is NH_3. Due to the dissociation of ammonia, stripping of NH_3 could then lead to secondary pH changes which could prove to be detrimental to growth (Wilke & Chang, 1955).

1.7. Genetic Regulation and Monitoring

According to the research on the regulation of lipase encoded genes in *Candida rugosa* by Lotti *et al*. (1998) it has been hypothesized that the lipase genes may be grouped into two classes, encoding for a constitutive and an inducible lipase respectively. These two sets of genes are controlled through different regulatory

pathways because of the inhibitory effect of glucose on the lipase production of cells grown with olive oil as an inducer. The synthesis of inducible enzymes is inhibited at the level of transcription by the addition of glucose and, conversely, oleic acid (major product of lipid hydrolysis) appears to hinder the synthesis of the constitutive lipase. A further type of regulation may appear in the form of intracellular accumulation, caused by a rate limiting step in the transport of the newly synthesized protein when growth conditions are supporting high levels of transcription. It is important to note that with the addition of glucose the lipase is expressed at a much slower rate, the cellular secretory machinery is not overloaded and no intracellular accumulation is found.

Valero *et al*. (1991) monitored the effect of glucose addition on transcriptional level by performing direct hybridization of cellular RNA samples with DNA probes encompassing the entire lipase isoenzyme encoding gene. (Northern Blotting) This analytical tool doesn't allow us to discriminate between which one of these iso-enzymes are expressed under which condition, but the existence of these complex regulation patterns were suggested by cross inhibition experiments using competing substrates.

In Fungal species like *Aspergillus* (Pokorny *et al*., 1994) and *Rhizopus* sp. (Nahas, 1988) lipases are constitutively induced. In *Pseudomonas* sp. the production of lipase has been shown to be strongly induced by triglycerides and detergents and not repressed by the addition of glucose or glycerol. On the other hand long chain fatty acids, such as oleic acid, strongly inhibited lipase production (Gilbert *et al.* 1991).

A linear relationship was found by Christiansen & Nielson (2002) between the specific production rate of enzyme (Savinase) and total RNA content. The stable RNA mainly consists of ribosomal RNA which reflects the translational capacity of the cells. The stable RNA content of the cells decreased with a decreasing production rate due to a decrease in specific growth rate brought on by a step

down in dilution rate. This indicated that the protein synthesizing machinery may be limiting the production rate.

1.8. Conclusions

It has been estimated that only approximately 1% of microorganisms observable in nature can be cultivated using standard techniques (Amman *et al*., 1995). For organisms isolated from extreme environments, in situ cultivation and large scale production can prove to be difficult or even impossible.

Currently, most of the lipases commercially available are produced by mesophilic yeast such as *Candida* sp., and these lipases are not stable under extreme conditions, especially high temperatures. For industrial applications an enzyme's thermal and pH stability is essential (Brady *et al*., 1988). Recombinant techniques proved not to be the answer for cultivation problems and subsequent difficulty in production of extremophilic enzymes. Fujiwara *et al*. (2002) showed marked differences between function and structures of native enzymes and those expressed in mesophilic hosts such as *E. coli.* High temperature itself seems to play an important role in determining the specific characteristics and three dimensional structure of thermostable enzymes.

To produce a thermostable lipase in its native host many considerations such as those discussed in this section should be considered and fundamental studies need to be conducted and parameters defined to not only increase levels of production but ultimately also if possible improve enzyme stability.

Chapter 2
Materials and Methods

2.1. Microorganism

The bacterium used in this investigation is a novel thermophilic bacterium isolated from fissure water collected approximately 3.1km below ground surface from a mine in East Driefontein (S.A.). It is an aerobic, rod-shaped, gram positive, spore forming bacteria which showed high lipase activity and a broad substrate specificity against triacylglycerides ranging from C4 to C18. This isolate was not only able to grow (specific growth rate of $2.5h^{-1}$) on olive oil as the sole carbon source, but also on a variety of other lipid substrates and even emulsifiers. This isolate was taxonomically characterized by molecular method and positively identified as *Geobacillus thermoleovorans* (De Flaun *et al.,* 2004, personal communication and submitted).

2.2. Culture media

2.2.1. R2A broth

Stock cultures were maintained on petri dishes containing R2A media at 4ºC and were sub-cultured at 3 month intervals.

R2A broth consisted of (per liter): 0.5g yeast extract, 0.5g peptone, 0.5g casamino acids, 0.5g glucose, 0.5g starch, 0.3g sodium pyruvate, 0.3g KH_2PO_4

and 0.05g $MgSO_4.5H_20$. The constituents were dissolved in distilled water and the pH adjusted to 7.5.

2.2.2. Tributyrin Agar

Tributyrin agar was used as plate screening method for lipase activity. Constituents and preparation are discussed in section 2.5.2.1

2.2.3. Designing a Lipase Production Media

Due to the lack of published information on the growth requirements of *Geobacillus thermoleovorans,* yield coeffients for *Klebsiella aeruginosa* was used in the design of the semi-defined media used for induction studies.

Table 2.1(a) The Preliminary media constituents calculated from *Klebsiella aeroginosa* yield coefficients with the added trace mineral solution consisting of substances shown in Table 2.1(b)

Table 2.1(a)

Constituent	Concentration g/l
Glucose **(A)**	5
Citric acid **(B)**	0.25
$(NH_4)_2SO_4$ **(C)**	2.5
K_2HPO_4	2.5
$MgSO_4.7H2O$	0.2
$CaCl_2.2H_2O$	0.01

(A) Carbon source **(B)** Chelating agent
(C) Nitrogen source

Table 2.1(b)

Constituent	Concentration mg/l
$FeSO_4.7H_20$	35
$MnSO_4.7H_2O$	7
$ZnSO_4.7H_20$	11
$CuSO_4.5H_20$	5
$CoCl_2.5H_2O$	2
$Na_2MoO_4.2H_2O$	1.3
$H_3BO_{3,}$	2
KI	0.35
$Al_2(SO4)_3$	0.5

2.2.3.1 Final Media Definition

The final media composition for growth and induction studies was determined by using a type factorial design focusing on different combinations of carbon sources (starch, glucose), nitrogen sources (proteose peptone, tryptone and $(NH_4)_2SO_4$) and types of inducers (Tween 20 (polyoxyethelene-sorbitan monolaurate), Tween 80 (polyoxyethelene-sorbitan monooleate), glycerol tributyrate, olive oil and stearic acid), that were individually varied and the effect on growth and activity monitored.

2.2.3.2. Medium Concentration Optimization

The effect of different concentrations of carbon (glucose), nitrogen (proteose peptone), inducer (olive oil or stearic acid) and the additional effect of NaCl concentration on lipolytic activity in a series of shake flasks containing lipase production media were determined as described in section 3.7.1. The constituent's concentrations were individually altered.

2.2.4. Growth and Induction Media

Unless otherwise stated, all growth and induction studies were performed in the following media obtained from section 2.2.3.1. Media consisted of (per liter): 5g glucose (slightly carbon limiting), 2.5g proteose peptone, 0.25g citric acid, 2g K_2HPO_4, 0.2g $MgSO_4.5H_20$, 0.01g $CaCl_2.2H_20$, 0.5ml trace mineral solution and 2.5g olive oil as inducer.

The trace mineral solution consisted of the constituents depicted in table 2.1(b) and was prepared by separately dissolving each in a 20X dilution of concentrated HCl.

For batch cultivations, 200 µl of a 60% (v/v) stock solution Durapol Antifoam (based on a Polyoxyethelene-polyoxypropylene copolomer) was added before autoclaving.

2.2.5. Final Optimized Media

From experimental work performed in section 3.7.1, the following alterations were made to the lipase production media mentioned in section 2.2.4.
The altered constituent concentrations were (per liter): 7.5g glucose, 4g proteose peptone, 1.6mM stearic acid and the addition of 3.75g NaCl.

2.3. Culture Methods

2.3.1. Optimum Temperature

2.3.1.1. Growth

Optimum temperature for growth was determined by using a temperature gradient incubator (Scientific industries, U.S.A.), consisting of a solid aluminum bar heated at one end and cooled at the other to produce a stable temperature gradient. The bacterial isolates were cultured in L-shaped test tubes made of optically selected glass and the growth was monitored directly by turbidity using a Photolab S6 Photometer (WTW, Weilheim, Germany). These tubes fit snugly into thirty sample wells across the created temperature gradient, and were capped by metal test tube caps. Aeration and agitation was provided by a rocking motion through an arc of approximately 30º at 35 oscillations per minute.

The temperature gradient incubator temperature limits were set at 42ºC - 70ºC and allowed to equilibrate for up to three days with appropriate temperature measurements taken every 12 hours until the gradient remained constant. At the

onset of every experiment the culture tubes, containing 10 ml of sterile lipase production media (section 2.2.4) (no inducer added), was placed in the temperature gradient incubator overnight to allow equilibration prior to inoculation.

Each culture tube was sequentially inoculated with 1ml of a shake flask culture of *Bacillus thermoleovorans* in the mid-exponential phase (measuring 65 Klett units) and grown in lipase production media (section 2.2.4) (no inducer) incubated at 55ºC on a rotary shaking incubator.

2.3.1.2. Lipase Production

Optimum temperature for lipase production was determined in three different shaking incubators set at different temperatures (45ºC, 55ºC and 65ºC respectively). A series of shake flasks were prepared containing lipase production

media (section 2.2.4). Lipolytic activity as well as metabolic activity (according to cellular ATP determination) was measured over time.

2.3.2. Induction Studies

2.3.2.1. Shake Flask Cultivation

Unless otherwise stated, shake flask cultivations were performed in 500 ml shake flasks containing 65ml of the appropriate induction media. These were inoculated from the same source, receiving 5 ml of an inoculum measuring 65 Klett units (using a Klett-Summerson colorimeter). Pre-inocula were all grown on the standard lipase production media (no inducer) as mentioned in section 2.2.4.

2.3.2.2. Bioreactor Cultivation

A Scientific Multigen F-2000 benchtop bioreactor (Edison, N.J, U.S.A) was used for batch cultivations. The temperature was thermostatically controlled at 55ºC and it was necessary at this high temperature to insulate the glass reactor vessel by using a space blanket (Cape Union Mart). The pH was monitored with a pH probe (Mettler Toledo, Halstead, U.K) and controlled at the specific set pH by the automated addition of 3M KOH (during exponential growth) and 3M H_2SO_4 (during stationary phase) (See section 3.6.2, figure 3.17). Especially during optimum pH studies the adjustment of final pH was performed after autoclaving to avoid precipitation of minerals at high pH (section 3.6.1).

A polarographic oxygen probe (Ingold AG, Urdorf, Switzerland) was used to measure dissolved oxygen tension. The dissolved oxygen tension was kept above 40% saturation using manually increased aeration rate and stirrer speed during exponential growth.

2.4. Confirmation of Strain Identity

2.4.1. PCR Amplification of 16s rDNA

Polymerase Chain Reaction (PCR) amplification of the 16s rDNA region was performed on whole cells. To aid in cell disruption and subsequent release of genetic material the cells were rapidly frozen at -70ºC and exposed to a 5 min (94ºC) hot start PCR. Both forward and reverse primers used were universal 16s rDNA bacterial primers.

The reaction mixture contained 5 µl of 10X PCR Buffer containing 15 mM $MgCl_2$, 300 nM of both the forward and reverse primers, 200 µM dNTP`s, 5 µl cell suspension (OD = 0.6) and 37.5 µl sterile Milli-Q water. After completion of the 5

min hotstart, 2.6 U Expand High fidelity enzyme was added, yielding a final reaction volume of 50 μl.

Thermal cycling was performed using an Eppendorf Mastercycler Temperature Gradient Personal with the following cycling program: Initial denaturing (hot start) of 5 min at 94ºC, 35 cycles of denaturing (94ºC for 15 sec), annealing (30 sec at 50ºC) and elongation (72ºC for 90 sec). After 35 cycles, a final elongation step of 20 min at 72º was added to ensure complete elongation of the amplified product.

The PCR product was visualized on a 1% (w/v) agarose gel containing 2.5 mg/μl ethidium bromide. The agarose gels were prepared in TAE buffer containing 100mM Tris (2-amino-2(hydroxymethyl)-1,3propanediol)-HCL (pH 8), 0.05 M EDTA (disodium ethylenediaminietetraacetic acid) and 100mM glacial acetic acid. Electrophoresis was performed at 5.6V/cm for 40 min in the above mentioned TAE buffer. DNA was visualized under a low radiation UV source, for isolation and purification of the DNA bands from the agarose gel.

The purified PCR product was ligated overnight into pGem-T easy plasmid vector. The transformation of competent *E. coli* Strain JM 109 cells was performed according to the method described by Shambrook *et al.* (1989). The transformation mixture was then plated out on LB (Luria-Bertani)-media plates supplemented with ampicillin (60mg/l), IPTG (isopropylthio-β-D-galactoside (10mg/l)) and X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside (40mg/l)). Plates were incubated at 37ºC for 20 hours. Blue-white colony selection was performed. White colonies were inoculated into tubes containing 5ml LB-media supplemented with the appropriate antibiotic and grown for 16 hours in a shaking 37ºC incubator.

DNA mini-preparation (plasmid extraction) was performed using the lysis by boiling technique as described by Shambrook *et al*. (1989). Screening for the

correct recombinant plasmid was performed by restriction analysis using *Eco*R1 restriction enzyme, followed by separation by electrophoresis as mentioned above. The insert was purified using the GFX™ PCR DNA and gel band purification kit and eluted into 50 μl elution buffer (10mM Tris-HCL, pH 8.5)

The purified restriction analysis products containing the 16s rDNA insert was then subjected to sequencing reactions. The 16s rDNA fragment was sequenced by using primers specific for the SP6 and T7 promotor regions of the pGem-T easy vector, using the ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready reaction kit v.3.0 or 3.1 (Applied Biosystems, U.S.A.) following the manufacturer's instructions. Approximately 50% of the purified sequencing PCR product was loaded onto a 4% Acrylamide gel, separated at 1.6 kV and the data collected on an ABI Prism® 377 DNA Sequencer (Perkin Elmer Biosystems, U.S.A). DNA sequence alignments were then performed using DNAssist v.2.0.

2.4.2. Transmission Electron Microscopy

Cells harvested in the late exponential phase of *Geobacillus thermoleovorans* was added to 1% agar solution and the embedded material was cut into small cubes. These cubes were then fixed using 5ml 3% gluteraldehyde solution prepared in 0.2M sodium phosphate buffer (pH 7) for up to 7days. The fixative was then carefully decanted and the cubes washed for 5 minutes with sodium phosphate buffer. The tubes were then covered in a 4% aqueous stock solution of OsO_4 for a fixation time of between 30 min and 2 hours until samples turned dark in color. The OsO_4 was then decanted and the samples repeatedly washed with sodium phosphate buffer.

Samples were then dehydrated with a series of 50-90% acetone for 10-20 minutes followed by a 100% acetone step for 30 minutes to one hour.

Epoxy resin was prepared freshly at the onset of the final dehydration step. All but approximately 1ml of the remaining 100% acetone was decanted and to this 1ml of epoxy was added. The mixture was stirred well and allowed to impregnate the samples for up to 8 hours. The 1:1 mixture (epoxy: acetone) was then removed and replaced by 1ml epoxy and this step repeated again overnight. The samples were then placed in a desiccator to remove all traces of the acetone.

Epoxy was prepared by weighing (not volumetric measuring) of the following components, 23g VCD (vinylcyclohexene dioxide), 62g NSA (nonenyl succinic anhydride), 14g DER736 (diglycidyl ether of polypropylene glycol) and 1g DMAE ((S1) dymethylaminoethanol) added together with constant stirring.

The moulds were dried in the embedding oven overnight. Samples and epoxy were transferred to the pre-dried and pre-heated moulds and allowed to polymerize at 70ºC for eight hours. Two days after polymerization the embedded material was sectioned by ultra-microtome, yielding sections of approximately 0.2µm.

Sections were then mounted on copper grids and stained using drops of stain solutions placed on a wax layer in a Petri dish. The uranium stain is prepared in the dark to prevent photo-oxidation (using a bell jar). The first step is a 20 minute stain with 6% (saturated aqueous solution) uranyl acetate, triple rinsing in three beakers with distilled water. The second stain was a 10 minute stain with lead citrate with the same rinsing procedure with fresh water. The addition of NaOH pellets in the Petri dish where staining procedure took place prevented the formation of lead-carbonate crystals contaminants.

The lead citrate stain was prepared as follows: 0.665 g lead nitrate, 0.880 g sodium citrate, 4 ml 1N sodium hydroxide. The lead nitrate was then dissolved in 15 ml water. The sodium citrate was added which immediately formed a milky-white precipitate. This was swirled continuously for 60 seconds (not shaking to

prevent the introduction of oxygen). The solution was allowed to settle for 30 minutes, stirred once every minute or two and the precipitate cleared with the introduction of the 1N NaOH solution.

2.5. Analytical Methods

2.5.1. Monitoring Growth

During induction studies monitoring growth proved to be difficult due to the presence of non-soluble (lipid) lipase inducers and emulsified oil droplets.

2.5.1.1. ATP Luciferase Kit

Measurement of metabolic activity was performed using the Luciferase ATP Biomass kit and Field Kit Luminometer (Thermo labsystems). The assay follows the release of light during the following reation:

$$\text{ATP} + \text{D-Luciferin} + O_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \textbf{LIGHT}$$

The ATP monitoring reagent produces a linear signal from a lower detection limit of 5 pg ATP/ml to an upper limit of 0.5 µg ATP/ml (according to manufacturers manual). Culture samples were appropriately diluted not only to work in this detection range but also to minimize possible interference from foreign components present in the culture medium.

To each 4 ml polystyrene cuvette was added 100 µl culture sample and 100 µl ATP releasing reagent, the solution was mixed gently and allowed to stand for 1 min. To this mixture was then added 500 µl ATP monitoring reagent and readings were immediately taken using the Luminometer. Internal calibration was

performed by adding 10 μl of a 10 μM ATP standard to a sample after a reading was taken and measuring the specific increase in light emission. If no internal interference is present this increase should remain constant.

External calibration was performed by creating a standard dilution series and plotting a standard curve of ATP concentration versus Luminometer reading (Figure 2.1). ATP concentrations of 1.6, 3, 7.4, 14.75, 40, 115, 204 μg/ml were prepared.

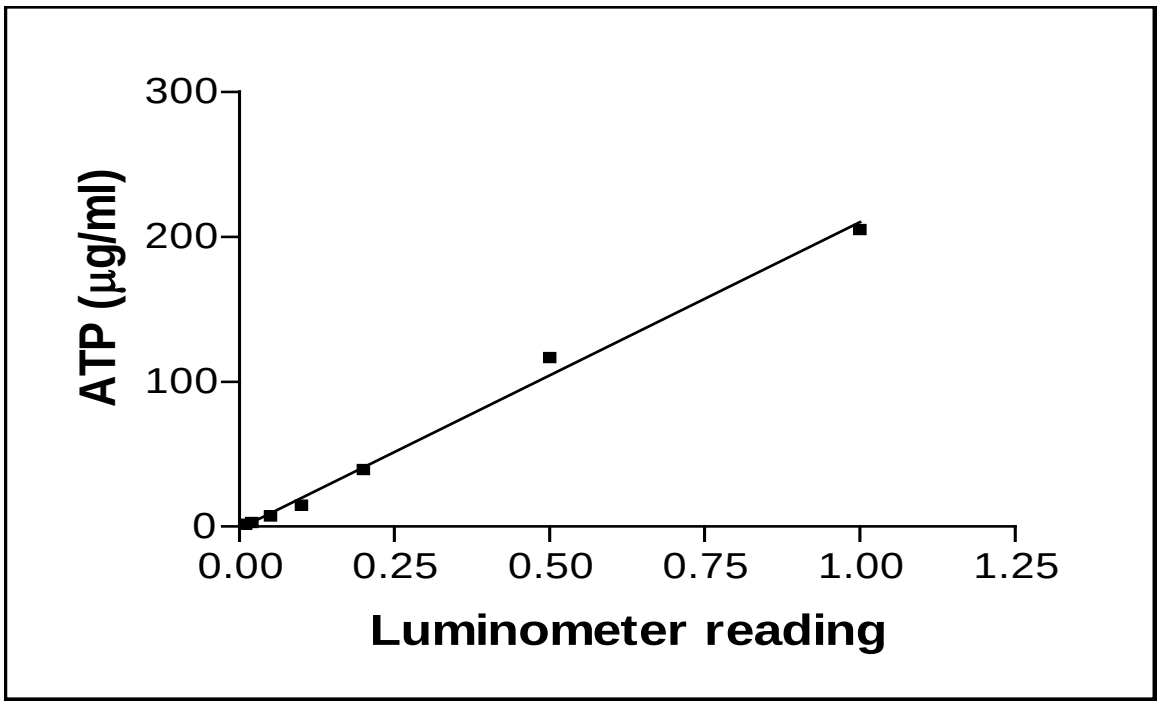

Figure 2.1 Standard curve of series of ATP concentrations versus Luminometer readings used for the calculation of cellular ATP levels during growth.

During growth studies a reagent blank was prepared by omitting the culture sample from the reaction mixture.

2.5.1.2. Biuret Assay

The Robinson-Hogden biuret assay method as used by Herbert *et al.* (1971) was used after slight further modification. Culture samples of 10 ml were centrifuged; cells were washed twice in saline solution and allowed to dry. Samples were then resuspended in 700 μl distilled water and transferred to 1.5 ml Eppendorf tubes. To each Eppendorf tube was then added 350 μl of a 3M NaOH solution, samples were placed in a boiling water bath for 5min. Each tube was rapidly cooled in ice water and allowed to stabilize at room temperature. To each sample was then added 300 μl 2.5% $CuSO_4.5H_2O$ solution, the resultant precipitate was broken by vortexing and the tubes were centrifuged at 4000 rpm for 10 minutes in a Beckman TJ-6 centrifuge. The absorbances of the supernatants were measured against a reagent blank at 555 nm in a Beckman DU 650 spectrophotometer.

A set of Protein standard solutions were prepared by dissolving Bovine serum albumin (Fraction V) (Sigma Chem. Co. St. Louis) in distilled water to final concentrations of 0, 10, 20, 30, 40 $mg.ml^{-1}$. These samples were subjected to the same treatment as cell samples. A standard curve of Absorbance (555nm) versus Standard Protein concentration was plotted (Data not shown).

A series of known biomass concentrations were also subjected to previously mentioned treatment and a standard curve of absorbance at 555nm (as measured after Biuret protein determination) versus Biomass (g/l) was constructed (Figure 2.2)

2.5.2. Lipase Screening and Quantification

2.5.2.1. Glycerol Tributyrate Plates

The lipase screening was performed by means of glycerol tributyrate agar plates This screening medium contained (per liter): 5g peptone, 3g yeast extract, 10g glycerol tributyrate and 12g bacteriological agar. This solution was sonicated to a milky emulsion with a Branson Sonifier cell disruptor B-30 and the pH set at 8 before autoclaving. The tributyrate agar was dispensed into sterile plastic petri-dishes and allowed to cool and solidify in a laminar flow hood.

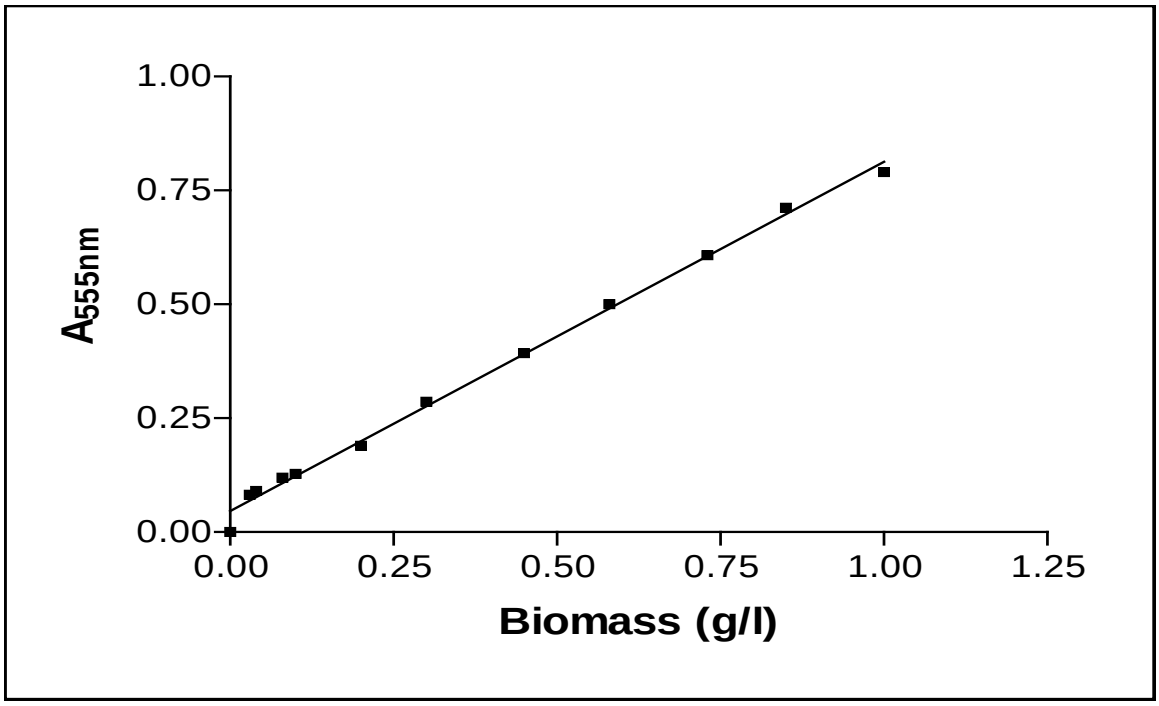

Figure 2.2 Standard curve of biomass (g/l) versus A_{555nm} as measured after Buiret protein determination.

2.5.2.2. *p*-Nitrophenyl Palmitate Assay

A synthetic ester; *p*-nitrophenyl palmitate (*p*-NPP) was used as substrate. This method measures both lipase and esterase activity.

Figure 2.3 Chemical structure of *p*-Nitrophenyl palmitate (Synthetic ester).

A slight modification of the *p*-NPP assay method as described by Winkler and Stuckmann (1979) was used to measure lipolytic activity. The *p*-NPP was dissolved in *iso*-propanol to a concentration of 0.3% (v/v). Final substrate dilution was performed by drop wise addition of the *p*-NPP solution to assay buffer containing 2g sodium deoxycholate and 0.5g Gum Arabic dissolved in 450ml of a 50mM Tris-HCL solution, pH 8. The final concentration of substrate was standardized to a reading of approximately 1.9 at 280nm.

The *p*-NPP assay reaction was started by addition of centrifuged culture supernatant after appropriate dilutions were made to keep reaction rate below 0.5 A/min. The reaction was followed over time on a Beckman DU 650 Spectrophotometer at 410 nm. The temperature of the cuvette was maintained at 55ºC using a water jacketed cuvette holder and a circulating water bath.

2.5.2.3. Olive Oil Assay

Assay methods based on the hydrolysis of synthetic esters such as *p*-NPP are usually limited by pH and type of fatty acid used. Therefore another lipase

specific activity assay was used in conjunction with the much more rapid *p*-NPP assay.

Olive oil emulsion (enzyme substrate) was freshly prepared by sonicating a solution containing 10% (v/v) olive oil and 1% (w/v) Gum Arabic with a Branson Sonifier cell disruptor B-30 until the mixture was milky. Centrifuged culture supernatant was then added to the assay mixture containing 500μl substrate and 500μl assay buffer consisting of 1mM Tris-HCL and 5mM $CaCl_2$. The assay mixture was then incubated at 55ºC in a shaking waterbath for 1 hour. The reaction was stopped by the addition of 2.5ml copper reagent which was prepared by separately dissolving 16.125g $Cu(NO_3)_2.3H_2O$ and 32.5ml tri-ethanolamine in 200ml distilled water. These solutions were mixed, the pH set at 7.5 with HNO_3 and the final volume adjusted to 500ml (Duncombe, 1963).

The complex formed between the released fatty acid and the copper was extracted by the addition of 5ml chloroform, vortexing for 30 seconds and centrifugation at 4000 rpm for 2 min. The aqueous phase was carefully removed and 2ml of the chloroform phase was transferred to new test tubes. To these test tubes was added a freshly prepared 0.25% (w/v) solution of sodium-diethyldithiocarbamate dissolved in 2-Butanol, and the absorbance was measured at 440nm in a quartz cuvette against a reagent blank containing chloroform. A substrate blank was prepared and subjected to the same treatment but no culture supernatant (enzyme) was added to the reaction mixture.

A set of fatty acid standards were prepared from a stock solution of 1mM stearic acid dissolved in chloroform. From this stock solution appropriate amounts were transferred to clean test tubes and the chloroform was allowed to evaporate. The standards were subjected to the same treatment as described above. A standard curve of known fatty acid (stearic acid) amount versus absorbance measured at 440 nm was constructed.

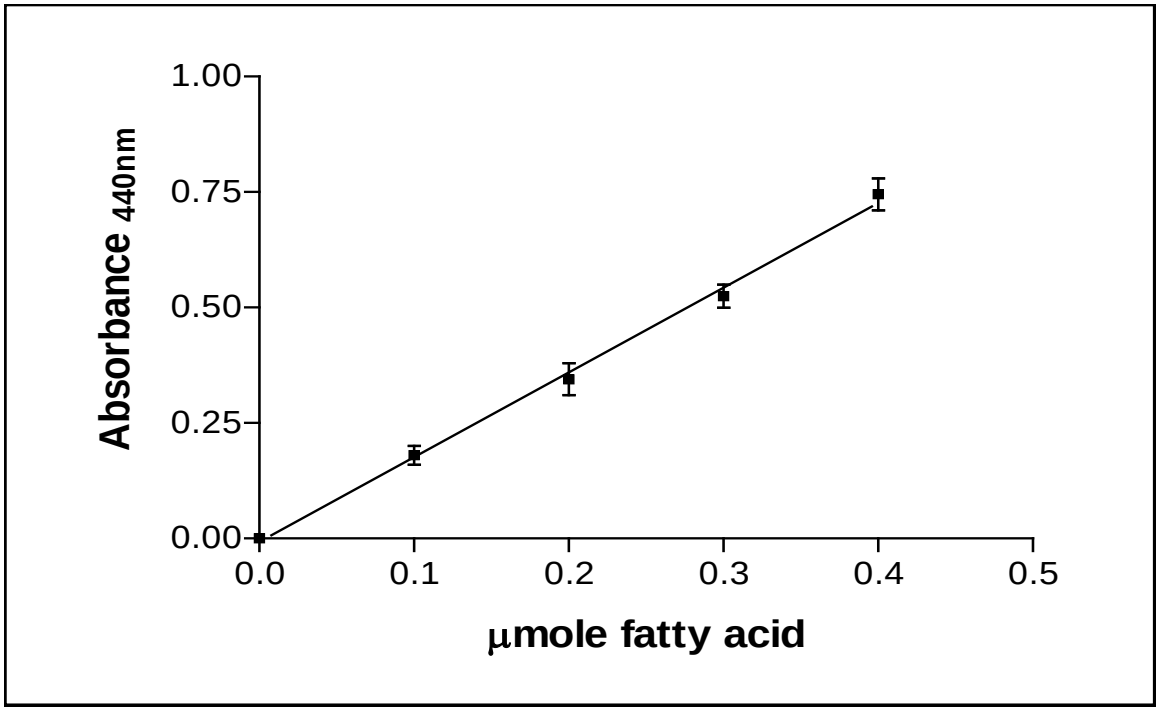

Figure 2.4 Standard curve for assay of fatty acid released during olive oil assay and for the quantification of free fatty acids present in medium due to lipase activity. Stearic acid was used as the standard.

2.5.3. Glucose Utilization

Glucose utilization in batch culture was monitored using a glucose oxidase assay kit (Randox™) as per manufacturer's instructions. Due to the very high sensitivity of the glucose oxidase kit, appropriate dilutions of clarified culture broth were made.

2.5.4. Fatty Acid Liberation and Utilization

The method used to monitor the free fatty acids present in the medium was a further modification of the method used for the olive oil (lipase specific) assay described in section 2.5.2.3. A 750 μl sample of culture supernatant, clarified by centrifugation was added to 2.5 ml copper reagent (constituents discussed in

section 5.2.3). The Cu^{2+}-fatty acid complex formed was then extracted with chloroform and developed by the addition of 0.25% (w/v) Sodium-diethyldithiocarbamate prepared in 2-Butanol. Fatty acid concentrations were then calculated by using a standard curve (Figure 2.4).

Chapter 3
Results and Discussion

3.1. Identification and Morphology

3.1.1 PCR Amplification and Sequence Analysis of 16S rDNA

The PCR amplification was done according to section 2.4.1. Figure 3.1 depicts the 16s rDNA PCR amplification product obtained from *Bacillus* GE-7 cells.

1 2 3 λIII

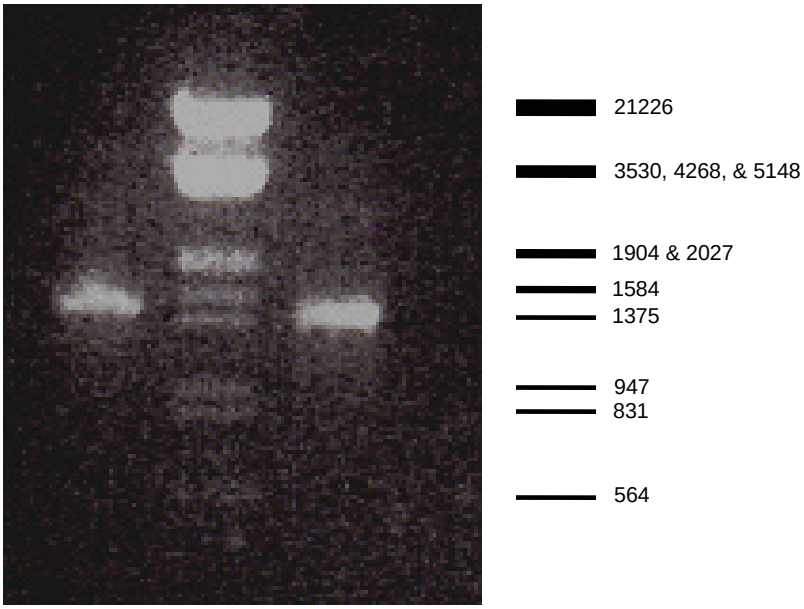

Figure 3.1 Gel electrophoresis of PCR amplification product of 16s rDNA of GE-7 strain. Lanes 1 and 3 are duplicate experiments and lane 2 is the loaded λIII size marker set. Adjacent to the right are the band sizes of the λIII marker set.

Lanes 1 and 3 (duplicate reactions) shows the isolated 16s rDNA and size of approximately 1500bp.

The 16s rDNA product was cloned into pGem-T Easy® cloning vector and competent *Escherichia coli* (Strain JM 109) were transformed and Minipreps performed to isolate the proliferated plasmid. Restriction digestion with *Eco*R1 enzyme produced two bands (approx. 900 and 600 bp indicated by A & B) and the pGem-T easy vector (Figure 3.2, Lane 3). This indicated the presence of an *Eco*R1 restriction site in the 16S rDNA fragment. Lane 1 shows the λIII-marker (the 947-and 564bp marker bands are not visible due to low marker DNA

concentrations). Lane 2 shows the untransformed pGem-T easy plasmid digested with *Eco*R1.

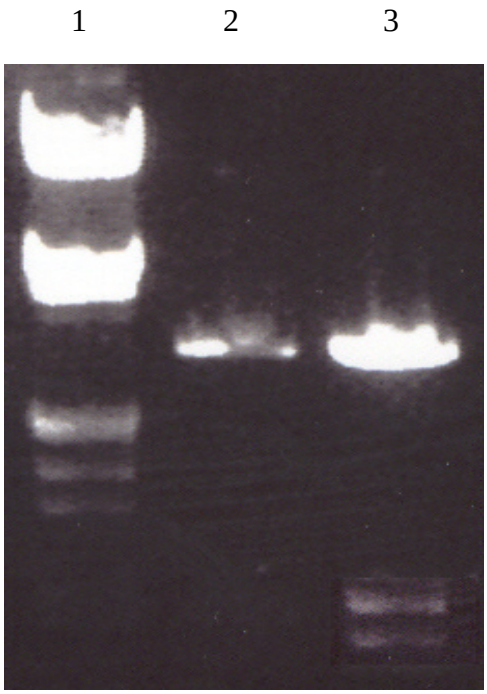

Figure 3.2 Gel electrophoreses of *Eco*R1 digest of 16s rDNA insert from pGem-T easy vector (Lane 3). Lane 1 shows the λIII marker and lane 2 the *Eco*R1 digest of untransformed pGem-T easy vector. Adjacent to the right is the band sizes of the λIII marker set.

Sequence analysis of the 1522bp 16s rDNA insert ligated into a pGem-T Easy® cloning vector gave a 413bp (forward sequence) and 490bp (reverse sequence) (Figure 3.3).

These were subjected to a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) where both the obtained sequences revealed a 99% identity with the known *Geobacillus thermoleovorans* T80 16S rDNA sequence.

The presence of the *Eco*R1 restriction site in the *Geobacillus thermoleovorans* T-80 16s rDNA sequence was confirmed by restriction mapping of the complete sequence obtained from http://www.ncbi.nlm.nih.gov/BLAST using DNAssist ver. 2.0. The exact position of the restriction site divided the 1522bp 16s rDNA fragment into a 678 and 844 fragments respectively. This correlates well with the band sizes obtained during our *Eco*R1 restriction analysis (Figure 3.2).

Approximately 60% of the complete 16s rDNA sequence was obtained but the high degree of identity with the known *Geobacillus thermoleovorans* T80 sequence, taken together with the presence of the single *Eco*R1 restriction site and the data of DeFlaun *et al.* (2004) was sufficient evidence to confirm the identity of this isolate.

3.1.2. Light and Transmission Electron Microscopy

The visible morphology and size of *Geobacillus thermoleovorans* compared well to the findings of DeFlaun *et al.* (2004). During transmission electron microscopy

(Figures 3.5(a&b)) cellular width was measured at approximately 0.5 µm. The measured length of 4 µm compared well with the reported length of 4-6 µm. Light microscopy (Figures 3.4(a&b)) shows the formation of long Bacilli chains when grown in shake flasks (Figure 3.4 (b)). These chains were prone to fragmentation

A

```
Ge7t7  1    CGGACTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGGATTAGAGTTTGATCA 65
GtT80  1                                                      TAGAGTTTGATCC 13

Ge7t7  66   TGGCTCAGGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCAAATCGGAGCT 130
GtT80  14   TGGCTCAGG-ACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCAAATCGGAGCT 77

Ge7t7  131  TGCTCTGGTTTGGTCAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCCGCAAGACCGGGA 195
GtT80  78   TGCTCTGGTTTGGTCAGCGGCGGACGGGTGAGTAACAGGTGGGCAACCTGCCCGCAAGACCGGGA 142

Ge7t7  196  TAACTCCGGGAAACCGGAGCTAATACCGGATAACACCGAAGACCGCATGGTCTTTGGTTGAAAGG 260
GtT80  143  TAACTCCGGGAAACCGGAGCTAATACCGGATAACACCGAAGACCGCATGGTCTTTGGTTGAAAGG 207

Ge7t7  261  CGGCCTTTGGCTGTCACTTGCGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTC 325
GtT80  208  CGGCCTTTGGCTGTCACTTGCGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTC 272

Ge7t7  326  ACCAAGGCGACGATGCGTAGCCGGCCTGAAGAGGGTGACCGGCCACACTGGGACTGAGACACGGC 390
GtT80  273  ACCAAGGCGACGATGCGTAGCCGGCCTGA-GAGGGTGACCGGCCACACTGGGACTGAGACACGGC 336

Ge7t7  391  CCAAACTCCTTACGGGAGGCAGCAGTAAGGGAATCTTCCGCAATGGGCGAAAGCCTGGACGGAAC 455
GtT80  337  CCAGACTCCT-ACGGGAGGCAGCAGTA-GGGAATCTTCCGCAATGGGCGAAAGCCTG-ACGGAGC 398

Ge7t7  456  AACCCCCCCTGAGC                                                 469
GtT80  399  GACGCCGCGTGAGCGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTGAGGGACGAAGGAGCGCC 463
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B

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GtT80   1025 GGGCGTTCCCCCTTCGGGGGGGACAGGGTGACAGGTGGTGCATGGTTGTCGTCA-GCTCGTGTCG 1087
Ge7sp6  1               TTCGGGGGGGACAGG-TGACAGGTGGTGCATGGTTGTCGTCAAGCTCGTGTCG 51

GtT80   1088 TGAGATGTTGGGTTAA-GTCCCGCAACGAGCGC-AACCCTCGCCT-CTAGTTGCCAGCACGAAG 1148
Ge7sp6  52   TGAGATGTTGGGTTAAAGTCCCGCAACGAGCGCCAACCTTCGCCTTCTAGTTGCCAGCACGAAG 115

GtT80   1149 GTGGGCACTCTAGAGGGACTGCCGGCGACAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATC 1212
Ge7sp6  116  GTGGGCACTCTAGAGGGACTGCCGGCAACAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATC 179

GtT80   1213 ATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCGGTACAAAGGGCTGCGAACCCGC 1276
Ge7sp6  180  ATGCCCCTTATGACCTAGGCTACACACGTGCTACAATGGGCGGTACAAAGGGCTGCGAACCCGC 243

GtT80   1277 GAGGGGGAGCGAATCCCAAAAAGCCGCTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATG 1340
Ge7sp6  244  GAGGGGGAGCGAATCCCAAAAAGCCGCTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATG 307

GtT80   1341 AAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 1404
Ge7sp6  308  AAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 371

GtT80   1405 ACACCGCCCGTCACACCACGAGAGCTTGCAACACCCGAAGTC-GGTGAGGCAACCCTTAAGGGA 1467
Ge7sp6  372  ACACCGCCCGTCACACCACGAGAGCTTGCAACACCCGAAGTCCGGTGAGGCAACCCTTACGGGA 435

GtT80   1468 GCCAGCCGCCGAAGGTGGGGCAAGTGATTGGGGTGAAGTCGTAACAAGGTAACCA          1522
Ge7sp6  436  GCCAGCCGCCGAAGGTGGGGCAAGTGATTGGGGTGAAGTCGTAACAAGGTAACCAATCGAATTC 499
```

Figure 3.3 Sequence alignments of *Geobacillus thermoleovorans* 16s rDNA obtained from using T7 (forward primer) (A) and SP6 (reverse primer) (B) specific for those promoters carried on the pGem-T easy plasmid vector. Alignments performed with DNAssist ver. 2.1

and the cellular length also shortened during prolonged growth in batch culture as seen in figure 3.4 (a).

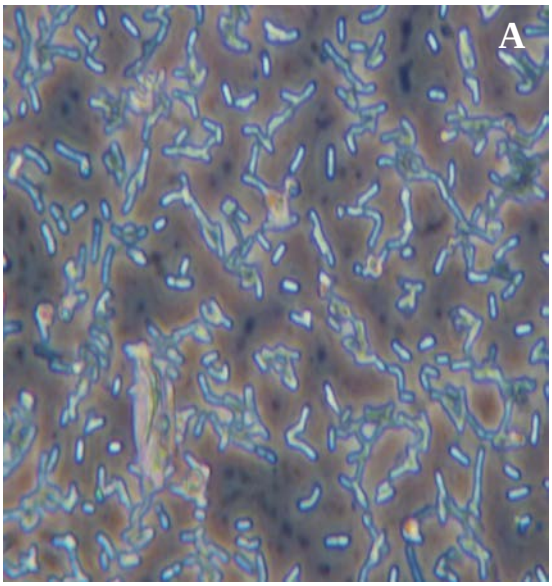


Figure 3.4 (a)
Light microscopy photograph of *Geobacillus thermoleovorans* during late exponential phase in batch culture.

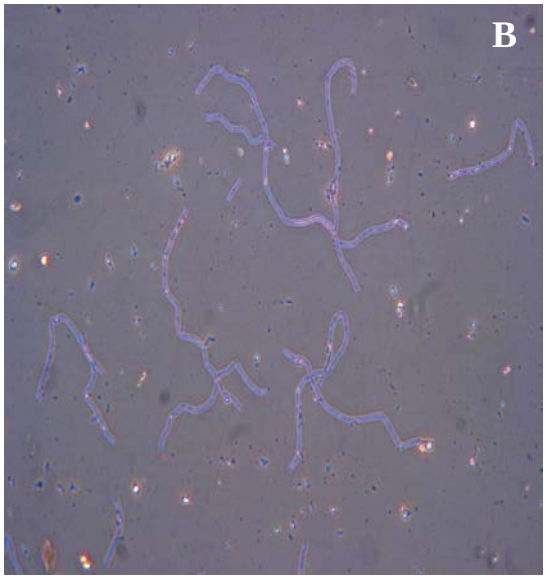


Figure 3.4 (b)
Light microscopy photograph of *Geobacillus thermoleovorans* during late exponential phase in shake flasks

There were clear differences between samples grown with the addition of olive oil inducer in comparison to those grown without added inducer. The transmission electron micrograph in figure 3.5(a) (grown without inducer) shows a clearly visible membrane structure with very little accumulated fat present. Figure 3.5(b) however shows the presence of accumulated saturated as well as unsaturated fats and the cellular membrane is not as prominent.

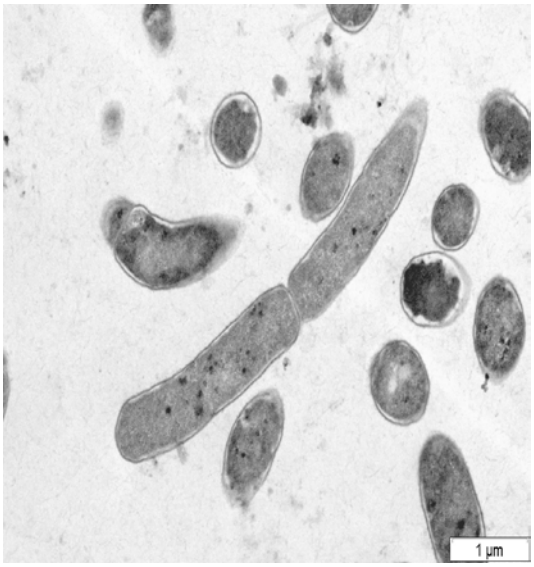


Figure 3.5(a)
Geobacillus thermoleovorans grown in lipase production medium (section 2.2.4). Harvested in mid-exponential phase (no inducer).

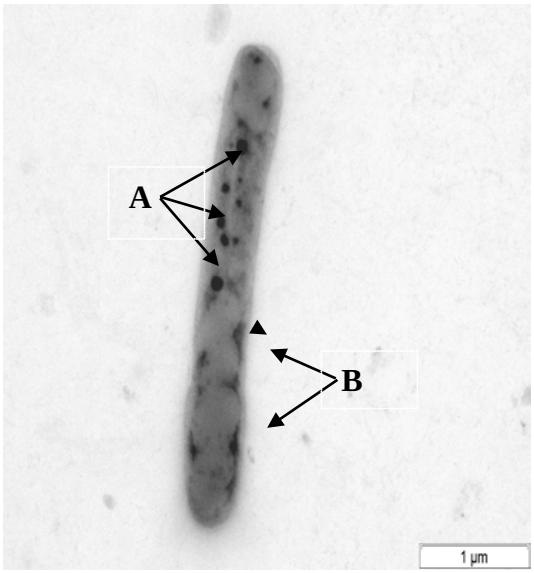


Figure 3.5(b)
Geobacillus thermoleovorans grown in lipase production medium (section 2.2.4). Harvested in mid-exponential phase (olive oil inducer).

In figure 3.5(b) the different dark shapes (indicated by A and B) represent lipid inclusion bodies (Personnel communication Prof. P.J.W. van Wyk, U.F.S). This correlates well to the results obtained by DeFlaun *et al*. (2004) regarding PHB (poly-hydroxybutyrate) accumulation by this strain.

The bacterial accumulation of lipid substrates reported by Olukoshi *et al*. (1994) was believed to be due to an excess of carbon and limited nitrogen rather then due to the presence of the lipid substrates present in the medium. Bacterial strains block lipid metabolism and β-oxidation due to the limitation in essential nutrients that occur during later stages of growth resulting in the possible accumulation of these lipids. With the addition of olive oil as an inducer and the presence of glucose as a secondary carbon source the utilizable carbon must be

in excess, and during stationary phase certain nutrients, specifically nitrogen could become limiting. This creates conditions for lipid accumulation.

The question that arises is why does extracellular lipase production commence during the stationary phase? These micrographs were both taken in the mid-exponential phase of growth where no nutrient limitation should be present but the accumulation still persisted, with the subsequent production and release of extracellular lipase during the stationary phase.

3.2. Lipase Screening and Quantification

3.2.1. Screening for Lipolytic Activity

Geobacillus thermoleovorans and a known non-producer of lipase (*E. coli* JM109) were streaked onto a petri-dishes containing glycerol tributyrate. A clear zone around the *G. thermoleovorans* colonies (A) indicates the presence of lipase activity (Figure 3.6).

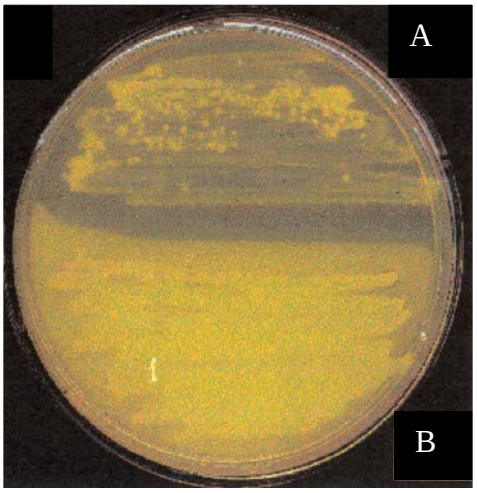


Figure 3.6 Glycerol tributyrate agar with *G. thermoleovorans* (A) and a known non-producer of lipase (*E. coli* JM109) (B)

Repeated sub-culturing of *G. thermoleovorans* on glycerol tributyrate plates caused the termination of lipase production. This was also seen in later shake flask cultivations and for this reason the pre-inocula (including those used in batch cultivations) were all grown in lipase production medium according to section 2.2.4 without added inducer.

3.2.2. Lipase Activity

Lipase activity was monitored using a fast colorimetric assay with *p*-nitrophenyl palmitate as substrate (section 2.5.2.2) or a discontinuous lipase assay with emulsified olive oil as substrate (section 2.5.2.3). These assays were correlated by evaluating the activity simultaneously in shake flasks prepared according to section 2.2.4. These flasks were inoculated with pre-inoculum grown to 65 Klett units without added olive oil inducer.

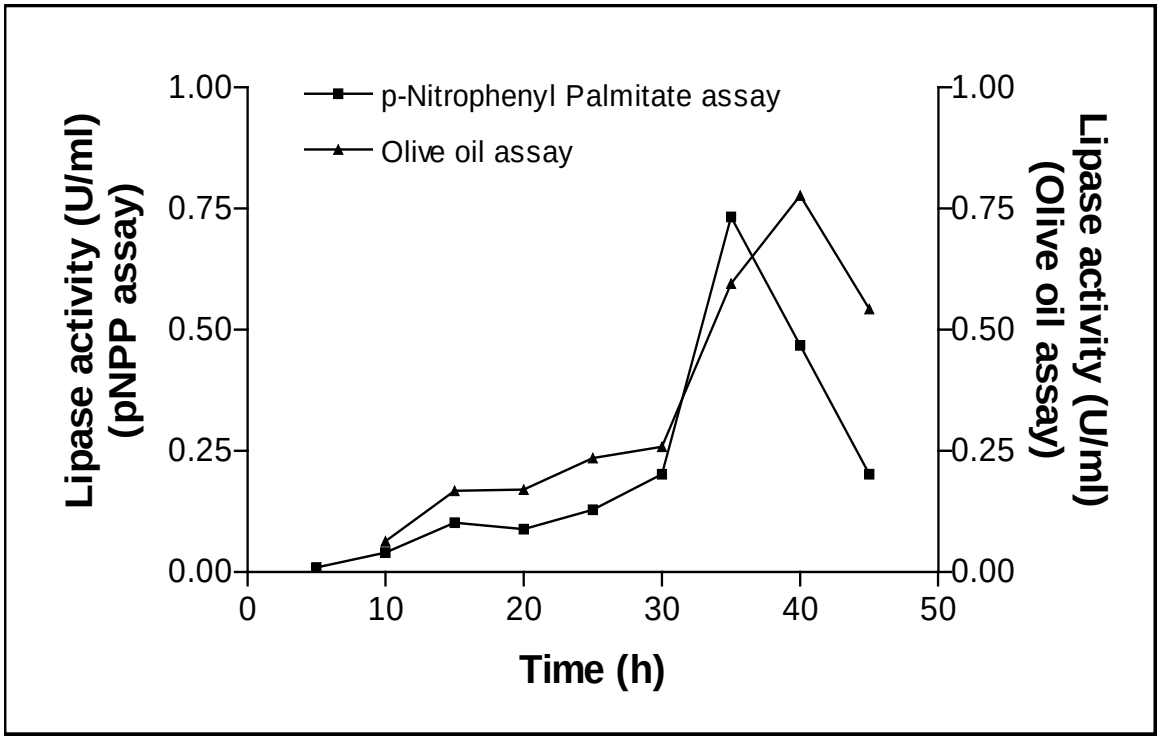


Figure 3.7 Activity obtained from *p*-Nitrophenyl-palmitate assay and olive oil activity assay. Cultivation was performed in lipase production media (section 2.2.4) with 2.5g/l olive oil as inducer.

The resultant activity profiles correlated well, and the *p*-nitrophenyl palmitate assay was used in all further induction experiments to quantify lipolytic activity.

3.3. Design of a Lipase Production Medium

Yield coefficients of *Klebsiella aeroginosa* were used as a start for the design of a medium specifically for the production of lipase by GE-7. Tan *et al*. (1984) and Nesbit *et al*. (1993) reported that the most important factors influencing lipase production are the carbon and nitrogen sources and the type of inducer. The importance of metal ions was proposed by Janssen *et al.* (1994), but in our media containing non-limiting amounts of Mg^{2+}, Ca^{2+}, and a trace mineral solution providing a variety of other metal ions, this should not be a limiting factor.

Series of shake flasks with base media composition were prepared as described in section 2.2.4 altering the specific constituents mentioned in section 2.2.3.1 and adding combinations of these constituents as seen in table 3.1.

Due to the fact that these experiments were performed in two parts because of the problems encountered with monitoring growth when lipid inducers were added, the actual amounts of biomass and lipase activity obtained could not be correlated. Therefore only the relative amounts of growth and lipase production are depicted in table 3.1.

Glucose proved to be the best second carbon source for growth as well as lipase production.

The *G. thermoleovorans* strain does not seem to be amylolytic, contrasting that reported by DeFlaun *et al*. (2004). Starch was reported by Sztajer and

Maliszeska (1988) to induce lipase production in various *Bacillus* sp. The small amounts of growth obtained when starch was added as the secondary carbon source was probably due to either the utilization of the inducer as a carbon source (when lipolytic activity was present), or the carbon delivered by the tryptone and peptone. Lipase yields obtained using starch as a secondary carbon source were in most cases negligible.

Table 3.1 Effect of different combinations of varying carbon (red), nitrogen (green) and inducer sources (blue) on growth and lipase activity (a: Relative growth, b: Relative lipase activity) (Sections 2.2.3.1).

		Inducer									
Nitrogen source		Tween 20		Tween 80		Tributyrin		Olive oil		Stearic acid			**Nitrogen source**
	Peptone	(a)+++	(b)-	+++	++	+++	-	+++	+++	+++	+++	Peptone	
	$(NH_4)_2SO_4$	-	-	-	-	-	-	-	-	-	-	$(NH_4)_2SO_4$	
	Tryptone	+++	-	+++	++	+++	-	+++	++	+++	++	Tryptone	
		Glucose		Glucose		Glucose		Glucose		Glucose			
		Carbon source									
		Starch		Starch		Starch		Starch		Starch			
	Peptone	+	-	++	+	+	-	++	++	++	++	Peptone	
	$(NH_4)_2SO_4$	-	-	-	-	-	-	-	-	-	-	$(NH_4)_2SO_4$	
	Tryptone	+	-	++	+	+	-	++	++	++	++	Tryptone	
		Tween 20		Tween 80		Tributyrin		Olive oil		Stearic acid			
		Inducer									

Of the five inducers tested, stearic acid and olive oil induced the highest lipase production, with Tween 80 stimulating some minor production. Lipase activity

with tributyrin and Tween 20 as inducers was negligible. Important to note is that the activity profiles for stearic acid and olive oil differed. Olive oil induced the appearance of lipolytic activity in mid-stationary phase, compared to its appearance in late exponential phase when stearic acid was used. Stearic acid induced lipase production was slightly higher, but was not sustained for long periods of time as was mostly the case with olive oil induction (See section 3.6.3.2, Figure 3.19).

G. thermoleovorans was unable to grow or produce extracellular lipase on inorganic nitrogen ($(NH_4)_2SO_4$). Growth and lipase production obtained on peptone and

tryptone (organic) nitrogen sources compared well with the work of Nesbit & Gunasekaran (1993) and Sztajer & Maliszeska (1988), with slightly higher lipase yields obtained from using peptone.

The best carbon and nitrogen combination was glucose (C-source), peptone (N-source) and either olive oil or stearic acid (Inducer). With this combination and the newly designed medium, an approximate 20-fold increase in lipase production compared to the R2A medium was obtained. Further optimization by investigating specific concentrations of carbon (glucose), nitrogen (proteose peptone), inducers (olive oil or stearic acid) and the effect of NaCl concentrations on lipase production follows in section 3.7.

3.4. Problems Associated with Monitoring Growth

With the addition of non-soluble lipid substrates as inducers, optical methods i.e. measuring Klett/Optical density readings is simply not viable for determining growth. Other methods, such as microscopic counts become tedious with the micellar droplet formation that occurs at high stirring and aeration in batch

culture. Dry biomass determination is problematic due to the lipids adhering to the cell pellet. For this reasons we looked at various alternatives and the following were used to monitor growth.

3.4.1. ATP-Luciferase

An ATP biomass determination kit (ATP-Luciferase) was used to monitor growth during shake flask cultivations as described in section 2.5.1.1. This method did not enable us to construct growth curves but did give us an accurate estimate of metabolic activity (Figure 3.9). The size of the samples required for the ATP determinations were very small and suited the small volumes used during shake flask cultivations.

3.4.2. Biuret Protein Determination

The bacterial cellular protein measured by the Biuret method showed a linear relationship to dry biomass (Figure 2.2). The lipid inducers did not interfere with the results of the Biuret protein determinations on wet biomass. The volume of the samples required were large (10ml) and therefore this method described in section 2.5.1.2 was only used during batch cultivations.

3.5. Optimum Temperature

3.5.1. Growth

Figure 3.8 shows that the maximal growth rates obtained in the temperature gradient incubator were very low ($\mu_{max} < 0.4\ h^{-1}$) in comparison with later batch cultivations ($\mu_{max} > 1.5\ h^{-1}$). Specifically, at higher growth rates the growth was linear rather than exponential highlighting the importance of aeration on the specific growth rate of *Geobacillus thermoleovorans.* The low aeration was

probably due to the low rate of agitation and aeration possible in an oscillating incubator and the fact that the culture tubes were filled to their maximum capacity to compensate for evaporation at high temperatures.

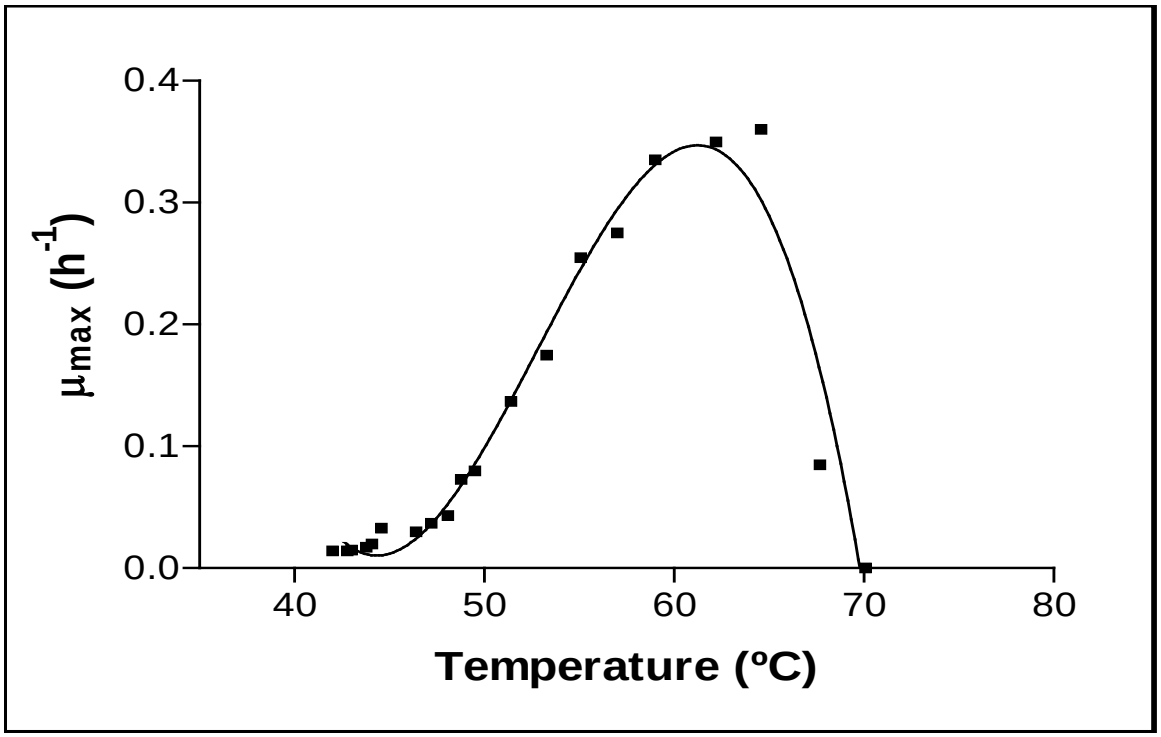


Figure 3.8 Effect of incubation temperature on the maximum specific growth rate of *Geobacillus thermoleovorans* cultivated in lipase production media (Section 2.2.4) (without added inducer).

The optimum temperature was found to be in the range of 60 - 65ºC which agrees with previous results obtained for this strain published by DeFlaun *et al.* (2004). Little growth was obtained below 44ºC with a very sharp decline in growth above 65ºC. The reason for this dramatic decrease in growth rate, according to Pirt (1975) is either due to a disruption in metabolic regulation or dominance of cell death when the upper temperature limit is exceeded.

3.5.2. Maximum Metabolic Rate

Three groups of shake flasks were inoculated and incubated at 45ºC, 55ºC and 65ºC in shaking incubators, respectively. Growth is expressed as metabolic rate using the ATP luciferase kit (Section 2.5.1.1).

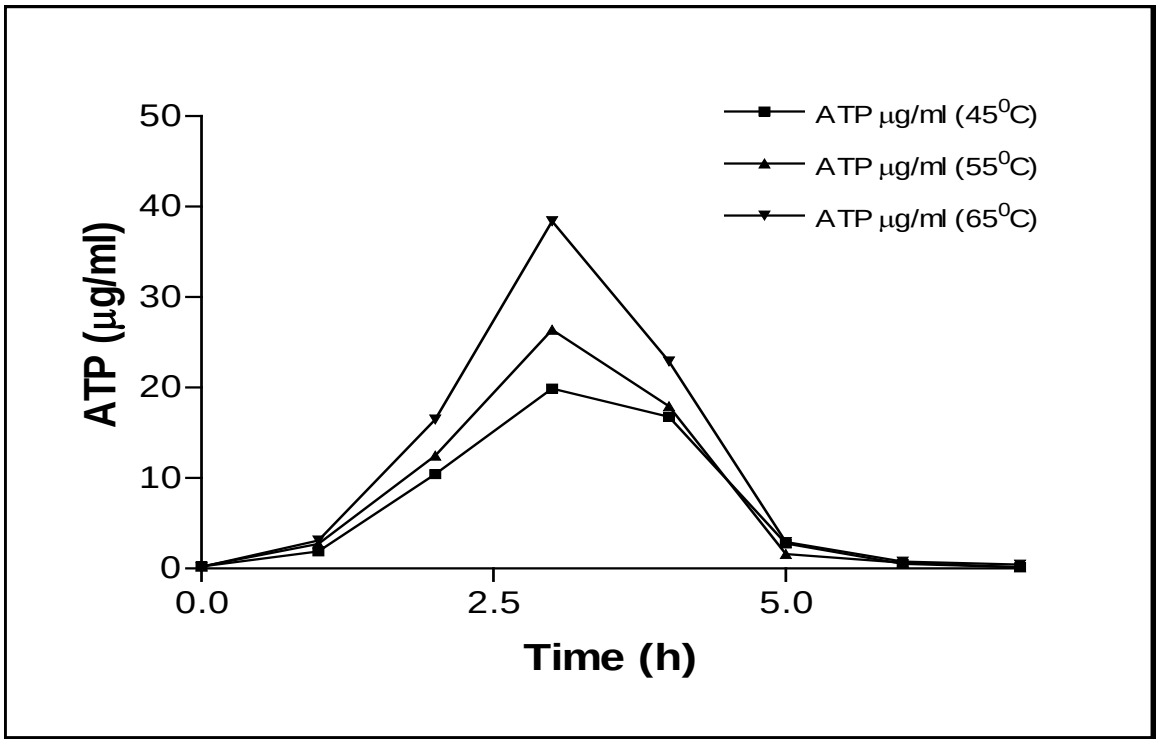


Figure 3.9 Effect of temperature on the metabolic rate of *Geobacillus thermoleovorans*. Cultivation was performed in shake flasks containing lipase production media (section 2.2.4) with 2.5g/l olive oil.

As expected, the highest metabolic rate (Figure 3.9) corresponded to the optimum temperature (65ºC) for growth obtained from the temperature gradient studies (Figure 3.8).

3.5.3. Lipase Production

The highest lipase yield was obtained at 55ºC with very little metabolic rate or lipase production at 45ºC (Figure 3.10). The metabolic rate of the culture at 65ºC was maximum but did however not yield any notable lipase production.

Unfortunately we later found that the luciferase detection kit for ATP (specifically the luciferase) is inhibited by the appearance of free fatty acids. Therefore the possibility exists that with the appearance of free fatty acids in the media as lipase is released, the ATP concentration is underestimated and β-oxidation could actually lead to even higher levels of ATP than those measured. The use of ATP as a method to measure cellular activity as a substitute for cell counts therefore was not a viable option and we had to resort to protein determinations to estimate biomass.

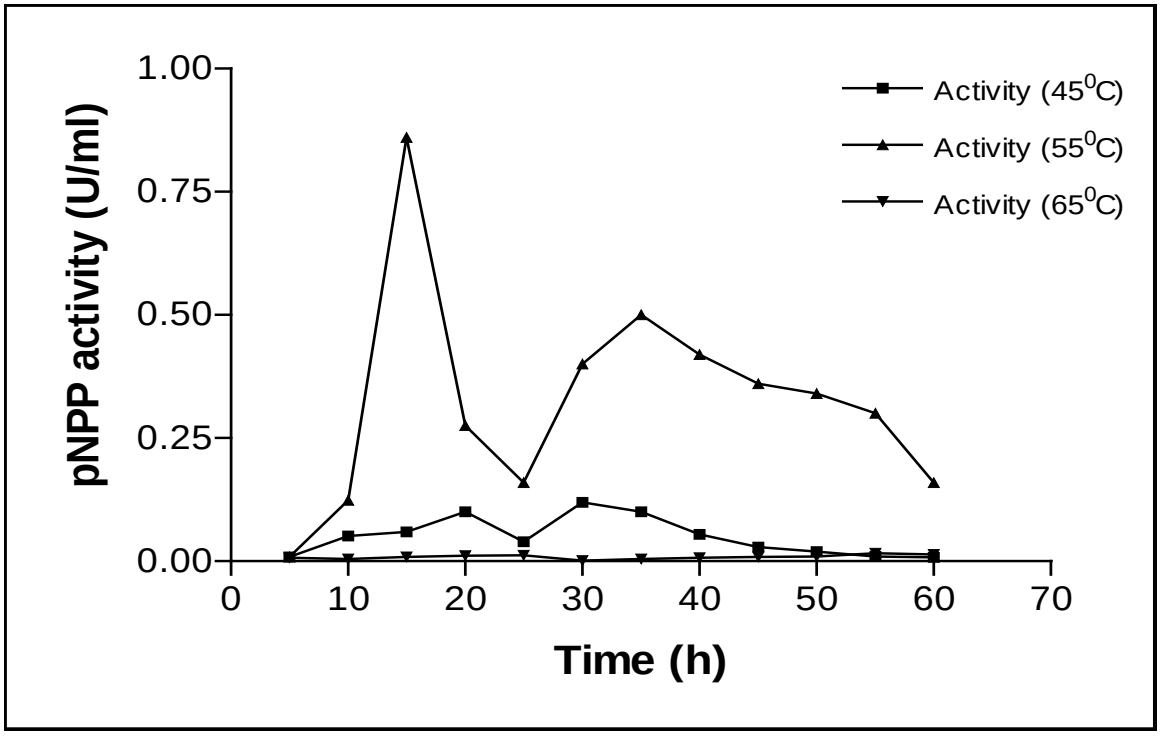


Figure 3.10 Effect of temperature on lipase production of *Geobacillus thermoleovorans.* Cultivation was performed in shake flasks containing lipase production media (section 2.2.4) with 2.5g/l olive oil.

3.6. Fermentor studies

3.6.1. Optimum pH

3.6.1.1. Growth

The Multigen F-2000 bioreactor was used for the cultivation of *Geobacillus thermoleovorans* at different pH values. The maximal specific growth rate was calculated during these batch cultivations from the linear regression performed on the exponential phase of growth. The medium composition is described in section 2.2.4 with olive oil (2.5g/l) used as the inducer. The pH adjustment was performed as described in section 2.3.2.2.

The pH range where *G. thermoleovorans* was capable of growth was between pH 5.5 and 7.5, with an optimum of pH 6.5 (Figure 3.11). This compared well with the pH range and optimum pH for growth reported by DeFlaun *et al.* (2004). These researchers however used the method for optimum pH determination adapted by Gerhardt *et al.* (1994) using a strongly buffered system in shake flasks containing R2A medium.

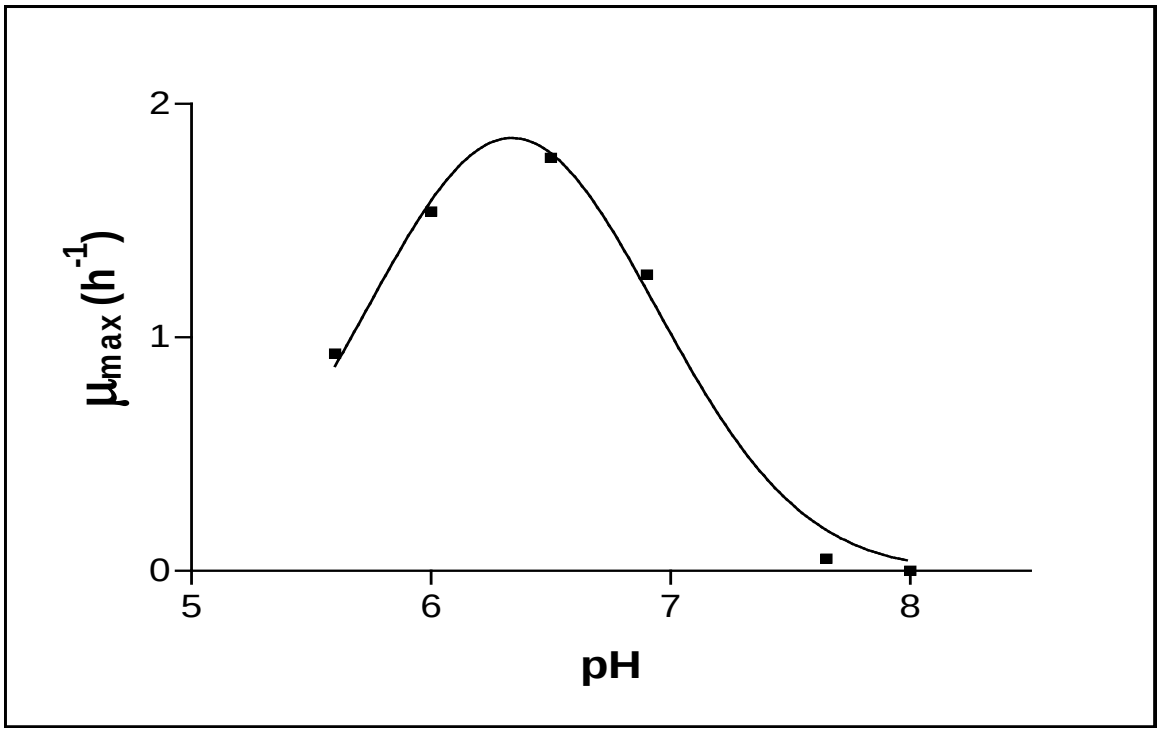


Figure 3.11 Effect of pH on the maximum specific growth rate of *Geobacillus thermoleovorans.* Cultivation was performed in lipase production media (section 2.2.4), with 2.5g/l olive oil and pH set according to section 2.3.2.2.

3.6.1.2. Lipase Production.

Optimum pH for growth and optimum pH for lipase production were both determined to be pH 6.5 (Figure 3.12). At higher pH the growth rate decreased significantly, but the production of lipase, measured when lipase secretion was maximal at or near the stationary phase, did not follow the same trend.

A possible explanation for the high lipase production at high pH (even with inhibited growth) can be seen in figure 3.17 where growth without pH control causes an increase in pH with the production of lipase and subsequent release of free fatty acids in the stationary phase. The higher pH value therefore may have an influence not only on the production of lipase but possibly on its secretion.

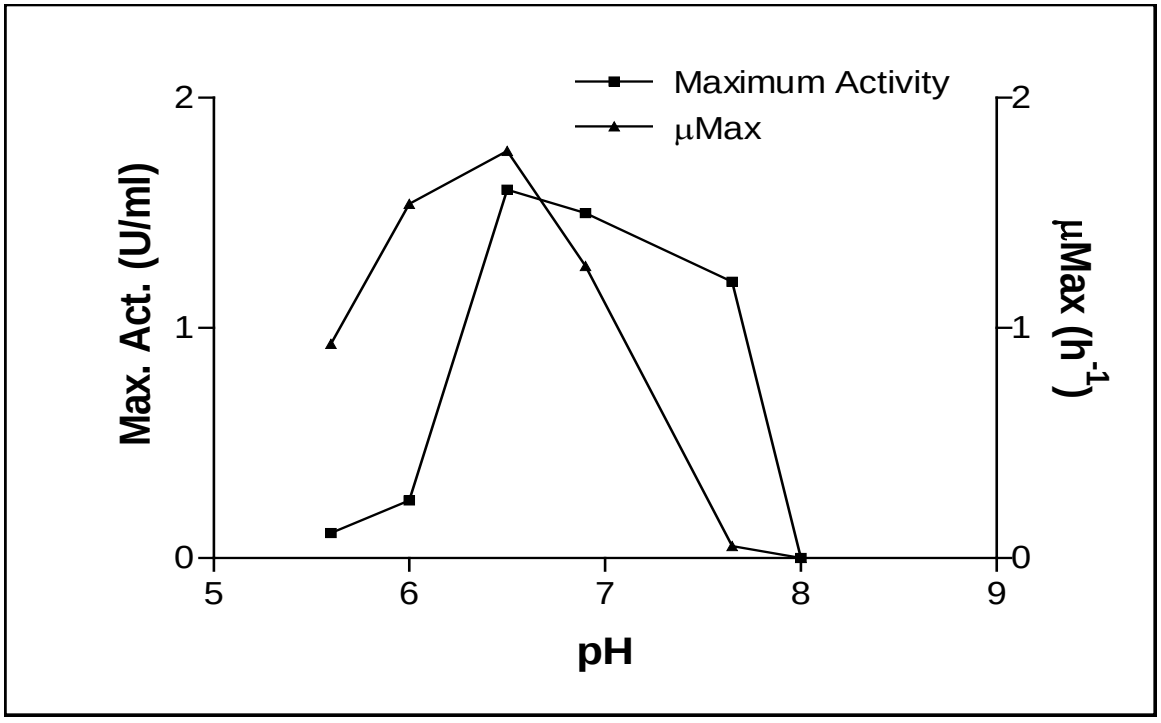


Figure 3.12 Comparison between maximum specific growth rate and maximum lipase activity at stationary phase obtained at different pH values. Cultivation was performed in lipase production media (section 2.2.4), with 2.5g/l olive oil and pH set according to section 2.3.2.2.

3.6.2. Physiology of Growth and Lipase Production

The following batch cultures were all performed in lipase production media as described in section 2.2.4. These experiments show the consumption of glucose together with the appearance of free fatty acids and lipase during cultivation. The initial exponential growth of the organism led to a rapid decline in glucose concentration, accompanied by an expected rapid consumption of oxygen (Figure 3.13).

In the late exponential to stationary phase, the rate of glucose consumption dropped. During stages of decelerated growth, as expected, the rate of oxygen

consumption also slowed. Low levels of lipase started to appear at this stage. It is unlikely that the culture experienced carbon limitation as the residual glucose levels were 4 - 5 mM and fatty acids started to appear.

Unfortunately, we do not have data for nitrogen concentrations as this would indicate if the cultures were experiencing nitrogen limitation. The slow rate of lipase production and release from time 5 - 15h produced small amounts of free fatty acids that appeared in the medium. A dramatic increase in lipase activity and free fatty acids, together with a second phase of rapid consumption of the remaining glucose and a second drop in the dissolved oxygen levels (<5%) followed in the early stationary phase. It is possible that the release of the initial low concentrations of fatty acids could act as a signal for further lipase production. This is supported by the data obtained in section 3.6.3.2, where it was shown that stearic acid was as good an inducer of lipase activity as olive oil with lipase activity peaking much earlier when stearic acid was used as the inducer. This is also the first indication that free fatty acids do not inhibit lipase production of *G. thermoleovorans* as was reported for *Pseudomonas* sp. by Winkler & Stuckmann (1979).

The rapid fatty acid liberation and subsequent consumption (the availability of free carbon) does not cause a secondary exponential phase (Diauxic growth); this may however be due to the lack of sensitivity of the Buiret protein determination (to determine biomass) or another critical medium limitation, possibly nitrogen limitation. Unfortunately the use of the ATP luciferase detection kit was ruled out due to the inhibition of the luciferase by free fatty acids.

The possibility still exists that β-oxidation could yield large amounts of ATP not necessarily utilized for biomass formation. On the other hand, our electron microscopy data (Figure 3.5(b)) showed that fatty deposits do accumulate in the cells in the presence of a lipid substrate. The drop in free fatty acid concentration could also be attributed to storage of the fatty acids in these lipid deposits.

The occurrence in nearly all of the shake flask and batch cultures of two (or multiple) lipase activity peaks is not understood. The appearance of lipolytic activity again corresponded to a fall in the dissolved oxygen tension in the stationary phase. This again indicated the possibility of β-oxidation occurring once free fatty acids appear. This fall of dissolved oxygen is used as an indirect sensor in industry to signal the start of lipase production (Destain *et al.,* 1997).

In figure 3.14 (the duplicate batch culture of figure 3.13) illustrates the reproducibility of results obtained. Both experiments show similar drops in dissolved oxygen with the appearance of free fatty acids and two lipase activity peaks.

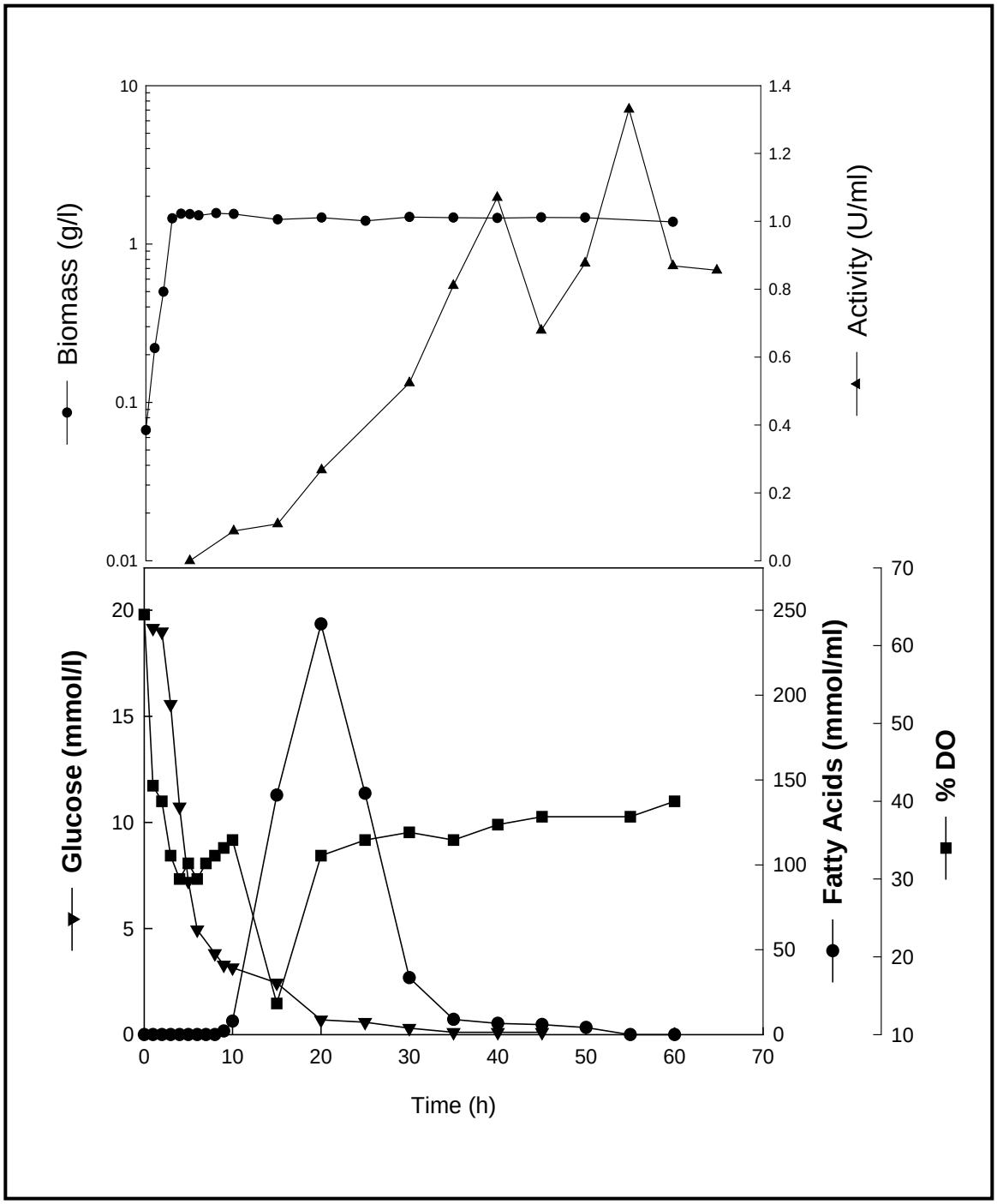


Figure 3.13 Graph showing the relationship between growth, glucose consumption, % dissolved oxygen (%DO), extra cellular lipase activity and the subsequent release and utilization of fatty acids. Cultivation was performed in lipase production media (section 2.2.4), 2.5g/l olive oil and pH 7

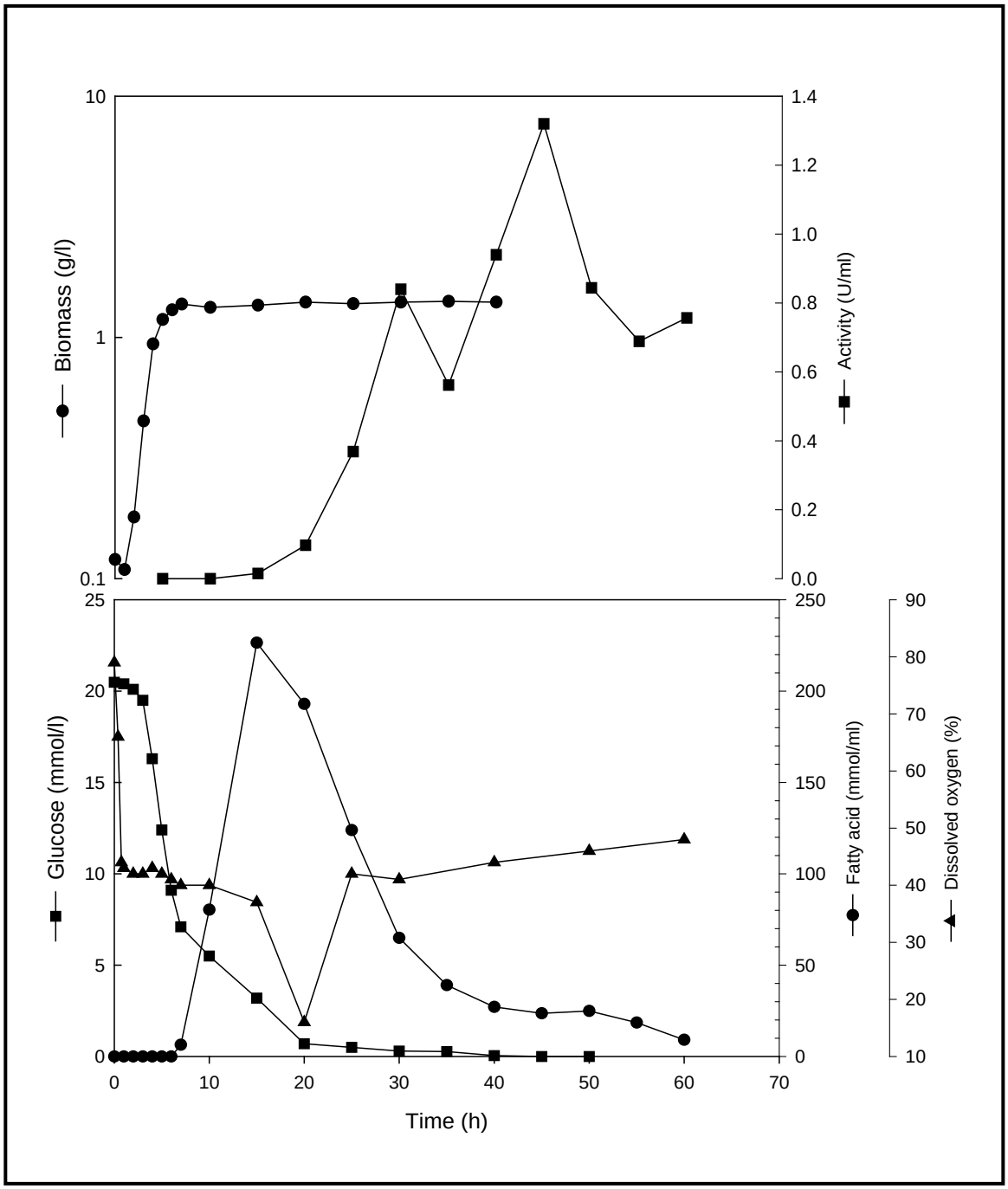
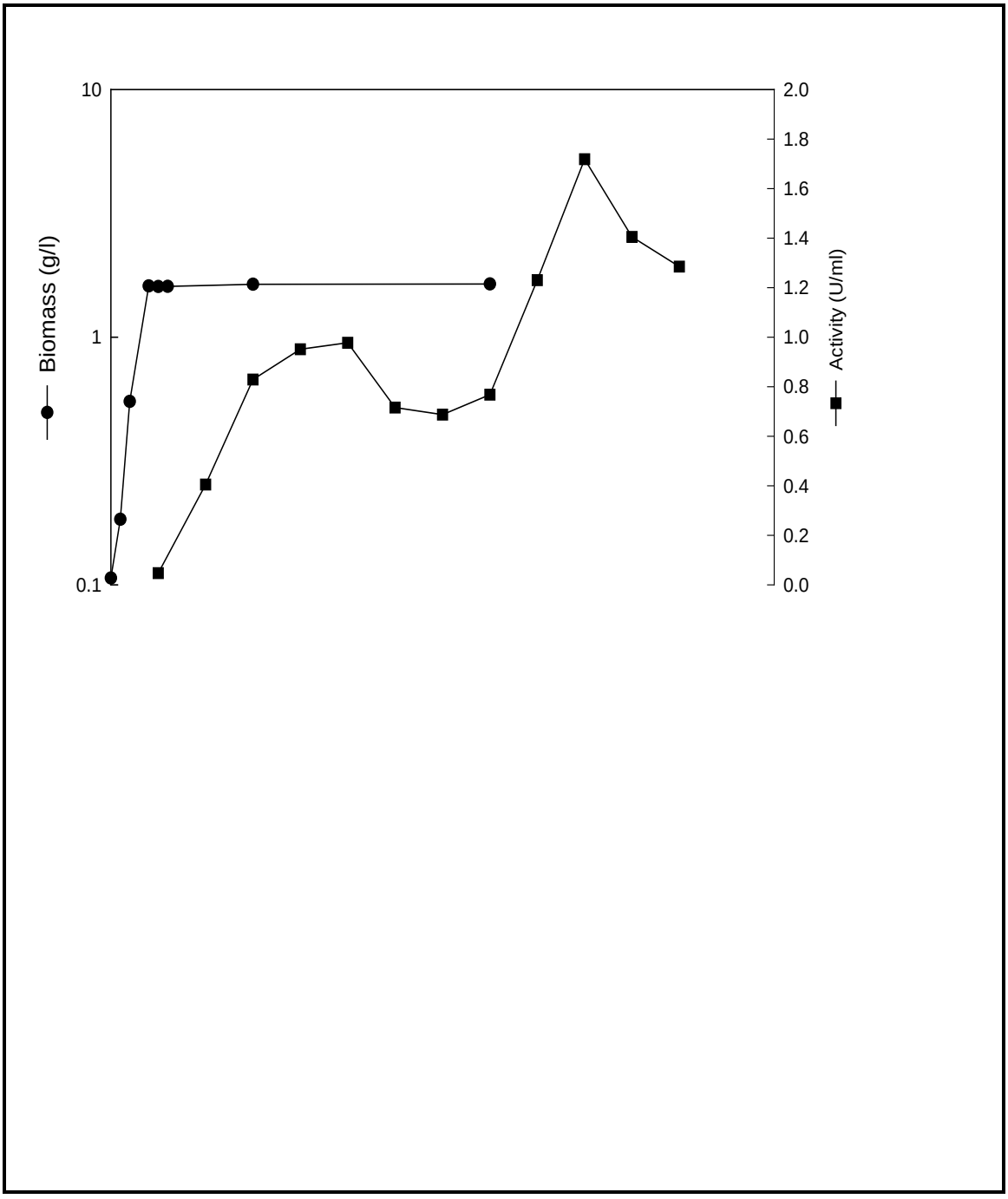

Biomass (g/l)
Activity (U/ml)
Glucose (mmol/l)
Fatty acid (mmol/ml)
Dissolved oxygen (%)
Time (h)
10
1
0.1
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
25
20
15
10
5
0
250
200
150
100
50
0
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70

Figure 3.14 Duplicate reactor run of that depicted in figure 3.13 showing excellent reproducibility of results.



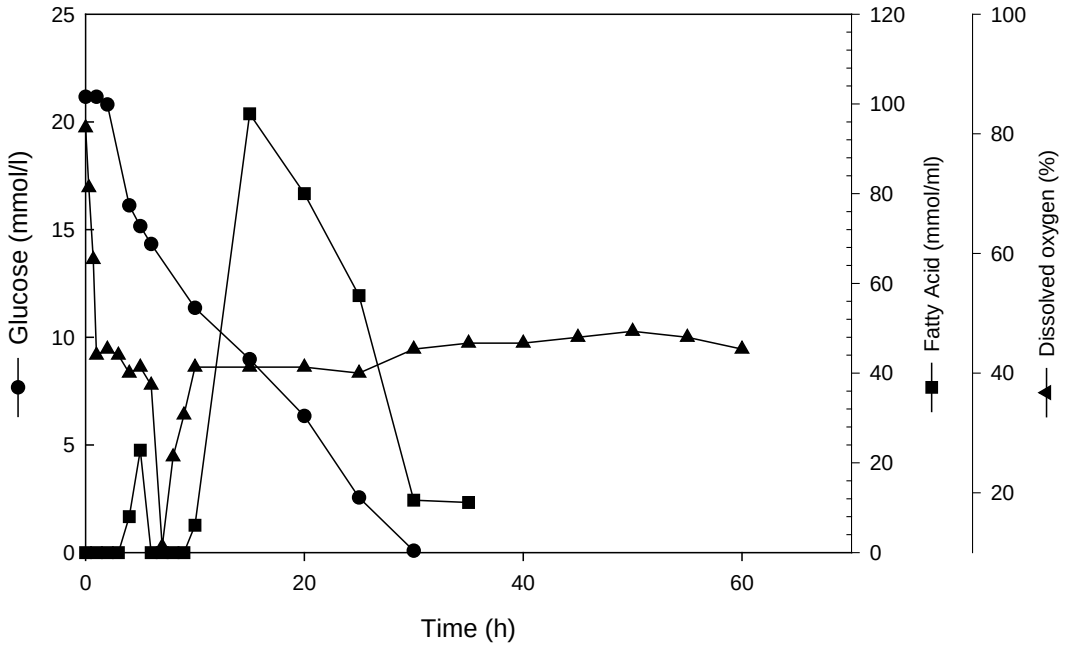


Figure 3.15 Batch culture at pH 6 highlighting difference in fatty acid and glucose consumption at lower pH. Cultivation was performed in lipase production media (section 2.2.4), with 2.5g/l olive oil.

Batch cultures performed at lower pH values (pH6) with the same media constituents showed differences in glucose and fatty acid consumption. Figure 3.15 shows the decrease in dissolved oxygen much earlier with the production of lipase commencing at a much faster rate than seen in figures 3.13 & 3.14. There is a small amount of free fatty acid accumulation at time 5 h and the consumption of glucose is more uniform in comparison to the clear two phases of glucose consumption discussed in figures 3.13 & 3.14. The drop in the dissolved oxygen tension once again coincides with the start of lipase production.

Batch cultures performed at higher pH values (pH 7.6) (Figure 3.16) show that the production of lipase was sustained for longer periods and the rate of production in early phases was much higher than usual. The rapid accumulation and slow decline of fatty acids indicates a very slow rate of fatty acid consumption. The possibility exists that the consumption of free fatty acids rather than its availability can have an influence in the quantity and stability of lipase production. The higher pH did have a slight effect on the rate of glucose consumption, but the availability and utilization of glucose did not seem to have an effect on lipase production. However the slower rate of fatty acid utilization did seem to have an effect on the time that lipase production continued.

During exponential growth the dissolved oxygen was allowed to fall from approximately 80% at inoculation to 40% during all batch cultivations. When the desired dissolved oxygen tension was obtained it was manually controlled by adjusting (increasing) stirrer as well as aeration rate. In figure 3.18 there is no secondary decrease in dissolved oxygen tension visible. Whether the secondary decrease in dissolved oxygen is due to lipase production or β-oxidation as a result of the appearance of free fatty acids is unclear. What is clear from the data described above is that the secondary decrease in dissolved oxygen (seen in figures 3.13-3.16) must have occurred during the exponential phase when olive oil was the sole carbon source. This demonstrated that a lack of utilizable carbon at the time of inoculation may prompt earlier lipase production.

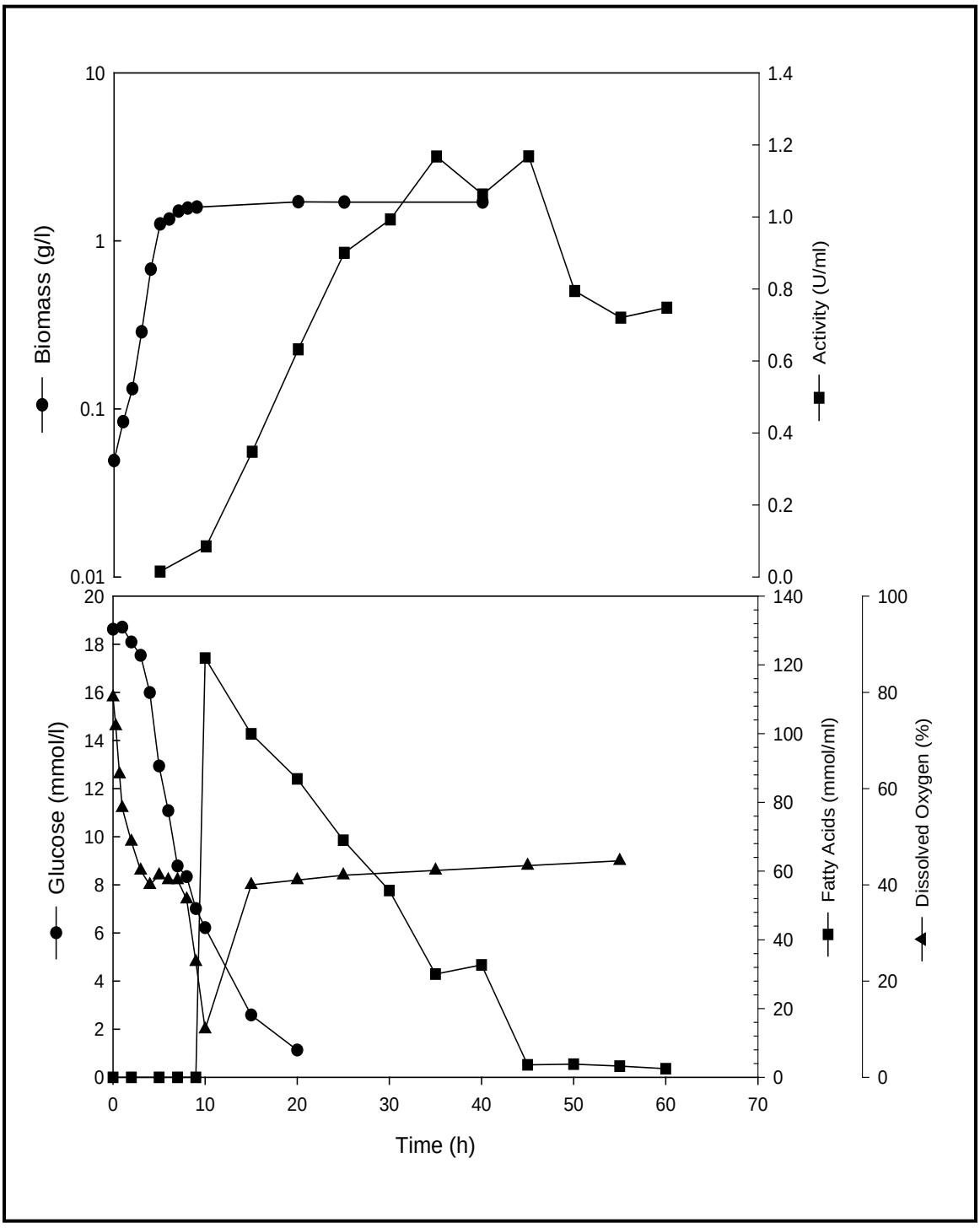

Biomass (g/l)
Activity (U/ml)
Glucose (mmol/l)
Fatty Acids (mmol/ml)
Dissolved Oxygen (%)
Time (h)
10
1
0.1
0.01
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
20
18
16
14
12
10
8
6
4
2
0
140
120
100
80
60
40
20
0
100
80
60
40
20
0
0
10
20
30
40
50
60
70

Figure 3.16 Showing the sustained production of lipase and the slower or inhibited consumption of free fatty acids at higher pH values (pH7.6). Cultivation was performed in lipase production media (section 2.2.4) with 2.5g/l olive oil as the inducer.

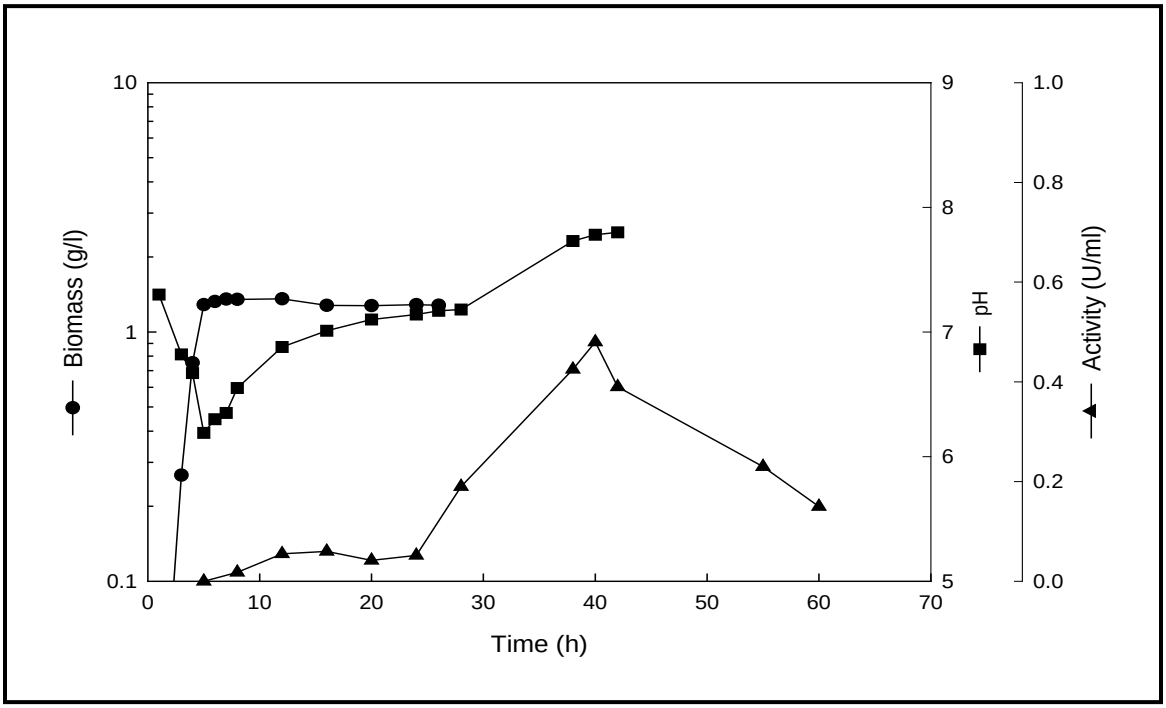
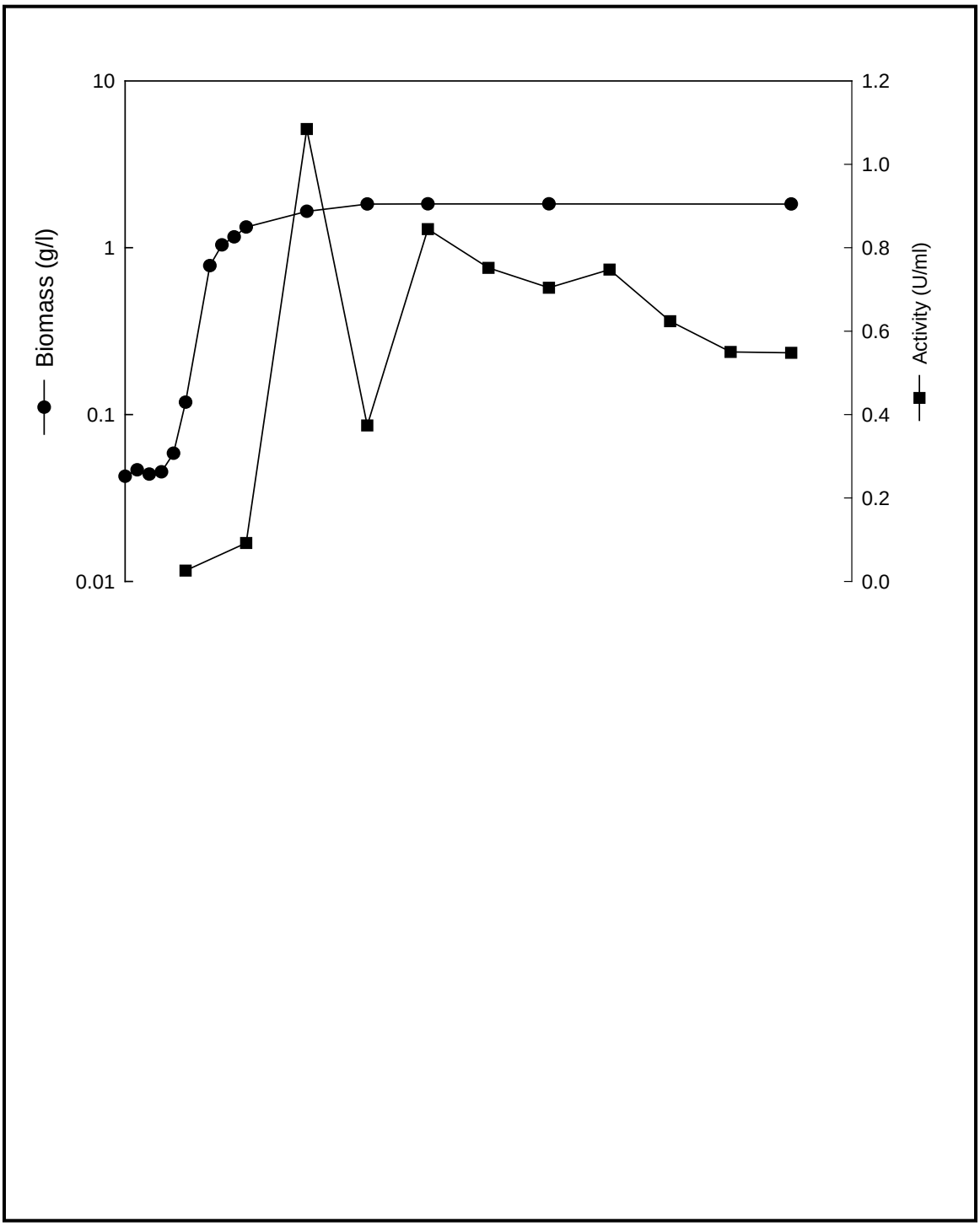


Figure 3.17 Batch culture performed in lipase production media (section 2.2.4) with no active pH control. The pH change was monitored over time.

Batch culture without titrated pH control showed a decrease in pH during exponential growth and a rise in pH at the onset of the stationary phase (Figure 3.17). A phenomenon that we cannot account for is the substantial rise in pH with the appearance of lipase and the subsequent release of free fatty acids. From optimum pH studies of growth and lipase production (Section 3.6.1, Figure 3.12) it was also clear that at higher pH growth was inhibited but lipase production continued. At lower than optimum pH for growth both growth and lipase production decreased.


10
1
0.1
0.01
Biomass (g/l)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Activity (U/ml)

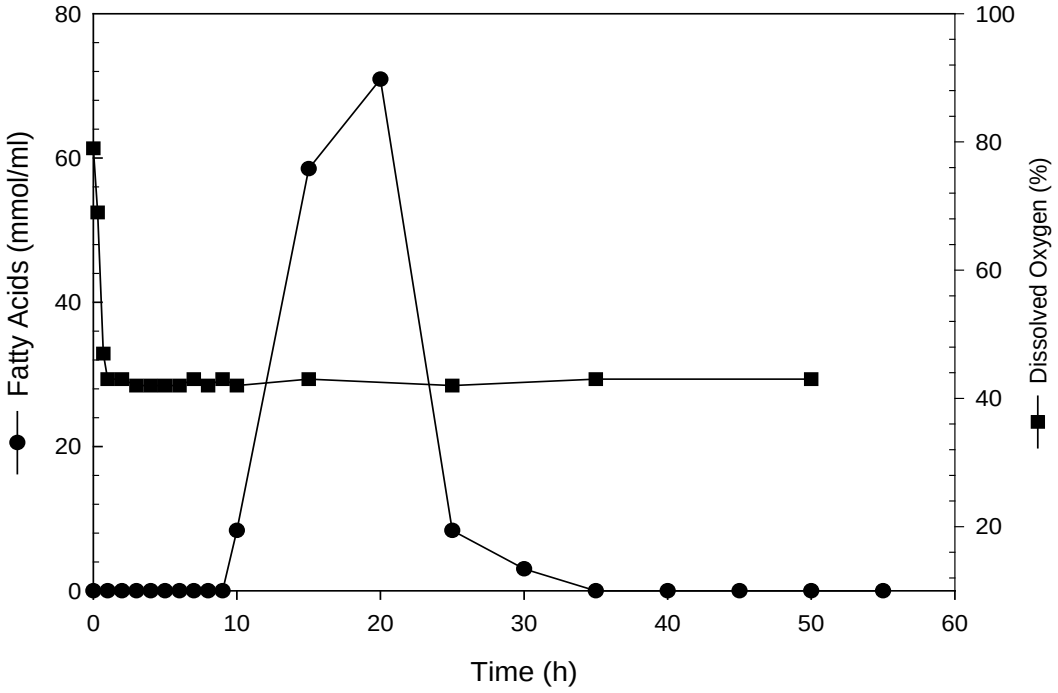


Figure 3.18 Effect of olive oil as sole carbon source on lipase production. Cultivation was performed in lipase production media (section 2.2.4) excluding glucose.

The consumption of fatty acids is faster due to the lack of other utilizable carbon sources and for this reason the production of lipase is sustained for longer periods. The amount of biomass is also comparable to that obtained with the addition of both glucose and olive oil (Figures 3.13-3.16) showing that there must be some other nutrient limitation present, or the use of carbon for pathways other than biomass formation.

Not clearly illustrated in figure 3.19 (due to the log scale used) is the slight increase in biomass from the normal 1.65 g/l to over 2g/l. Indicating that in previous batch cultures even with the addition of glucose and olive oil as carbon sources the available carbon was still the biomass limiting nutrient. In figure 3.19 the effect of higher (5g/l olive oil as opposed to the usual 2.5g/l) olive oil can be seen. Lipase activity was severely inhibited (10 fold) probably by the excess olive oil, growth however was not. A large amount of fatty acid accumulation persisted even after lipase production ceased. The fall in dissolved oxygen seen between hour 10 and 15 correlates with a small increase in biomass, possibly due to fatty acid oxidation. The large amount of fatty acid accumulation seen during later stages can be explained due to the excess olive oil, large amounts of lipase still present in the media and metabolically inactive cells incapable of free fatty acid utilization.

The effect of stearic acid as an inducer (Figure 3.20) will be discussed in greater detail in section 3.6.3.2. Note that there is no secondary fall in dissolved oxygen, again indicating that the production of lipase or β-oxidation that normally cause the fall in dissolved oxygen must have taken place during exponential growth.

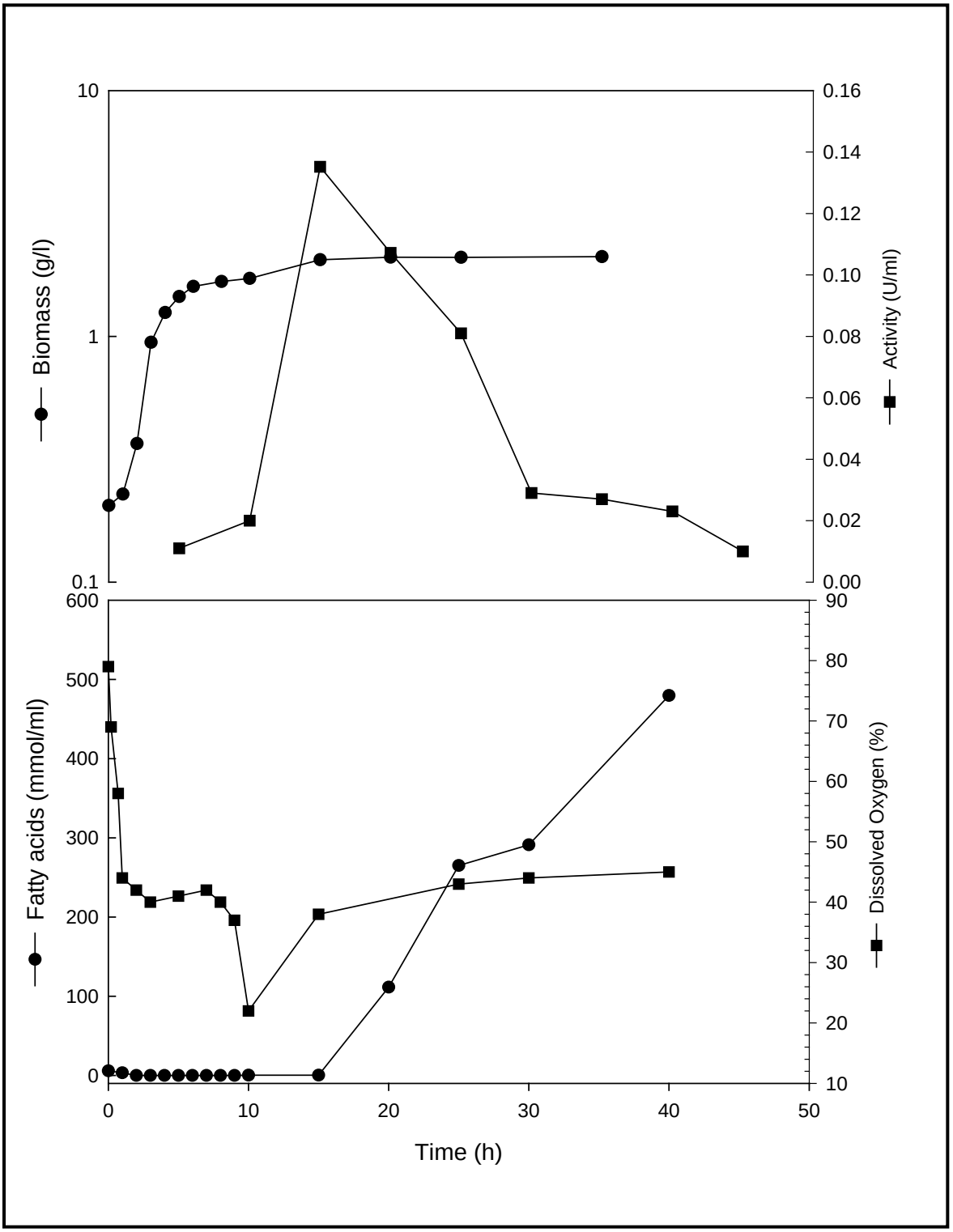

Biomass (g/l)
Activity (U/ml)
10
1
0.1
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Fatty acids (mmol/ml)
Dissolved Oxygen (%)
600
500
400
300
200
100
0
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
Time (h)

Figure 3.19 The effect of higher concentrations of olive oil (5g/l) as the sole carbon source. Cultivation was performed in lipase production media (section 2.2.4) excluding glucose.

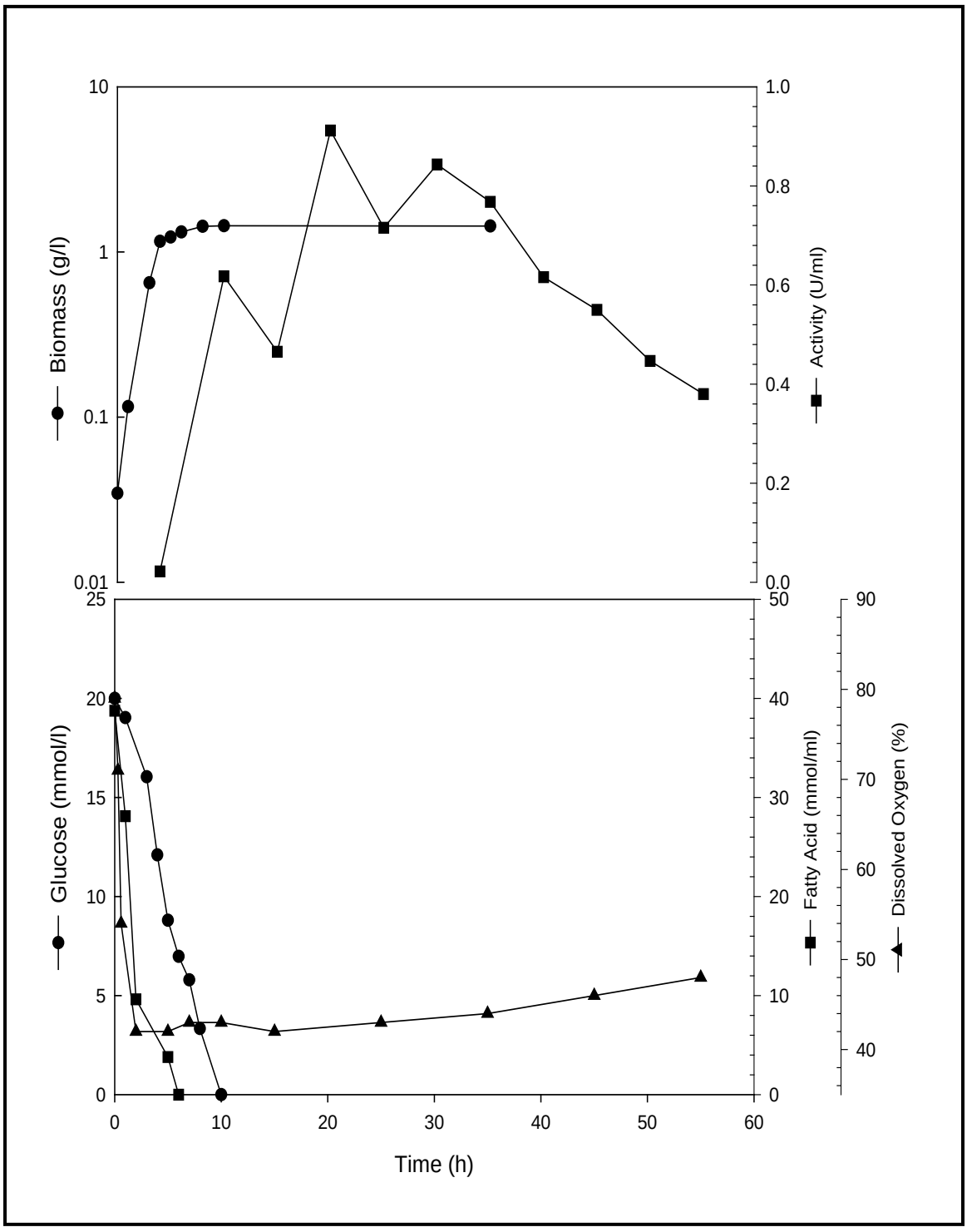


Figure 3.20 Batch culture of induction with stearic acid showing the simultaneous consumption of glucose and stearic acid. Cultivation was performed in lipase production media (section 2.2.4) with an initial 40mM stearic acid.

3.6.3. Effect of the Carbon Source

3.6.3.1 Inducer as Sole Carbon Source

The addition of glucose as an added (2^{nd}) carbon source did not have any noticeable effect on growth rate or biomass production, but the lack of utilizable carbon (when glucose was absent) during early stages of growth did have an effect on the speed at which lipase production commenced after inoculation (Figure 3.21). There were almost always two (or multiple) peaks of lipase activity present.

The inoculum size for these two experiments unfortunately differed. A longer lag phase was seen when glucose was absent indicating the organism's need for utilizable carbon during early stages of growth. This need for utilizable carbon most probably prompted the much earlier production of lipase when glucose was absent, rather then the theory that the presence of glucose represses lipase production.

Production rates of lipase and free fatty acid release and utilization will also be discussed in further detail in section 3.7.2 using comparative data compiled in Table 3.2

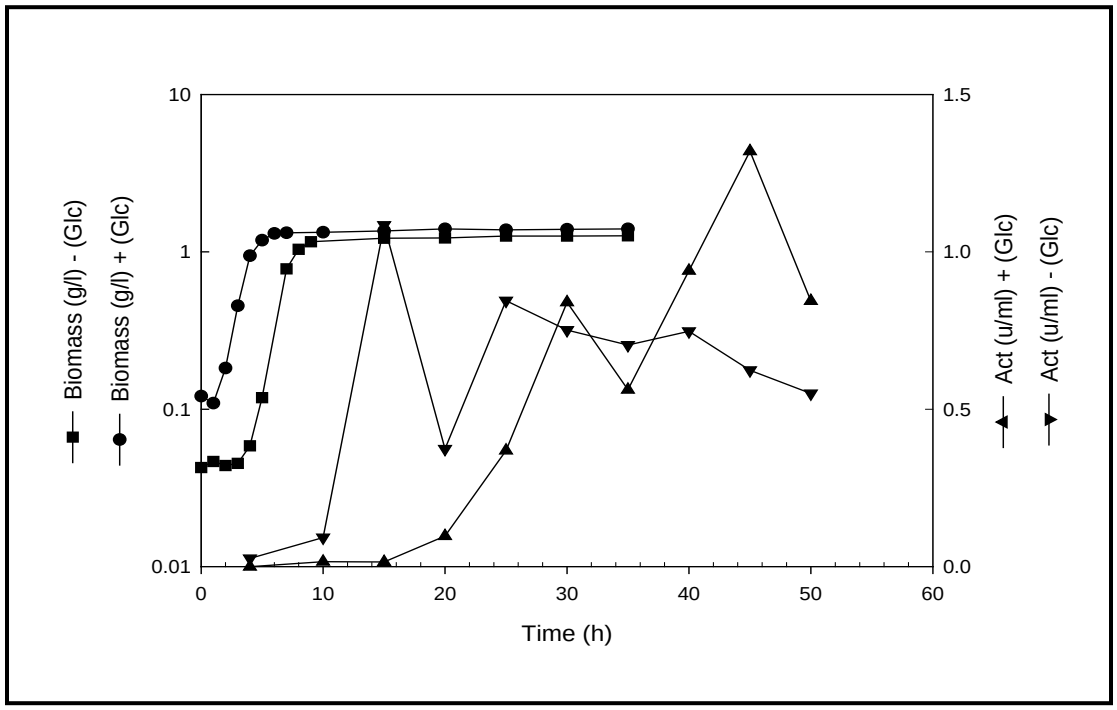


Figure 3.21 Comparison between growth and lipase activity in lipase production media (section 2.2.4) with and without the addition of glucose as a second carbon source. Olive oil (2.5 g/l) was used as the inducer.

3.6.3.2. Induction by Fatty Acid

To test whether free fatty acids induce lipase activity of *G. thermoleovorans* we used stearic acid instead of oleic acid (the major product of olive oil hydrolysis). Oleic acid is subject to oxidation due to the presence of its double bond. Stearic acid is a solid (melting point 69ºC) and oleic acid is liquid at room temperature.

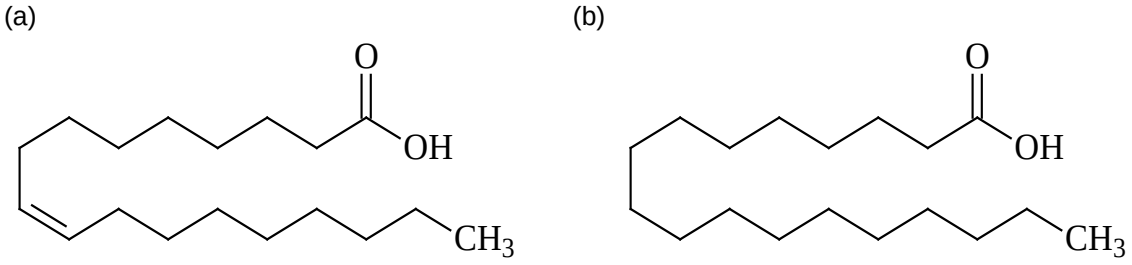


Figure 3.22 The structural differences between oleic acid (a) and stearic acid (b)

Stearic acid proved to be an effective inducer of lipase activity as seen in figure 3.23 with the production of lipase peaking much earlier (10h) in comparison to 15-30 hours obtained with olive oil in section 3.6.3.1. This again supports the idea that the fatty acids rather than the presence of olive oil acts as the signal for lipase production.

The utilization of stearic acid and glucose (Figure 3.24) are simultaneous and the amount of biomass formed is comparable to that obtained with olive oil as inducer (sole carbon-source), even though the amounts of inducers added were not comparable (2.5g/l olive oil and 40uM stearic acid). The rate of stearic acid consumption was found to be higher than that of glucose (Table 3.2) making it clear that its consumption seems to be stimulated by the presence of glucose.

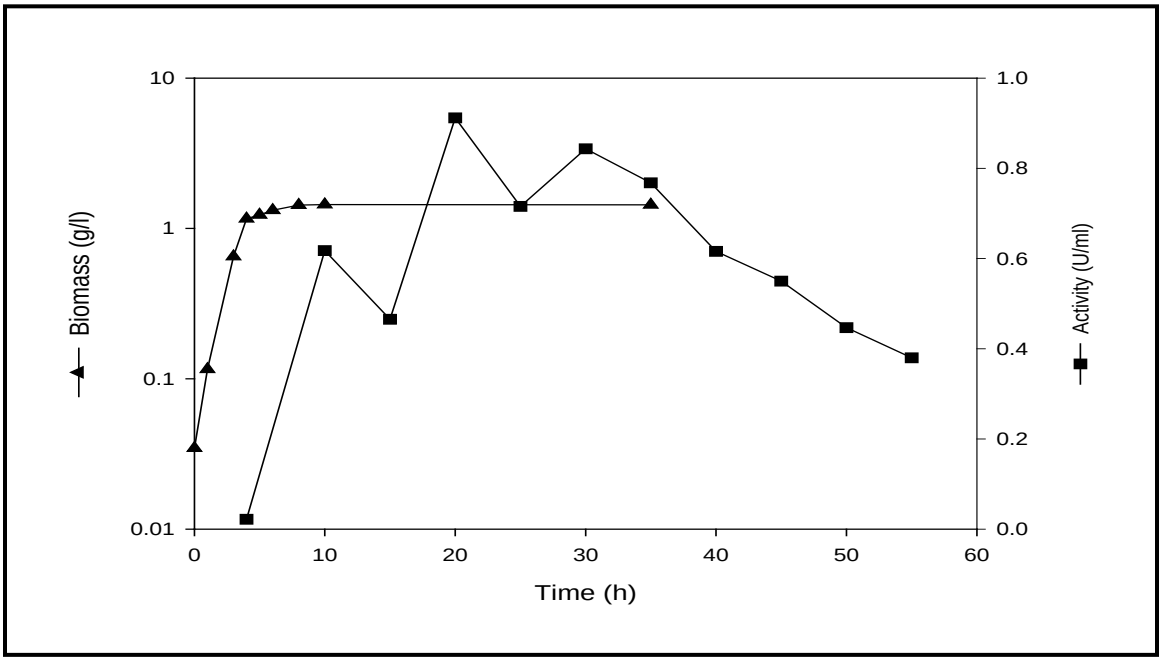


Figure 3.23 Biomass formation and lipase activity in lipase production media (section 2.2.4) with stearic acid as the inducer and glucose as the second added carbon source.

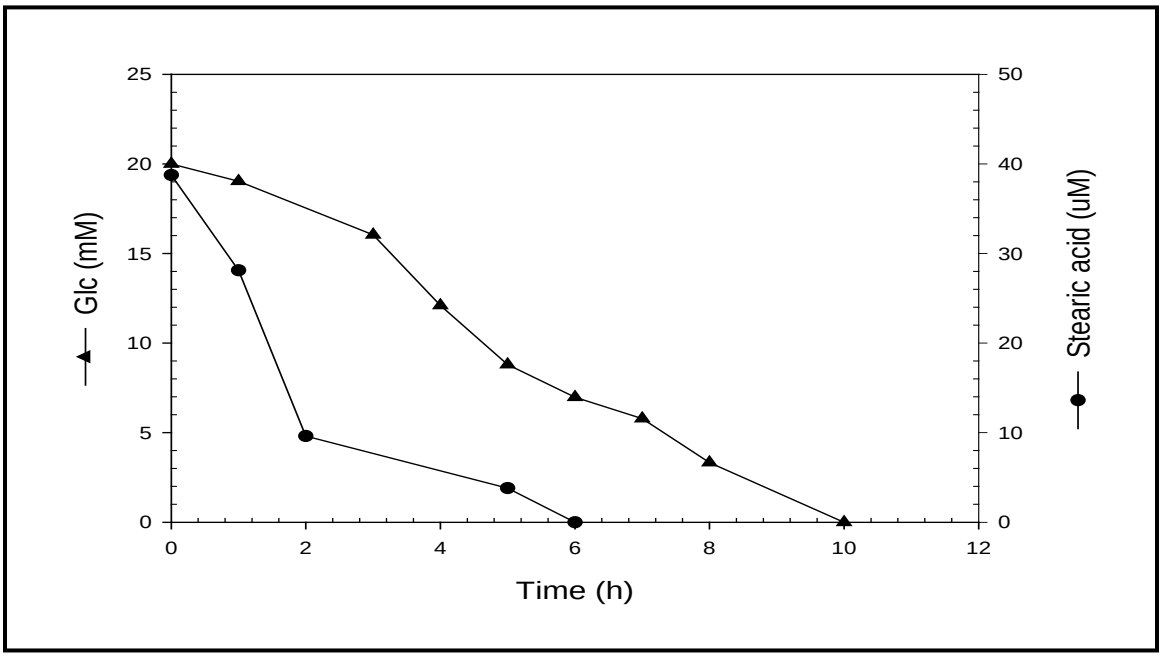


Figure 3.24 The simultaneous consumption of glucose and stearic acid as carbon sources during stearic acid induction (figure 3.23) of lipase production.

Importantly at time 6h there are no fatty acids present in the medium but the lipase activity only starts to decline after 30hours. Therefore the presence of glucose does not repress the start of lipase production; neither does the continued presence of free fatty acid seem to be necessary for continued lipase production.

3.7. Optimizing Lipase Production

3.7.1. Concentration of Medium Constituents

From the factorial-like design and *Klebsiella aeroginosa* yield coefficients (section 2.2.3.1) used to design the lipase production media it was clear that not only carbon, nitrogen and inducer source, but also the concentration of each plays a role in the physiology of lipase production. An enhancing effect of NaCl on the amount of lipase activity obtained was reported by Tan *et al.* (1988). Whether the NaCl enhances the production or merely acts as a stabilizer for the enzyme was however not clear.

The following experiments were performed in shake flasks as described in section 2.2.3.2. This was the final procedure in optimizing conditions for lipase production. As always, monitoring biomass during shake flask cultivations proved to be problematic. These experiments therefore focused only on increasing lipase yield. If this increase in yield was only due to an increase in biomass, the final batch culture with optimized concentrations was designed to show that conclusively.

3.7.1.1. Effect of Glucose Concentration

Higher concentrations of glucose yielded linearly higher activity; this could be due to an increase in biomass if glucose (available carbon) was the limiting factor. Unfortunately the experimental work was performed in shake flasks and biomass could not be determined.

According to the yield coefficients of *Klebsiella aeroginosa* used for the preliminary design of the lipase production media 5g/l glucose should be slightly limiting. The linear increase in lipase activity was only up to 7.5 g/l, this is either an inhibitory effect of the higher glucose concentration on lipase production, or due to no further increase in biomass due to other nutrient limitations.

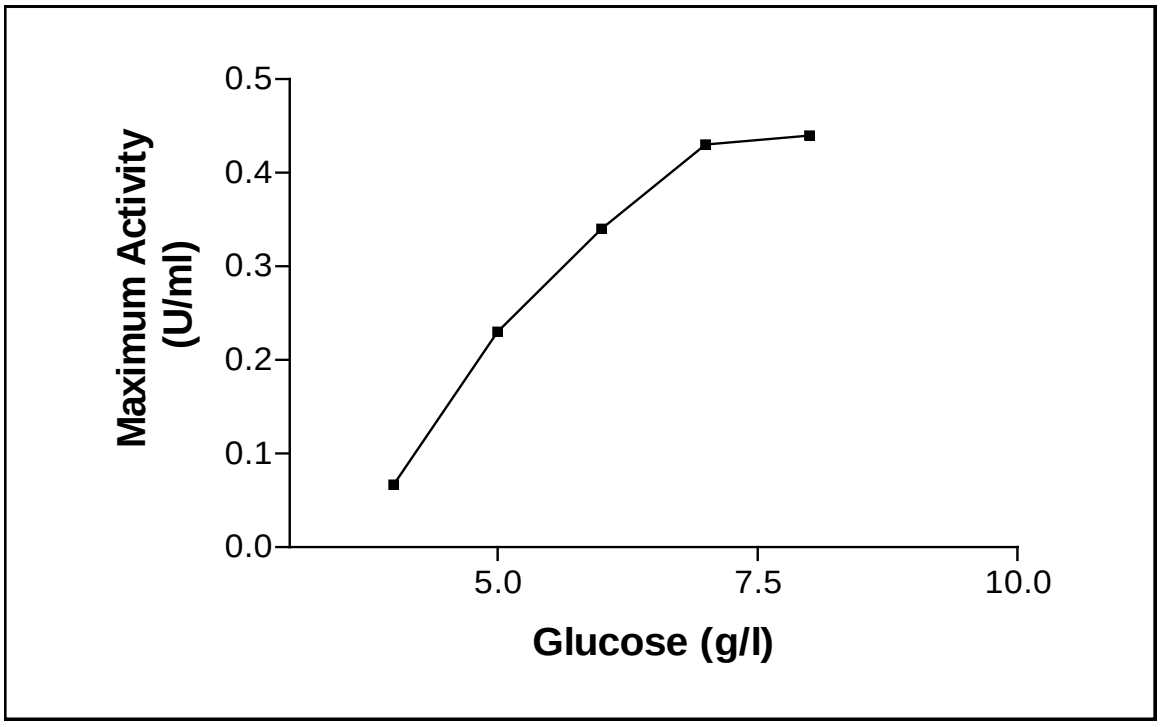


Figure 3.25 Graph of maximum lipase activity obtained in shake flasks in lipase production media (section 2.2.4) with olive oil as the inducer. Glucose concentrations varied according to section 2.2.3.2.

3.7.1.2. Effect of Protease Peptone Concentration

Increase in protease peptone concentration also caused an increase in the amount of lipase activity, possibly also due to an increase in biomass if peptone (available nitrogen) was the growth limiting nutrient.

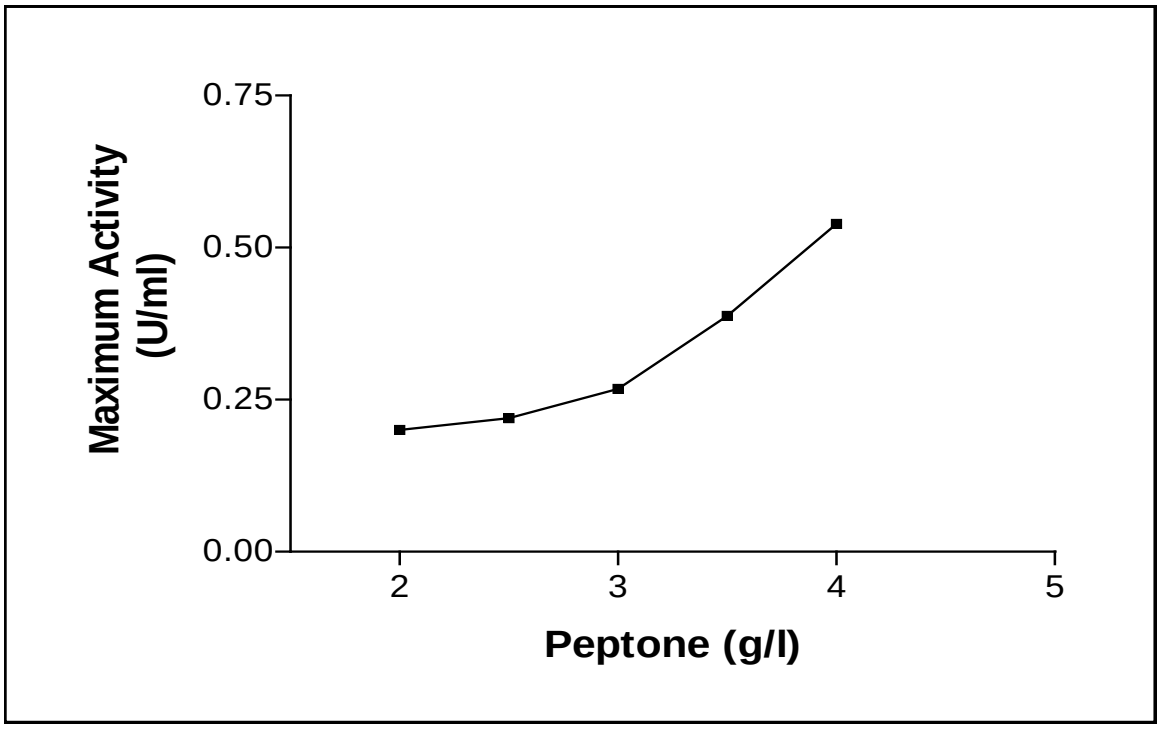


Figure 3.26 Graph of maximum lipase activity obtained in shake flasks in lipase production media (section 2.2.4) with olive oil as the inducer. Peptone concentrations altered according to section 2.2.3.2.

3.7.1.3. Effect of NaCl Concentration

The enhancing effect of NaCl reported by Tan *et al*. (1988) was also seen in *Geobacillus thermoleovorans* with an increase in lipase activity up to a NaCl concentration 3.75 g/l, with a subsequent decrease at 4.25 g/l possibly due to inhibition of growth. Due to the increasing salt concentrations, the lag time of the cultures also increased. (Data not shown due to problems encountered with monitoring growth in shake flasks).

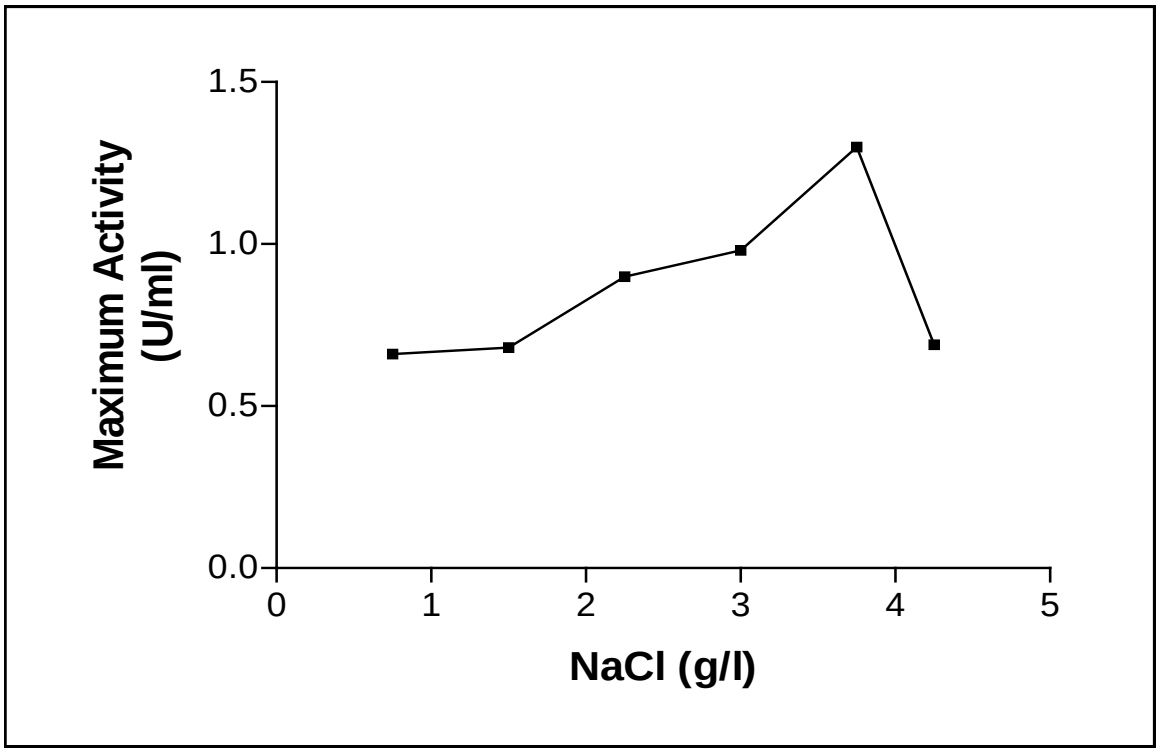


Figure 3.27 Graph of maximum lipase activity obtained in shake flasks in lipase production media (section 2.2.4) with olive oil as the inducer. NaCl concentrations altered according to section 2.2.3.2.

3.7.1.4. Inducers

3.1.7.4(a) Effect of Stearic Acid Concentration

An increase in lipase activity was seen with increasing stearic acid concentrations. During preliminary studies the lipase production regularly commenced during mid stationary phase. Induction with lower final concentrations of stearic acid showed that the presence of free fatty acids induced lipase production even during exponential growth.

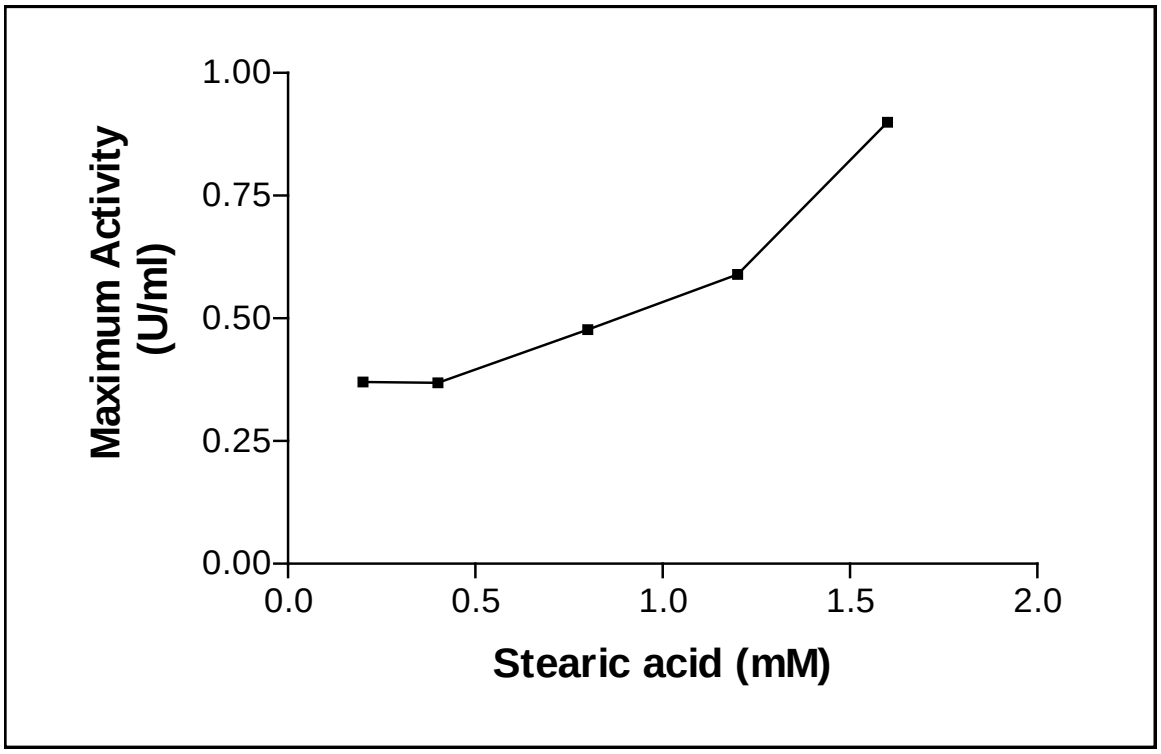


Figure 3.28 Graph of maximum lipase activity obtained in shake flasks in lipase production media (section 2.2.4) with olive oil as the inducer. Stearic acid concentrations altered according to section 2.2.3.2.

3.7.1.4(b) Effect of Olive Oil Concentration

No clear pattern emerged with increasing olive oil concentrations as inducer. Olive oil is insoluble and forms a second phase for a major part of the incubation. The different amounts of olive oil could lead to different amounts of emulsification as the enzyme is slowly released and leads to variable results.

Importantly, the maximum activities with stearic acid as inducer were slightly higher and experimentally more reproducible than that obtained with olive oil.

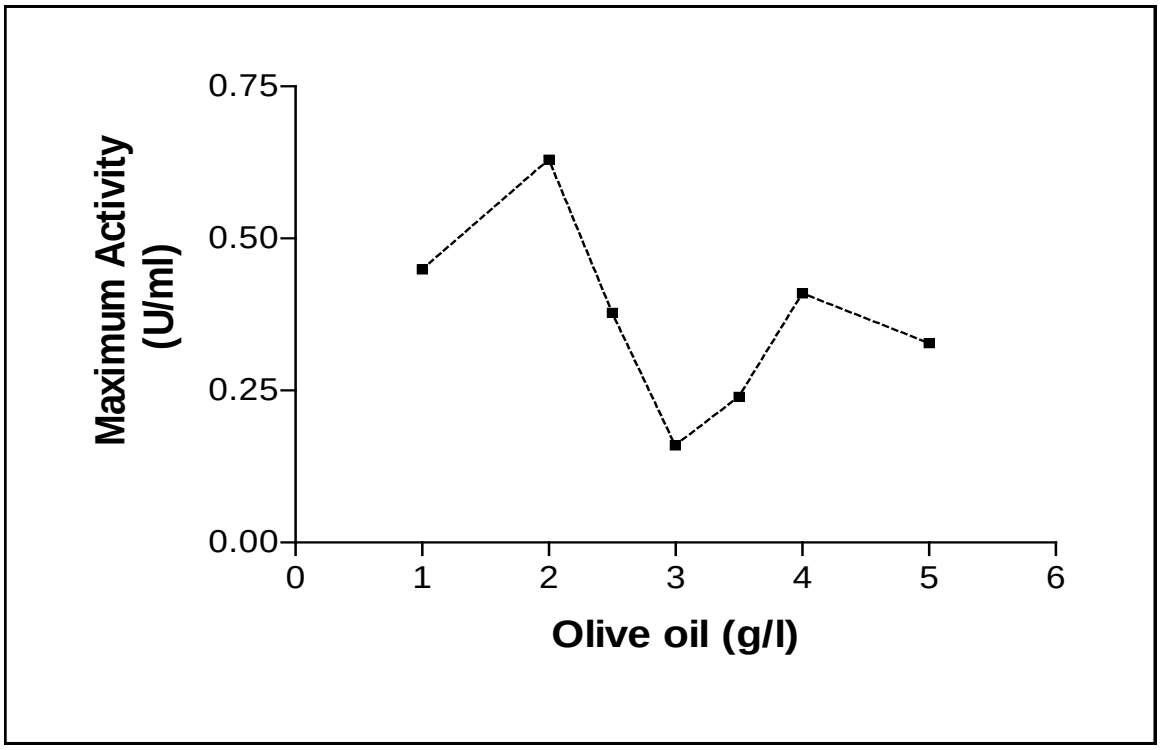


Figure 3.29 Graph of maximum lipase activity obtained in shake flasks in lipase production media (section 2.2.4) with olive oil as the inducer. Olive oil (inducer) concentrations altered according to section 2.2.3.2.

3.7.2. Optimum Batch Culture

The final optimum batch culture was performed with the base medium composition with just the alterations for glucose, protease peptone, stearic acid with the extra addition of NaCl, at optimum determined concentrations (As obtained from concentration optimization experimental work (section 3.7.1) and specified in section 2.2.5.)

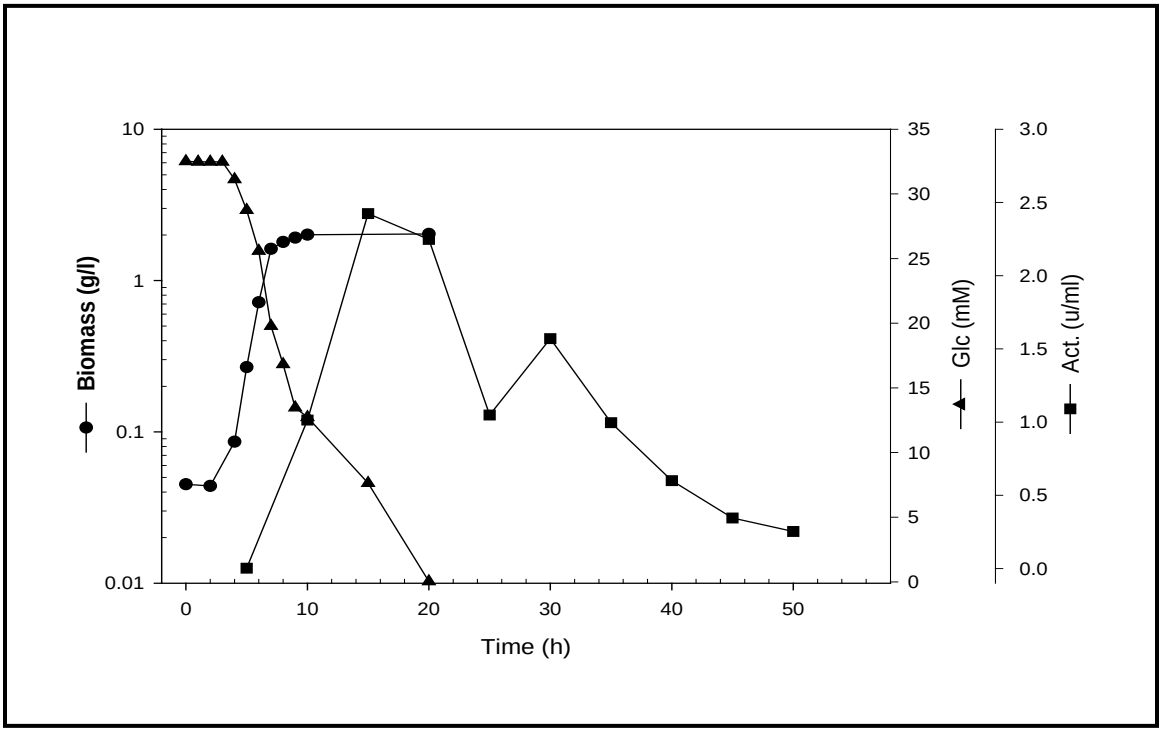


Figure 3.30 Batch growth of *Geobacillus thermoleovorans* in final optimized medium for maximum lipase production as described in section 2.2.5 obtained from experimental work performed in section 3.7.1.

The biomass and lipase yield was substantially higher, proving that neither the available nitrogen nor carbon was the growth limiting factor and that either is an inhibitor of lipase production. The plot does not show the utilization of the stearic acid (inducer) due to problems encountered with the fatty acid analysis possibly due to the addition of NaCl.

The slightly longer lag phase observed is probably due to the higher NaCl concentrations or possibly even due to the higher stearic acid concentrations.

This experiment demonstrated again that the presence of fatty acid at the point of inoculation caused a much faster lipolytic response then olive oil. In the cases where olive oil was used, the lipase activity began only after the culture had reached stationary phase and free fatty acids had started to accumulate. In this

final batch growth experiment, lipase activity began during the exponential growth phase and at glucose concentrations of more than 10 mM.

Table 3.2 Table showing comparative batch data highlighting the differences in growth, enzyme production and carbon utilization with different carbon sources and combinations (n/a (Red); Not available data). Highlighted in blue is not relevant data (n/r) as these components were not present in the media.

Parameter	**+Glc (Olive oil)**	**-Glc (Olive oil)**	**+Glc (Stearic acid)**	**Optimum Conditions**
X Max (g/l)	1.44	1.33	1.44	2.03
μMax (1/h)	0.82	0.87	0.88	1.06
Y x/glc	0.06	n/a	0.07	0.12
Y x/Stearic acid	n/r	n/r	0.12	n/a
Q Glucose util. (g/l/h)	0.21	n/r	2.14	3.2
Q Oleic acid form. (mmol/l/h)	27.68	19.33	n/r	n/r
Q Oleic acid util. (mmol/l/h)	10.26	6.25	n/r	n/r
Q Stearic acid util. (mmol/l/h)	n/r	n/r	4.14	n/a
Q Enz.prod. (U/ml/h)	0.04	0.04	0.03	0.24
Max act. (U/ml)	1.3	1.08	0.84	2.4

In most cases Delmau *et al.* (2000) found that mixed carbon sources only improved biomass yield and did not improve on lipase yields obtained when lipid substrates were used as the sole carbon source. Table 3.2 shows that the difference in the amount of biomass formed with or without the addition of glucose as a second carbon source is negligible. The optimized conditions did however yield higher total biomass proving that either the available carbon or nitrogen was the growth limiting nutrient.

The maximum specific growth rates also compared well with a slightly higher rate obtained at optimum conditions, probably due to the higher rate of glucose uptake. The presence NaCl seemed to enhance the bacterium's affinity for glucose due to the slightly higher yield obtained when NaCl was present.

The lipase production rate with or without the addition of glucose as a second carbon source also compared well and highlighted the fact that the presence of glucose is not an inhibitor of lipase production.

When olive oil was used as an inducer the specific rates of oleic acid (the major product of olive oil hydrolysis) formation and utilization differed substantially as a result of the presence or absence of glucose. Both the rate of utilization and formation was higher when glucose was present highlighting the organism's affinity for large amounts of utilizable carbon.

The rate of enzyme production also increased with the increase of growth rate and biomass found at optimum conditions. This means that contrary to a report sighted in literature, a semi-starved state and carbon or nitrogen limitation (Suzuki *et al.* 1988) is not preferential to lipase production of *Geobacillus thermoleovorans.* There was an increase in biomass of approximately 25% with the optimum conditions as opposed to the other tested conditions. On average however there was a 100% increase in lipase production indicating that the increase in lipase production was not a stepwise increase due to higher biomass yield.

Chapter 4
Conclusions

1. The PCR (Polymerase chain reaction) performed to amplify the 16s rDNA region of the *Geobacillus thermoleovorans* strain and the subsequent sequencing thereof proved to be a useful and accurate method of verifying the identity of this bacterial strain. Even though only 60% of the complete gene sequence (yielding a 99% identity) was obtained, the additional presence of a single *Eco*R1 site at a specific position in a part of the gene which we did not sequence, identified our bacterial strain as *Geobacillus thermoleovorans* T-80.

2. The electron microscopy data showed the accumulation of lipid material intracellularly. This took place at or close to the onset of lipase production and the liberation and utilization of free fatty acids. There is a question of whether free fatty acids are utilized via β-oxidation or are merely intracellularly accumulated in lipid inclusion bodies, but we do not have sufficient evidence to clarify this.

3. The design of the lipase production media and its further optimization was successful in improving the lipase production obtained in R2A media by approximately 30-fold. *Geobacillus thermoleovorans* was found to require rich sources of organic nitrogen (Peptone) not only for lipase production but also for growth. This raised the question as to how this bacterium

survived in an environment where the major nutrient sources were of mineral origin with little to no organic nitrogen present.

4. The problems encountered with monitoring growth were largely overcome by monitoring cellular ATP and indirect biomass estimation via cellular protein determination. Unfortunately the ATP Luciferase was inhibited by free fatty acids and the possibility that large amounts of ATP could be released by β-oxidation of fatty acids could not be confirmed.

5. Optimum temperature for lipase production was found to be 10ºC lower than the optimum growth temperature, with the optimum pH for growth and lipase production found to be identical (pH 6.5).

6. The oxygen demand was not studied in detail but the organism's need for high rates of aeration was highlighted during growth studies at different temperatures where growth rates were low and linear instead of exponential, due to low rates of aeration.

7. The dissolved oxygen tension during bioreactor cultivation could be used as a signal for the commencement of lipase production. Whether this second phase of oxygen consumption is due to the production of lipase or the oxidation of free fatty acids via β-oxidation is not clear.

8. The pH value of the medium had a marked effect on the consumption of free fatty acids liberated by enzymatic activity. The possibility that not only the presence but also the rate of utilization of fatty acids plays an important role in the production of lipase exists.

9. Different concentrations of non-soluble lipids (specifically olive oil) in the aqueous environment of the culture media had variable effects on lipase

production, probably due to the uneven aggregation and inconsistent emulsification of the lipid.

10. When the enzyme inducer olive oil was used as the sole carbon source it showed that the appearance of putative cellular signals for the commencement of lipase production speeds up probably due to the lack of utilizable carbon at inoculation.

11. During induction of enzyme activity by stearic acid the presence of free fatty acids rather than the nutrient limitations at stationary growth phase was shown to be the signal for lipase production. The presence of glucose together with stearic acid had no effect on lipase production, proving that glucose is not an inhibitor of the inducible lipase of *Geobacillus thermoleovorans*

12. The optimum conditions for lipase production, which consisted of increased concentrations of carbon (glucose), nitrogen (proteose peptone), fatty acid (stearic acid) and inclusion of NaCl, increased the formed biomass by 25%, with a 100% increase in lipase yield. This proved that the presence of specific nutrients is not only necessary for the formation of biomass but also for the production of lipase.

13. We propose that for lipase production, sufficient biomass must be accumulated, which can be achieved by providing a rich medium for growth. The primary signal for lipase release appears to be the presence of free fatty acids in the media. We propose that the organism produces a basal level of lipase which will release small amounts of fatty acids from a suitable lipid substrate, if present. The small amounts of free fatty acids released then provide the signal for high rate of enzyme production, possibly by activating a lipase gene, which leads to a rapid release of fatty

acids from the substrate with subsequent uptake by the organism. These lipids can be stored or utilized as an energy source.

14. Further experiments should include determining the signaling pathway involved and the possibility of increased lipase gene transcription as a result of a free fatty acid signal.

Summary

The aim of this study was to elucidate factors, specifically media composition and growth conditions that would improve the thermophilic lipase production by *Geobacillus thermoleovorans*.

A semi-starved state where carbon and or nitrogen becomes limiting was found not to be conducive to optimum lipase production. Instead, the notion that lipase activity peaks at these limiting conditions, normally during stationary phase was disproved as we could get a 25% increase in biomass obtained under optimized conditions, yielding a 100% increase in lipase production. This showed that the availability of specific nutrients has a direct positive effect on the amount of enzyme produced. The occurrence of free fatty acids (products of lipolysis) under these conditions was found to be the main signal for the commencement of lipase production. This was shown by the almost immediate production of lipase once free fatty acids were present at the initial point of inoculation (stearic acid induction), showing that the presence of free fatty acids does not repress the production of inducible lipases by *Geobacillus thermoleovorans* but that it rather acts as a signal for lipase induction.

The presence of glucose as a second utilizable carbon source at inoculation was not inhibitory to lipase production. The consumption of glucose and stearic acid occurred simultaneously, with the consumption of free fatty acids being enhanced by the presence of glucose.

The pH of the media also had a noticeable effect on the physiology surrounding lipase production, specifically effecting the rate of uptake of free fatty acids. This indicated that not only the presence but also the rate of uptake of free fatty acids probably plays a direct role in the regulation and/or secretion of lipases.

The optimum temperature for lipase production and the optimum for growth differed. The optimum temperature for lipase production was found to be 55ºC with little or no lipase production occurring at 65ºC, which is the optimum growth temperature.

The addition of NaCl increased lipase yield and/or activity further. It is however not clear whether the presence of NaCl has an effect on lipase production or merely acts as a stabilizer of lipase activity.

We propose that the presence of free fatty acids in the culture media is the primary signal for lipase production, possibly by inducing the transcription of an inducible lipase gene.

Opsomming

Die doel van hierdie studie was om die faktore, meer spesifiek die kwekingsmedium samestelling en groei kondisies te onder soek wat die maksimale produksie van die termofiele lipase van *Geobacillus thermoleovorans* sal lewer.

Dis is gevind dat `n semi-uitgehongerde toestand waar koolstof en stikstof beperkend is nie optimale produksie van lipase bevorder nie. Die opvatting dat lipase poduksie gewoonlik tydens die stasionêre groeifase geskied is as verkeerd bewys. ‘n Verhoging van 25% in biomassaopbrengs is gekry onder die optimum toestande met `n 100% verhoging in die totale lipase opbrengs. Dit dui daarop dat die beskikbaarheid van spesifieke media komponente `n direkte, positiewe invloed het op die hoeveelheid ensiem wat geproduseer word. Die teenwoordigheid van vrye vetsure (die produk van lipolise) was as die hoofoorsaak van lipase produksie geïdentifiseer. Daar is aangetoon dat daar ‘n byna onmiddelike produksie van lipase plaasvind as vrye vetsure by die aanvang van groei teenwoordig was (stariensuur induksie). Ons kon ook aandui dat die teenwoordigheid van vrye vetsure nie `n onderdrukker van lipase produksie deur *Geobacillus thermoleovorans* is nie.

Daar is ook aangedui dat die aanwesigheid van glukose as `n tweede koolstofbron by die aanvang van groei nie `n onderdrukker van lipase produksie blyk te wees nie. Glukose en stariensuur is gelyktydig verbruik en die teenwoordigheid van glukose het die opname van stariensuur versnel.

Die pH van die kweking het ook `n effek gehad op die fisiologie van lipase produksie, meer spesifiek op die verbruik van vrye vetsure. Daar is aangetoon dat die teenwoordigheid van vetsure asook hulle verbruik `n direkte rol speel in die regulering van lipase produksie of -sekresie.

Die optimum temperatuur vir lipase produksie en die optimum temperatuur vir groei het verskil. Vir lipase produksie was die optimum temperatuur 55ºC, met min of geen lipase produksie by 65ºC, wat die optimum vir groei is.

Die bevoeging van NaCl het die lipase opbrengs en/of aktiwiteit verder verhoog. Dit is nie seker of die teenwoordigheid van NaCl `n effek het op die produksie van die lipase en of dit net optree as `n stabiliseerder van lipase aktiwiteit nie.

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