A kinetic and molecular study of the lipase from *Geobacillus thermoleovorans* GE-7

by

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Submitted in fulfilment of the requirements
for the degree of

Philosophiae Doctor

in the

Department of Biotechnology
Faculty of Science
University of the Free State
Bloemfontein
Republic of South Africa

June 2005

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ACKNOWLEDGEMENTS

I wish to express my gratitude to:

Prof. D. Litthauer, for his invaluable guidance, constructive criticism and patience. You taught me many invaluable lessons, both personal and professional, and you will never be forgotten.

Dr. Esta van Heerded, thank you for 6 wonderful years of friendship and patience. You will always have a special place in my heart.

Dr. Robert Verger and Dr. Frédéric Carriére and all the members at the “Centre National de la Recherche Scientifique”, for the opportunity to work and learn from the specialists.

Dr. Lizelle Piater for her friendship and for always supporting me and listening with infinite patience to all my ideas.

All the members of the Department of Biotechnology for interest shown, smiles and making everyday an adventure, especially the fellow students from Extreme Biochemistry for making the working days seem most sociable.

Special thanks to Christelle, Jaqui, Michel, Sanet, Lalie, Walter, Olga and Koos for all the support during the years. You made the tough times seem like a breeze.

The National Research Foundation for funding of the project.

The Ersnt and Ethel Erikson Trust for the financial support.

The Oppenheimer Trust for financial help with the work done in France.

To Shaun Knoesen for his friendship and all his help with the Microbiology. I will miss the long hours talking and laughing in the lab.

To my family for their love and invaluable prayers during all my years of study. Without you I would not have made it this far.

To God, my Creator and the Creator of all life, be the glory.

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LIST OF ABBREVIATIONS

µm	micrometer
BCA	bicinchoninic acid
BSA	bovine serum albumin
Cetrimide	(hexadecyltrimethyl-ammoniumbromide)
CHAPS	3-cholamidopropyldimethyl-ammonio-1-1-propane sulfonate
cmc	critical micellar concentration
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
FA	fatty acid
g	acceleration due to gravity
h	hour
HCl	hydrochloric acid
HGL	human gastric lipase
HPL	human pancreatic lipase
IEF	isoelectric focusing
IPTG	isopropylthio-β-D-galactoside
kbar	kilobar (pressure)
kDa	kilo dalton
kJ/mol	kilojoules per mole
m	metre
min	minute
M_w	molecular weight
mM	millimolar
mN	millinewton
$(NH_4)_2SO_4$	ammonium sulphate
OD	optical density
PCR	polymerase chain reaction
pI	isoelectric point
PLRP	pancreatic lipase related protein
*p*NP	*para*-nitrophenol

*p*NPP	*para*-nitrophenol palmitate
rpm	revolutions per minute
S.I.	stereoselectivity index
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sec	second
sn-x	stereospecific numbering, where x is any position on the glycerol
TE	50 mM Tris and 10 mM EDTA buffer (pH = 7.8)
TPEN	N,N,N',N'-tetrakis(2-pyridylmethyl)ethylene-diamine
Tris	Tris(hydroxymethyl)aminomethane
U/ml	activity expressed in Units per millilitre
µg	microgram
µl	microlitre
µmole	micromole
V.I.	vicinity index
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1

Literature Review

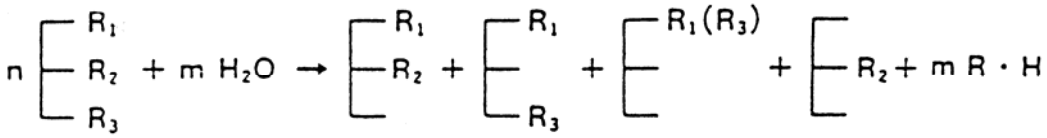
1.1 General introduction

Glycerol ester hydrolases (E.C. 3.1.1.3) or lipases are enzymes that act on the carboxyl ester bonds present in acylglycerols to liberate organic acids and glycerol (Jaeger *et al*., 1994). Lipases are physiologically important since they catalyse the hydrolysis of oils and fats to free acids and partial acylglycerols, which are essential for metabolic processes such as fatty acid transport, digestion, oxidation, and resynthesis of acylglycerols and phospholipids (Shahani, 1975). Although naturally occuring triacylglycerols are normally the preferred substrates, the enzyme can hydrolyse a wide range of insoluble fatty acid esters. It is well demonstrated that the reaction is reversible and that the enzyme can catalyse ester synthesis from various alcohols and acids and transesterification, often in nearly anhydrous organic solvents (Figure 1.1) (Kurashige *et al*., 1989).

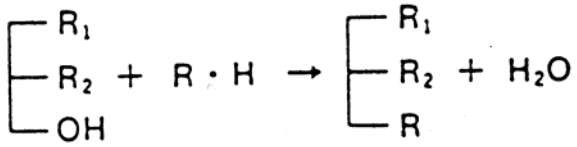
The hydrolysis reaction involves an attack on the ester bond of glycerides in the presence of water molecules to produce both an alcohol functionality and a carboxylic acid (Figure 1.1, Reaction 1). The hydrolysis of fats and oils (triacylglycerols) can be reversed by modifying the reaction conditions. The equilibrium between the forward and reverse reactions is controlled by the water content of the reaction mixture, so that in an environment with low water activity lipases catalyse ester synthesis reactions. Different types of ester synthesis can be distinguished: common ester synthesis from glycerol and fatty acids (Figure 1.1, Reaction 2) and the biotechnologically more important transesterification reactions in which the acyl donor is an ester (Figure 1.1, Reactions 3.1 – 3.3). Transesterification involving fats and oils can further be categorised depending on the type of acyl acceptor. Acidolysis refers to the exchange of acyl radicals between an ester and an acid (Figure 1.1, Reaction 3.1). Alcoholysis and

glycerolysis refer to the transfer of an acyl group from a triacylglycerol to either an alcohol or specifically, glycerol (Figure 1.1, Reaction 3.2). In interesterifications, the acyl group is exchanged between acylglycerols (Figure 1.1, Reaction 3.3).

1. Hydrolysis of ester

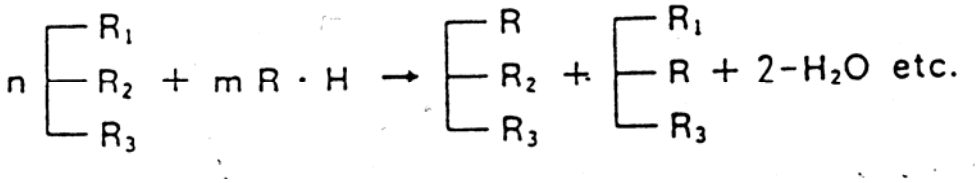


2. Synthesis of ester

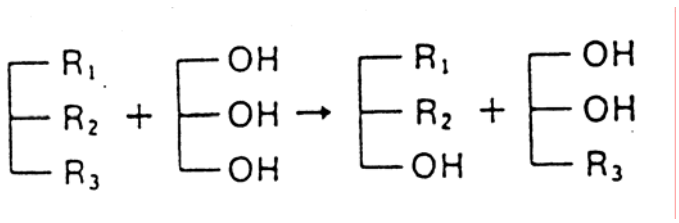


3. Transesterification

3.1. Acidolysis



3.2. Alcoholysis (Glycerolysis)



3.3. Interesterification

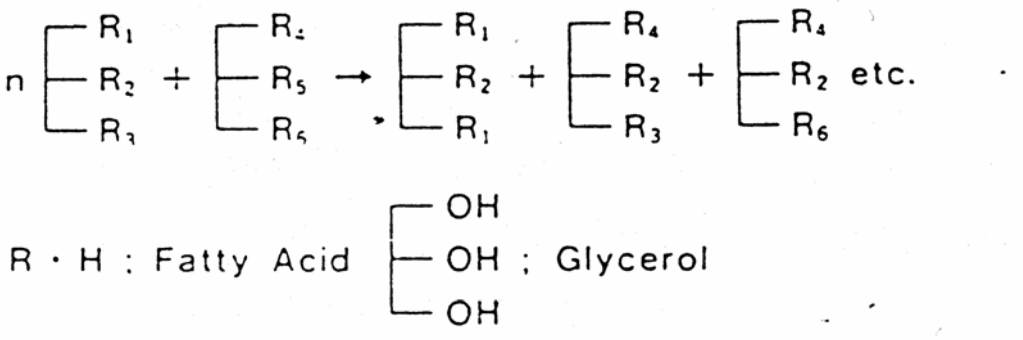


Figure 1.1: Schematic diagrams of lipase-catalysed reactions (Taken from Kurashige *et al*., 1989).

The lipase enzymes have a wide range of properties, with respect to substrate specificity, pH optimum and thermostability depending on its source. The fact that lipases remain active in organic solvents significantly broadens their biotechnological applications.

1.2 Classification by kinetics of ester hydrolysis

1.2.1 Esterases

Enzymes that hydrolyse ester bonds in general are esterases (E.C. 3.1.1.1). Esterase enzymes show normal Michaelis-Menten kinetics with respect to substrate concentration. The activity of esterase enzymes does not increase at substrate concentrations exceeding solubility (Bornsheuer, 2001).

1.2.2 Cutinases

Lipases and esterases have been found to be closely related to cutinases, enzymes that degrade the cuticle (the insoluble lipid-polyester matrix covering the surface of plants) and are capable of hydrolysing triacylglycerols. Cutinases differ from classical lipases in that they do not have “lids” covering the active site of the enzyme and they are active on both soluble and emulsified triacylglycerols (Martinez *et al*., 1992) and show no “interfacial activation” (Promper *et al*., 1999). Cutinases can be classified as true lipases since they are able to hydrolyse insoluble triglycerides. Suberinase is an esterase which hydrolyses suberin. Although there is functional similarity, cutinases and suberinases differ structurally (Derewenda *et al*., 1994).

1.2.3 Lipases

Figure 1.1 shows reactions that are catalysed by lipases. Esterase enzymes also catalyse the very same types of reactions. It thus becomes difficult to distinguish between a lipase and an esterase as these two groups of enzymes show considerable overlap in substrate specificities. However, lipases have been

characterised in kinetic terms using the “interfacial activation” phenomenon, a statement that no longer holds any ground (Beisson *et al*., 2000; Sarda and Desnuelle, 1958).

Long-chain triacylglycerols, which are the normal substrates of lipase, have hydrophobic properties. In aqueous environments, they form emulsions (lipid-water interfaces) at points of maximum concentration. By contrast, short-chain triacylglycerols posses a distinct solubility due to a higher hydrophilicity. They yield monomers at low concentrations and micelles in more concentrated solutions. It has been shown that whereas the rate of breakdown of a dilute solution of triacylglycerol by a lipase is very slow, the enzymatic activity increases dramatically once the substrate solubility is exceeded (Verger, 1980). This phenomenon was wrongly referred to as "interfacial activation" and was thought to demonstrate a fundamental difference between an esterase and a lipase based upon the presence or absence of “interfacial activation”.

1.3 Catalytic properties of lipases

Lipases have been purified from a number of sources in order to describe their catalytic properties. Properties of purified and crude forms of lipases have been described in literature. The properties of interest included substrate (positional, fatty acid, glyceride) specificities, effects of metals and detergents.

1.3.1 Substrate specificity

The glycerol molecule as the basic building block of the lipase substrate triacylglycerols contains two primary and one secondary hydroxyl groups. Although the molecule has plane of symmetry, the two primary groups are sterically distinct. Substitution of these hydroxyl groups with two different substituents leads to optically active derivatives. In a generally adopted nomenclature (IUPAC-IUB Commission on Biochemical Nomenclature), glycerol is written in a Fischer projection with the secondary hydroxyl group to the left, and the carbon atoms numbered *sn*-1,2 and 3 from top to bottom, thereby allowing the

unambiguous description of isomeric glycerides. The substrate specificity of a lipase is defined by its potential specificity, its preference for longer or shorter-chain, saturated or unsaturated acids or by its stereospecificity (Sanz and Olias, 1990).

1.3.2 Positional specificity and stereospecificity

Several groups have reported on positional selectivity of microbial lipases. Omar *et al*. (1987) reported that the lipase of *Humicola lanuginosa* has a *sn*-1,3 positional specificity and Sugihara *et al*. (1991) reported that the lipase of a *Bacillus* specie also has a *sn*-1,3 positional specificity. Studies done on lipase from *Geobacillus* species indicated a similar preference for the sn-1,3 position (Schmidt-Dannert *et al*., 1994; Rua *et al*., 1997; Dharmsthiti and Luchai, 1999; Lee *et al*., 2001). Several other bacterial lipases were depicted as *sn*-1,3 positional specific (Okeke and Gugnani, 1989; Muderhwa *et al*., 1986).

Sztajer *et al*. (1992) however felt that the lipase from the fungus *Penicillium simplissimum* was positionally non-specific which meant that this lipase hydrolyses any of the three bonds of the triacylglycerols. Similar results were reported by Lee *et al*. (2001), showing the lipase BTID-B from *Bacillus thermoleovorans* ID-1 as being positionally non-specific. The lipase from *Geotrichum candidum* (Sugihara *et al*. 1993) and the *Geotrichum* sp. FO401B lipase C (Ota *et al*., 2000) were reported to have a preference for the *sn*-2 position on a triacylglyceride molecule. These positional specificities were all determined with the thin layer chromatography (TLC) technique using a variety of substrates. The problem associated with these lipid-water emulsion experiments is that the interphase is ill defined and that acyl migration in aqueous media can make interpretation of the data difficult (Schmidt-Dannert *et al*., 1994). Application of pseudolipids containing non-ester linkages in some positions provided an alternative approach (Rogalska *et al*., 1990). The determination of positional and stereospecific preference of lipase acting on triacylglycerols analogs is however subject to problems: the non-ester bond could have a distinct effect on the interaction between the lipase and substrates as the exact stereochemical configuration of the linkages are not identical. Stadler *et al*.

(1995) demonstrated that even minor structural differences at the *sn*-2 position of a triacylglycerols could have strong effects on the stereoselectivity of microbial lipases.

The monolayer film technique has proven to be the preferred method in chiral recognition studies with lipid monolayers as substrates (Ransac *et al*., 1990; Rogalska *et al*., 1995). The technique allows one to monitor several physiochemical characteristics of a lipid monomolecular film independently (Ransac *et al*., 1991). The most important advantage of the technique is that it is possible to vary and control the "quality of the interphase". Thus one can modulate the organization and conformation of the lipid molecules, the molecular charge and charge density, or water structure by changing the lateral surface pressure. Biological lipids, which self-organize and orientate at interfaces, are chiral molecules and their chirality plays an important role in the molecular interactions between proteins and biomembranes. Monomolecular films, which can be seen as half-membranes as compared to bi-layered biological membranes, provide an attractive model system for investigating the influence of stereochemistry and the physicochemistry of the substrate on enzymatic hydrolysis (Rogalska *et al*., 1995).

The mechanism whereby an enzyme differentiates between two enantiomers of a chiral substrate may be influenced by physicochemical properties such as temperature (Holmberg and Hunt, 1991), solvent hydrophobicity (Wu *et al*., 1990; Matori *et al*, 1991; Nakamura *et al*., 1991), hydrostatic pressure (Kamat *et al*., 1993) or surface pressure (Rogalska *et al*., 1995), which can effect the lipase reaction stereoselectivity (Rogalska *et al*., 1995). Although not much literature is available on the subject of lipase stereoselectivity towards acylglycerols, a rather large body of literature deals with the preparation of chiral esters and alcohols employing lipase mediated kinetic resolution of racemic (non-triacylglycerol) substrates. Given the nature of enzymes as chiral catalysts with sophisticated molecular architecture, one might expect selectivity to be the norm, and non-selectivity to be an exception (Sonnet, 1998). Gupta and co-workers (2004) reported the lipases from *S. aureus*, *S. hyicus*, *Corynebacterium acnes* and *Chromobacterium viscosum* to be non-

specific, acting randomly on the triacylglycerol resulting in complete hydrolysis of the substrate.

1.3.3 Fatty acid specificity

Lipases often exhibit a particular ability to release fatty acids whose chain lengths fall within well-defined ranges (Malcata *et al*., 1992). Microbial lipases have been investigated for chain length specificities and diverse results have been reported. Lipases derived from *Pseudomonas aeruginosa* MB 5001 (Chartrain *et al*., 1993), *Penicillium caseicolum* (Alhir *et al*., 1990) and *Candida deformans* (Murderhwa *et al*., 1985), were found to hydrolyse triacylglycerols containing short-chain fatty acids more readily than those containing long-chain fatty acids. In contrast lipase from *Neurospora crassa* readily hydrolysed triacylglycerols with C16 and C18 fatty acids, but hydrolysed short chain fatty acids (C4 - C10) at a very slow rate (Kundu *et al.*, 1987).

The distribution of activities of some lipases relative to various triacylglycerols changes with temperature, as temperature is increased, the rates of release of long-chain fatty acids increase faster than those of the corresponding short-chain acids. Lipases isolated from *Fusarium heterosporum* and *Bacillus* species showed different preference towards fatty acid chain length depending upon the reaction temperature. At 30°C the lipase enzyme from *Fusarium heterosporum* hydrolysed triacylglycerols of short-fatty acids with a much higher velocity than the others (Shimada *et al.*, 1993). Elevation of the reaction temperature increased the activity towards the longer fatty acid chain triacylglycerols. The same results were obtained with the studies of the lipase derived from a *Bacillus* species which showed low activities towards triacylglycerols of long chain length (more than 12 carbons) at 30°C, but these substrates were readily subjected to enzymatic hydrolysis at 50°C when this substrate became liquidized (Sugihara *et al*., 1991).

For the same chain length of the fatty acid residue, the rate of attack by some lipases seems to increase with the number of double bonds in the hydrocarbon backbone (Malcata *et al*., 1992). Lipolytic activity of lipase from *Pseudomonas*

aeruginosa MB 5001 increased as C18-unsaturated fatty acid content of the oils increased (Chartrain *et al*., 1993). Low activity was obtained with lard oil (18:0 and *cis*-18:1(Δ^9) rich) and olive oil (rich in *cis*-18:1(Δ^9)), while higher activity was achieved with sunflower oil (*cis,cis*-18:2($\Delta^{9,12}$) and *cis,cis,cis*-18:3($\Delta^{9,12,15}$) rich). Similarly, a higher lipolytic activity was obtained with trilinolein (*cis,cis*-18:2($\Delta^{9,12}$)) than with triolein (*cis*-18:1(Δ^9)) (Chartrain *et al*., 1993). The rate of triacylglycerol hydrolysis by a lipase from *Pythium ultimum* was also found to increase with an increasing number of double bonds per molecule (Mozaffar and Weete, 1993).

One explanation for the above type of specificity involves the concept of induced fit (Malcata *et al*., 1992). Although many substrates can bind at the active site, only a few can release a proper amount of binding energy required for the change in the conformation of a lipase to a form which is a much more efficient catalyst. Substrates that are too small or possess too few double bonds are not able to release enough binding energy. In such cases the change in conformation of the native lipase to the desired catalytically active conformation does not occur or is, at best, incomplete. Hence, the reaction will proceed slowly. Substrates that are too long or possess too many double bonds are able to release enough binding energy that would in principle be sufficient to effect the desired conformational change. However, some of this energy becomes unavailable for this purpose because it is required to change the conformation of the substrates to make it fit into the active site. Hence only a small fraction of the energy released by the binding process will actually be available to drive the conformational change of the enzyme. Consequently, optimal activity will not be achieved.

The presence of two and especially three double bonds in the 18-carbon fatty acid chains reduced the rate of triacylglycerol hydrolysis by some other lipases. Lipase derived from *Candida deformans* hydrolysed triacylglycerols with *cis,cis*-18:2($\Delta^{9,12}$) and especially with *cis,cis,cis*-18:3($\Delta^{6,9,12}$) at a slower rate than those with 18:0 and *cis*-18:1(Δ^9) (Muderhwa *et al*., 1985). Similarly, *Humicola lanuginosa* No. 3 lipase catalysed hydrolysis of polyethylene sorbitan monooleate (Tween 80) to a higher extent than triolein (*cis*-18:1(Δ^9)) and showed low hydrolytic activity towards esters

of a higher degree of unsaturation such as methyl linoleate (*cis,cis*-18:2($\Delta^{9,12}$)) and methyl linolenate (*cis,cis,cis*-18:3($\Delta^{9,12,15}$)) (Omar *et al*., 1987).

A special kind of fatty acid specificity has been reported for lipase B from *Geotrichum candidum* which showed high specificity for esters of fatty acids with *cis*-Δ^9 double bonds (Jacobsen and Poulsen, 1991; Charton and Macrae, 1991). This feature is represented by the lipase isolated from *Galactomyces geotrichum* which displayed preference for long chain fatty acids containing a *cis*-Δ^9 double bond (Phillips and Pretorius, 1991).

Other lipases can equally hydrolyse saturated and unsaturated triacylglycerols. For example *Neurospora crassa* lipase hydrolysed tripalmitin (16:0), tristearin (18:0), tripalmitolein (16:1(Δ^9), triolein (*cis*-18:1(Δ^9)) and trilinolein (*cis,cis*-18:2($\Delta^{9,12}$)) at the same rates (Kundu *et al.*, 1987). A lipase isolated from lupin seed was found to be more active on saturated than on unsaturated fatty acids (Sanz and Olias, 1990). Lipase enzyme from *Fusarium oxysporum f.*sp.*lini* exhibited a higher affinity to the ester bond of saturated fatty acids than that of unsaturated fatty acids (Hoshino *et al*., 1992). This preference was exploited in the concentration of poly-unsaturated fatty acid (n-3 PUFA) content of partially hydrolysed glycerides obtained from fish-oil. The lipase gave increases in n-3 PUFA concentration as the hydrolysis progressed. In general, the lipases from *Geobacillus* species tend to preferably hydrolyze the saturated short-chain fatty acids (Rua *et al*., 1997; Sinchaikul *et al*., 2001).

1.3.4 pH

Changes in pH profoundly affect the degree of ionisation of the amino, carboxyl and other ionisable residues in protein. Since ionisable amino acid residues may be present in the active site of the enzyme, and other ionisable groups may be responsible for maintaining the protein conformation, it is not surprising that the pH of the solution may markedly affect enzyme activity. Moreover, since many substrates are ionic in character, the active site of an enzyme may require particular ionic species of the substrate for optimum activity. These effects are

probably the main determinants of the shape of the curve that represents enzyme catalytic activity as a function of pH (Conn *et al*., 1987). Usually, the catalytic activity of the lipase changes with pH in a bell-shaped fashion, thus yielding a maximum rate at the optimum pH (Zaks and Klibanov, 1985). The plateau of the bell-shaped curve usually is small and the rates decrease rapidly with pH on either side of the maximum. The rate decrease represents changes in the state of ionisation of groups on enzyme or the substrate, or both, that are critical, with regard to the state of ionisation, to the enzyme-catalysed reaction (Conn *et al*., 1987).

As with other enzymes each lipase has its own optimal pH, ranging from acid to neutral to alkaline (Yamane, 1987). There exists a great diversity in the pH optima of microbial lipases. Development of alkaline and acid lipase is important, particularly in the use of the enzyme in laundry detergents to enhance cleaning (alkaline lipases) and as a substitute for pancreatic lipase in digestive medicine(acid lipases) (Lengsfield *et al*., 2004; Yamane, 1987). Shifts in the pH optimum after immobilisation for various lipases have been observed. After immobilisation, the optimum activity of the lipase from *Candida rugosa* increased to a more alkaline value (Montero *et al*., 1993). Shifts in pH optima of immobilised lipases have been reviewed by Malcata *et al*., (1992). The maxima in the rates of reaction catalysed by immobilised lipases were observed at pH values between 4 and 10. With very few exceptions, the pH optima for the immobilised lipases are equal to or higher than those for their free counterparts. Hence, the immobilisation procedure seems to render catalytically important amino acid residues more basic. An explanation consistent with these results and with the experimental evidence is that upon immobilisation the active site becomes more exposed to the solvent than it was in the globular, folded soluble lipase form. Hence, proton transfer to the amino acid residues at the active site becomes less hindered.

The pH also affects the stability of enzymes. Some lipases are stable over a wide pH range, examples are the lipases from *Pseudomonas cepacia* (which retained 100 % activity after incubation over a pH range of 3 - 11.5 for 24 hours at 30°C) (Sugihara *et al*., 1992) and *Fusarium heterosporum* (stable over a pH range of 4 -

10 at 30°C for 4 hours) (Shimada *et al*., 1993). Braddoo and co-workers (1999) reported similar results for the *B. stearothermphilus* SB-1 lipase being active over a broad pH range (pH 3 - 12). The gastric lipases found in animals are not only active but also stable in acidic environments (Lengsfield *et al*., 2004)

1.3.5 Temperature

The Arrhenius equation relates the specific reaction rate or rate constant, k, to temperature

$$k = Ae^{-E_a / RT} \qquad [3.1]$$

where A is a proportionality constant, E_a is the activation energy, R is the gas constant, and T the absolute temperature. The equation predicts that the rate of the reaction, enzyme-catalysed or not, will increase with increasing temperature. However, since enzymes are proteins and many proteins will be denatured if the temperature is raised sufficiently, enzyme catalysed reactions show an increase in rate with increasing temperature only within relatively small and low temperature ranges. The optimum temperature of enzyme-catalysed reactions depends on several factors including how long the enzyme is incubated at the test temperature before the substrate is added and the type of organism from which the enzyme was derived (Conn, *et al*., 1987).

Heat stability is a useful attribute if the lipase is to be used commercially either as a fat splitting enzyme (e.g. as an enzyme additive to the detergents) or in transesterification reactions where little water or solvent is present and the reaction therefore depends upon the substrates being in the liquid phase (Ratledge, 1989). The melting point of fat is very variable and can in some cases be as high as 50°C, but enzymatic catalysis on solid substrates is limited and therefore becomes difficult for less thermostable enzymes to catalyse the required reactions (Sigurgìsladòttir *et al*., 1993).

Lipases from plants and animals are in general, not thermostable. Relatively thermostable microbial enzymes have been purified and characterised. Optimum temperatures of 55°C or above have been reported for bacteria belonging to the *Pseudomonas* species (60°C) (Yamamoto and Fujiwara, 1988), *Pseudomonas. cepacia* (55 - 60°C) (Sugihara *et al*., 1992) and *Pseudomonas. aeruginosa* MB 5001 (55°C) (Chartrain *et al*., 1993). A fungus identified as *Humicola lanuginosa* S-38 was reported to produce a heat stable lipase (Arima *et al*., 1972), and the optimal activity of a lipase from *Humicola lanuginosa* No.3 was found to be 45°C and retained 100 % activity for 20 hours at 60°C (Omar *et al.*, 1987). A thermophillic *Bacillus* species has been reported to produce a thermostable lipase (Kambourova and Manolov, 1993), and an optimum temperature of 60°C was reported (Sugihara *et al.*, 1991).

Enzymes, being proteins, are susceptible to heat denaturation. At elevated temperatures the Arrhenius model breaks down due to extensive irreversible denaturation of the lipase (Malcata *et al*., 1992). The inactivation temperature of lipases is influenced by the composition of the medium in which the inactivation is being determined. For example, it has been shown that in milk higher temperatures and longer times are needed to achieve destruction of lipases than in buffer systems (Law, 1979). This is probably due to the availability of the substrate of the enzyme which removes excess water from the vicinity of the enzyme and thus restricts its overall conformational mobility (Malcata *et al*., 1992) or changes its conformation towards a more stable one.

The lipase from *T. thermophilus* HB 27 was reported to be stable at 85ºC for several hours without any significant losses in activity (Fuciňos *et al*., 2005) and Bradoo *et al*. (1999) reported on the lipase from *B. stearothermophilus* with a half-life of 10 min at 100ºC. Thermostabillity of lipases have been enhanced by the addition of additives, such as ethylene glycol, sorbitol and glycerol (Gupta *et al.,* 2004) and Nawani and Kaur (2000) could enhance the stabillity of a *Bacillus* sp. to retain activity after 150 min at 70ºC with the addition of these additivies. Palamo and co-workers (2004 a and b) could stabilize the enzymes from *T. aquaticus* and *B. thermocatenulatus* by immobilising the enzymes on Octadecyl-Sepabeads-TAL.

This not only lead to enhanced stabillity, 100 % activity after 70 hours at 70ºC in both cases, but the activity of the lipases could be enhanced by a 50-fold factor at mesophillic temperatures.

1.3.6 Effects of metals

Numerous studies have been made concerning the effects of various salts on lipase activity and diverse results have been obtained. Most lipases are inhibited by heavy metals (Co^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, Sn^{2+}, Ni^{2+} and Ag^{+}). However, the lipase isolated from *Penicillium simplicissimum* was found to be resistant to most of the heavy metals tested (Sztajer *et al.*, 1992). It was significantly inhibited by Zn^{2+} and a minor reduction was observed with Ag^{+}.

In most cases, the monovalent cations, Na^{+}, K^{+} and Li^{+}, have been found to have stimulatory or no effect on the rate of lipase -catalysed reactions. A 50 % inhibitory effect by K^{+} was reported on the activity of a lipase isolated from *Pseudomonas* species (Yamamoto and Fujiwara, 1988). Light divalent cations (Mg^{2+} and Ca^{2+}) appear to stimulate the activity of most of the enzymes studied. A significant inhibitory effect by Mg^{2+} was observed on a lipase isolated from *Aspergillus oryzae* (Ohnishi *et al*., 1994). Often the lost activity can be restored via the addition of metal-chelating agents (Malcata *et al*., 1992). The lipase enzyme isolated from castor bean lipid bodies was stimulated 40 fold by 30 mM free Ca^{2+} (Hills and Beevers, 1987).

It is generally known that free fatty acids tend to inhibit lipase-catalysed hydrolysis probably by accumulating at the lipid/water interface, thereby blocking access of the enzyme to the unreacted triacylglycerol molecules (Benzonana and Desnuelle, 1968). The positive effects of metal ions could be due to the formation of complexes with ionised fatty acids which change their solubilities and behaviour at interfaces, whereas negative effects can be attributed to competitive inhibition at the active site (Gupta *et al*., 2004)

1.3.7 Effects of bile salts and detergents

Most studies on the effect of bile salts on lipases have been made with lipase enzymes derived from animal sources, probably due to the role they play as micellular solobilisation of lipolytic products in animal intestinal tracts. In most cases bile salts were found to have stimulatory effects on the activity of animal lipase (Tiruppathi and Balasubramanian, 1982; Gargouri *et al*., 1986; Carrière *et al.*, 1991; Lowe, 2002). Some animal lipases are characterised by being bile-salt dependent for their activity, particularly lipases purified from milk (Wang, 1991) and from the pancreas of human (Mas *et al*., 1993) and cod (*Gadus morhua*) (Gjellesvik *et al*., 1992). It has been shown that *in vitro* pancreatic lipase action on long chain triacylglycerols is inhibited early by the hydrolysed fatty acids and soaps. Bile salts and Ca^{2+} do not increase the initial rate but, rather, counteract the inhibitory effect of the soaps (Shahani, 1975).

Bile salts have also been shown to enhance the activity of lipases purified from *Pseudomonas putida* 3SK (Lee and Rhee, 1993), *Pseudomonas aeruginosa* MB 5001 (Chartrain *et al*., 1993), *Staphylococcus simulans* (Sayari *et al*., 2001) and *Staphylococcus xylosus* (Mosbah *et al*., 2005). When the activity of a lipase from *Penicillium caseicolum* was tested using tributyrin as a substrate, sodium taurocholate, sodium deoxycholate and $CaCl_2$ inhibited the enzyme, but with butter oil as a substrate, the bile salts enhanced the activity, while $CaCl_2$ weakly inhibited the activity (Alhir *et.al*., 1990). The activity of *Pseudomonas* sp lipase was enhanced by the addition of sodium cholate and sodium deoxycholate (Yamamoto and Fujiwara, 1988) whereas the activity of *Pseudomonas* sp KW I-56 lipase was inhibited by these bile salts (Iizumi *et al*., 1990).

The effect of other detergents on lipase activity has been widely studied. Different detergents affect lipases differently. In most studies, anionic detergents inhibited lipase activity while non-ionic detergents (Tween 20 and 80, Triton X-100) enhanced activity (Yamamoto and Fujiwara, 1988; Hoshino *et al*., 1992; Mozaffar and Weete,1993; Lin *et al*., 1996). Lipases from *Pseudomaonas* sp KW I-56 (Iizumi

et al., 1990) and *Brassica napus* (Weselake *et al*, 1989) were inhibited by non-ionic detergents. Cetyltrimethyl-ammonium bromide, which is a cationic detergent inhibited *Brassica napus* lipase (Weselake *et al.,* 1989) and *Pseudomonas* sp lipase (Yamamoto and Fujiwara, 1988). CHAPS, a zwitterionic detergent, enhanced activity of *Pythium ultimum* lipase (Mozaffar and Weete, 1993) and *Bacillus thermocatenulatus* lipase (Schmidt-Dannert *et al*., 1994).

1.4 Occurrence and classification of lipases

Lipases are widely distributed in nature, being found in plants, animals and micro-organisms. They have been classified according to their sources, kinetic properties and substrate specificities. More recently, studies on three-dimensional structures of lipases enabled a better classification of lipases.

1.4.1 Microbial lipases

Lipases are found in abundance in bacteria and fungi including yeasts. The initial studies on lipases concentrated on animal lipases, but over the last two decades much attention has been focused on microbial lipases due to their biotechnological potential (Jaeger and Eggert, 2002). Many lipases from microbial sources have been purified and sequenced. The number of amino acids ranges from about 200 in *Bacillus* species to more than 600 in *Staphylococcus* species. Comparison of amino acid sequences between microbial lipases often revealed no similarities beyond the pentapeptide Gly-X-Ser-X-Gly, which contains the catalytically active Ser residue.

Although microorganisms produce both intracellular and extracellular lipases, most of the studies have concentrated on the latter. Extracellular lipases are secreted through the external membrane into the culture medium, and this has facilitated their recovery from fermentation vessels. The extracellular nature of most lipases has enhanced their scope of application in biotechnology, as they can remain active under extreme catalysis conditions.

Microbial lipases can be subdivided into bacterial and fungal lipases. In the field of biotechnology, much attention has been paid to the use of fungal or yeast lipases (Pandey *et al*., 1999). This, however, does not imply the inferior properties of bacterial lipases, as it has been shown in some reviews that bacterial lipases are as good as, or sometimes to be preferred to their eukaryotic counterparts (Jaeger *et al*., 1994; 1998; Schiraldi and De Rosa, 2002). The interest in bacterial lipases has overgrown the initial attempts to classify them (Gilbert, 1993; Jaeger *et al*., 1994). Bacterial lipases were formerly classified as *Pseudomonas* group 1, 2 and 3 lipases because *Pseudomonas* lipases were the first studied due to their industrial importance (Arpigny and Jaeger, 1999). Some of the *Pseudomonas* species were re-classified as *Burkholderia* and it became evident that there was a need for a new classification system for lipases. Arpigny and Jaeger (1999) devised a classification system based on amino acid sequence similarities and biochemical properties grouping, bacterial lipolytic enzymes into 8 families (Table 1.1). The bacterial true lipases (Group 1) were subdivided into six families, which were further expanded in 2002 by Jaeger and Eggert to seven subfamilies (Jaeger and Eggert, 2002). Families 1.1, 1.2 and 1.3 contain the true lipases from Gram-negative bacteria with the Gram-positive lipases divided into families 1.4, 1.5 and 1.6.

Family 1.1 includes lipases from *Vibrio cholera*, *Acinetobacter calcoaceticus*, *Proteus vulgaris* and *Pseudomonas fluorescens* and includes lipases with molecular masses of 30 - 32 kDa that show higher sequence similarity to *Pseudomonas aeruginosa* lipase. Family 1.2 lipases are characterised by a slightly larger lipase (33 kDa) owing to an insertion of in the amino acid sequence leading to an anti-parallel β-strand at the surface of the molecule and shows a high homology to the *Burkholderia glumae* lipase. Other species showing lipase activity found in this group includes *Pseudomonas luteola* and *Chromobacterium viscosum*.

Table 1.1: Adaptation of the lipase classification system reported by Arpigny and Jaeger (1999) and Jaegert and Eggert (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			

Family 1.3 lipases contain the enzymes from at least two distinct species: *Pseudomonas fluorescens* and *Serratia marcescens*. These lipases have in common a higher molecular mass than lipases from family 1.1 and 1.2 (*P. fluorescens*, 50kDa and *S. marcescens*, 65 kDa) and the absence of an N-terminal signal peptide and of Cys residues. According to the original classification system, Arpigny and Jaeger (1999) divided the *Bacillus* and *Staphylococcus* lipases into two subfamilies. With the discovery and cloning of novel lipases coupled to the reclassification of certain *Bacillus* species to *Geobacillus* (Nazima *et al*., 2001). Jaeger and Eggert (2002) restructured these lipases into three subfamilies. Family 1.4 contains the enzymes from *B. pumilus*, *B. licheniformis* and *B. subtilis*. These lipases have in common that the first glycine in the conserved pentapeptide is replaced with an alanine (Ala-X-Ser-X-Gly).

Family 1.5 contains the *G. stearothermophilus*, *G. thermocatenulatus* and *G. thermoleovorans* lipases. These organisms are thermophiles and produce enzymes of approximately 45 kDa. Family 1.6 organisms produce the largest lipases (75 kDa), which is secreted as precursors and cleaved in the extracellular medium by a specific protease, yielding a mature lipase of approximately 400 amino acid residues. Included in this family are the *Staphylococcus* lipases.

The last subfamily (1.7) contains only two lipases from *Propionibacterium acnes* (339 amino acid residues) and *Streptomyces cinnamoneus* (275 amino acid residues) and show significant similarity to each other.

The rest of the families consists of the GDSL family (II) lipolytic enzymes that do not exhibit the conventional pentapeptide sequence but rather display a Gly-Asp-Ser-Leu motif containing the active site serine residue (example *Photorabdus luminescens*). The *Streptomyces exfoliates* extracellular lipase resides in family III and shows a 20 % similarity to human PAF-AH's.

Family IV lipolytic enzymes (example *Archaeoglobus fulgidus* carboxylesterase) resemble the human hormone-sensitive lipases. Family V enzymes show sequence similarity to various bacterial non-lipolytic enzymes such as epoxide hydrolases and dehalogenases. An example of this family is the *Haemophilus influenza* putative esterase. Family VI contains the smallest esterases known (23-

26 kDa) while family VII has some of the biggest esterases (55kDa) showing similarity with the eukaryotic acetylcholine esterases. Lastly the family VIII contains three enzymes with striking similarity to the class C β-lactamases.

The number of bacterial lipolytic genes that are cloned and isolated are increasing steadily, and it is hoped that the revised classification would serve as the basis and would evolve into a more complete classification (Jaeger and Eggert, 2000).

1.4.2 Animal lipases

Animal lipases were originally classified into three groups according to their source organs (tissues) and sites of lipolytic action (Aires-Barros *et al*., 1994). Firstly, the digestive lipases including lingual, pharyngeal, gastric and pancreatic lipases; secondly the tissue lipases contained in serum, heart, brain, muscle, arteries, kidney, spleen, lung, liver and adipose tissue. The third group comprises the milk lipases produced by lactating mammary glands and play a major role in neonatal fat digestion. The success achieved in the cloning and sequencing of genes encoding animal lipases has enabled their classification into pancreatic and hormone sensitive lipase families, based on primary structure analysis and biochemical properties (Carrière *et al*., 1998; Osterlund, 2001). Another important family of animal lipases are the acid lipase gene family comprising the gastric lipases preferring the more acidic environments (Lengsfeld *et al*., 2004)

1.4.2.1 The pancreatic lipase gene family

The cloning and sequencing of genes encoding the three major animal lipases namely, the pancreatic lipase (PL), lipoprotein lipase (LPL) and hepatic lipase (HL) revealed that they are derived from a common ancestral gene and they share structural similarities (Ben-Zeev *et al*., 1987; Warden *et al*., 1993; Connely, 1999). The overall pancreatic gene family has now been divided into eight subfamilies based on amino acid identity and homology (Figure 1.2) (Carrière *et al*., 1998).

HL is synthesized primarily in the liver (Connelly, 1999), while LPL is predominantly synthesized in heart, muscle and adipose tissue (Scow *et al*., 1998). HL is

distinguished from LPL by its resistance to inhibition by 1 M NaCl or protamine sulphate and the absence of a requirement for an apolipoprotein activator (Bruin *et al*., 1992; Connelly, 1999). LPL and HL are about 30 % homologous to pancreatic lipases and play an important role in the metabolism of phospholipids and triacylglycerols present in the core of chylomicrons and very-low-density lipoproteins (Carrière *et al*., 1998).

The lipases secreted by the pancreas have been divided into three subgroups sharing about 70 % amino acid identity: (i) the classical pancreatic lipases; (ii) pancreatic lipase-related proteins 1 (PLRP 1) and (iii) the pancreatic lipase related proteins 2 (PLRP 2) (Carrière *et al*., 1998). The lipases within each subgroup have been biochemically characterized. PLRP 1 display no significant activity on triacylglycerols and their physiological role has not yet been explained (Hjorth *et al*., 1993). The PLRP 2 proteins have been investigated in human (Giller *et al*., 1992) as well as in animal species (De Caro *et al*., 1998; Thristrup *et al*., 1994).

There is a high sequence homology between PLRP 1 and PLRP 2 but somewhat lower homology with the pancreatic lipases. All the PLRP 2s characterized do not exhibit the so-called “interfacial activation” phenomenon. Because of high phospholipase and galactolipase activity of PLRP 2, and inhibition by bile salts that cannot be overcome by colipase, it has been suggested that PLRP 2 function as phospholipases (Thirstrup *et al*., 1994) and galactolipases (Sias *et al*., 2004) *in vivo*.

The phospholipases A1 from vespid venoms (hornets and yellow jackets) have been identified as members of the pancreatic lipase gene family (Soldatova *et al*., 1993; Connelly, 1999). These enzymes are relatively small and share about 40 % homology with the N-terminal catalytic domain of pancreatic lipases and their lipase activity is very low.

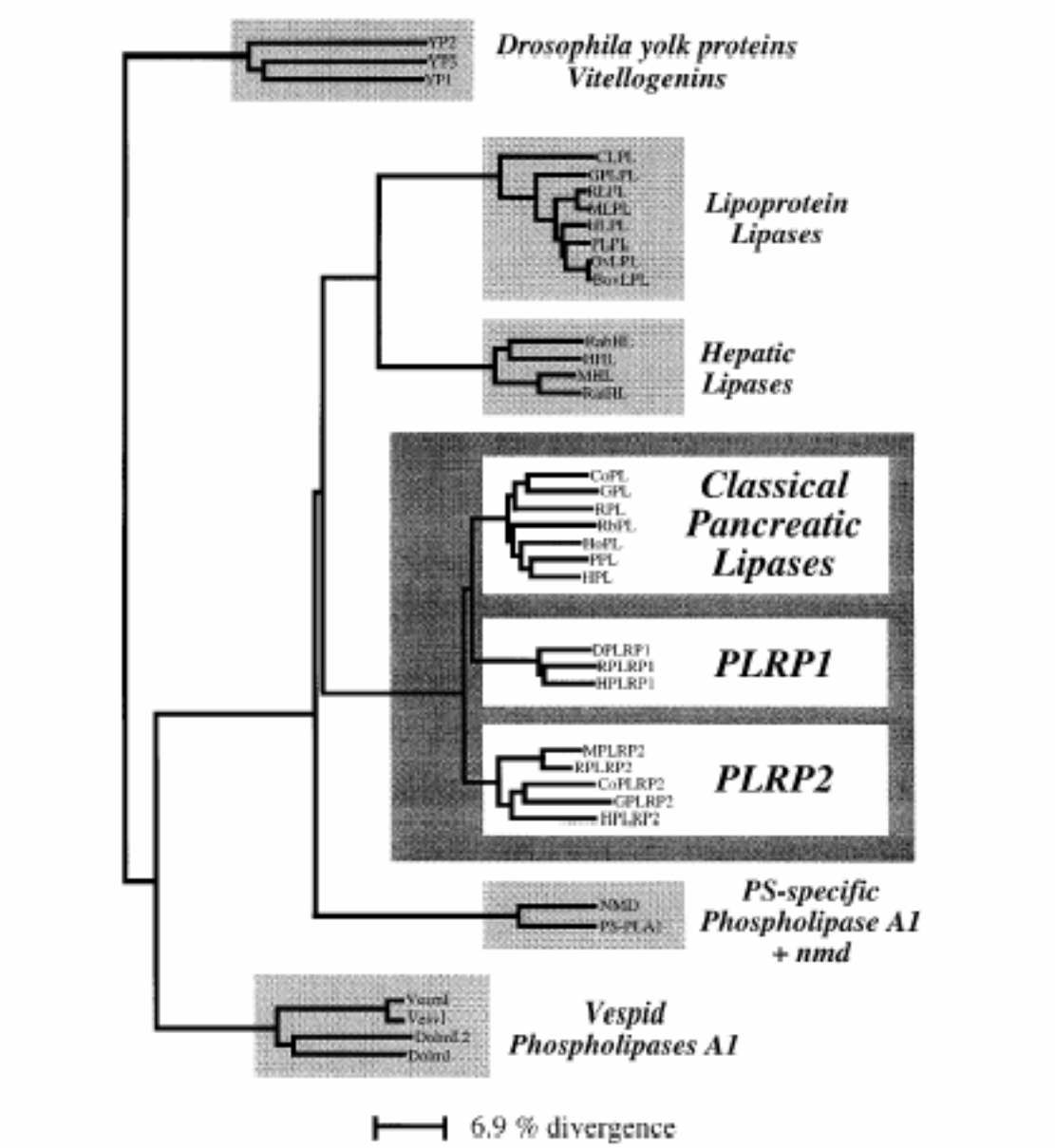


Figure 1.2: Dendogram of sequence alignment of the eight lipase gene famly: (1) the yolk proteins from *Drosophilia melanogaster* (YP1, YP2, YP3); (2) the lipolytic lipases, from chicken (CLPL), guinea pig (GPLPL), rat (RLPL), mouse (MLPL), human (HLPL), pig (PLPL), ovine (OvLPL) and bovine (BovLPL); (4) the classical pancreatic lipases from coypu (CoPL), guinea pig (GPL), rat (RPL), rabiit (RbPL), horse (HoPL), pig (PPL) and human (HPL); (5) the RP1 pancreatic lipases from dog (DPLRP1), rat (RPLPR1),and human (HPLPRP1); (6) the RP2 pancreatic lipases from mouse (MPLRP2), rat (RPLRP2), coypu (CoPLRP2); (8) the vespid phospholipases A1, from the yellow jackets (*Vespula maculifrons*, Vesm1, and *Vespula vulgaris*, VesVI) and the white-faced hornet (*Dolichovespula maculata*, DolmI and DolmI.2). (Taken from Carrière *et al*., 1998).

Phospholipase A1 secreted by rat platelets (Sato *et al*., 1997) and NMD, a protein found to be expressed in human melanoma cell lines (van Groningen *et al*., 1997) constitutes another subfamily. The two proteins share 80 % amino acid identities and show about 30 % homology with pancreatic lipases, LPL and HL. Whereas the biochemical properties of NMD have not yet been reported, the phospholipase A1 from rat platelets specifically hydrolyzes the ester bond at *sn*-1 position of lysophosphatidylserine and phosphatidylserine, but has no significant activity towards phosphotidylcholine, phosphotidylethanolamine, phosphotadylinositol, phosphatidic acid and triacylglycerols (Sato *et al*., 1997).

A distant amino acid homology relationship was also obtained with non-enzymatic yolk proteins (vitellogenins) from the *Drosophila* fruit fly. The vitellogenins do not contain the lipase/esterase catalytic triad and therefore do not display lipase activity. The conserved amino acid residues between yolk proteins and pancreatic lipase however surround the active site where interactions with lipids take place (Bownes, 1992). The likely reason for this sequence homology in the yolk proteins is to bind a steroid hormone and to store it in an inactive form until it is released during embryogenesis of *Drosophila* (Carrière *et al*., 1998).

1.4.2.2 Hormone sensitive lipases

Hormone sensitive lipases constitute a family of their own; they share no homology with other animal lipases. They catalyse the rate-limiting step in the hydrolysis of adipocyte triacylglycerols, and are therefore key enzymes in lipid metabolism and overall energy homeostasis (Osterlund, 2001). The activity of hormone sensitive lipase is under strict hormonal and neuronal control through reversible phosphorylation. Hormone sensitive lipase exhibits high enzyme activity towards cholesteryl esters, an unusual property of lipases and has, together with the relatively high level of expression in steroigenic tissues, led to the proposal that hormone sensitive lipase plays an important role in steroidogenesis (Holm *et al*., 1994).

1.4.2.3 Acid lipases

Gastric lipases form the major part of this family was not originally thought to play an essential role in the hydrolysis of dietary fats. The contrary was shown when it was found that pancreatic lipases do not readily hydrolyse triglyceride droplets covered with phospholipids. The gastric lipase was able to perform this function. These lipases are found in humans, dogs cat, pigs etc. The human gastric lipase is a 50 kDa highly glycosylated protein produced in the fundic mucosa of the human stomach. The lipase is stable and active in acidic conditions and can hydrolyse all three ester bonds in triacylglycerols (Lengsfield *et al*., 2004).

1.4.3 Plant lipases

It is known that lipases exist in several plant tissues, with most of the studies done on plant lipases have been devoted to seed lipases. During the initial phase of germination, seeds contain a large amount of triacylglycerols, which serve as a compact source of energy for the newly emerging plant, and a small amount of water (Adlercreurtz *et al.,* 1997). Examples of isolated plant lipases are the lipase from lupin seed (Sanz and Olias, 1990), *Brassica napus* and *Vernonia galamensis* (Adlercreurtz *et al.,* 1997), castor beans (Eastmond, 2004) and buckwheat seed (Suzuku *et al*., 2004) and *Carica papaya*.

1.5 Nutritional factors affecting microbial lipase production

Although lipases may be obtained naturally from different organisms, their production is influenced by culture conditions. A variety of conditions have been described which stimulate or repress the production of lipases by micro-organisms. Lipase production can be induced by the addition of various triacylglycerol substrates to the growth medium. When a *Pseudomonas* strain was cultivated in medium without oil, which consisted of glucose (1 %), peptone (1 %), urea (0,2 %) and minerals, lipase activity was scarcely detected in the culture supernatant in spite of the good growth of the micro-organism (Narasaki *et al*., 1968). Addition of olive oil resulted in significant lipase activity indicating a stimulation of lipase

production by the olive oil. Since then, olive oil has been used to induce lipase production by various micro-organisms (Yamamoto and Fujiwara, 1988; Suzuki *et al*., 1988; Phillips and Pretorius, 1991; Christakopoulos *et al*., 1992). Other oils that have been used to stimulate microbial lipases include soybean oil, corn oil, and sunflower oil (Chistakopoulos *et al*., 1992). The type of oil used is important for lipase production for a given micro-organism (Espinosa *et al*., 1990; Hatzinikolaou *et al*., 1996).

Addition of compounds containing hydrolysable ester groups such as Tweens and Spans in culture medium was found to increase lipase production by other micro-organisms. Of all the carbon sources tested, Tween 80 was by far the best inducer of lipase production by a *Bacillus* specie MC7 (Emanuilova *et al*.,1993). When Tween 80 was replaced by glycerol, glucose or starch, the lipase level was very low and could only be detected by the most sensitive fluorometric assay. The presence of Tween 80 in a culture medium for *Rhizopus delemar* increased lipolytic activity to a level of twice that with olive oil or butyric acid (Espinosa *et al*., 1990). This effect was postulated to be due to the possible double effect of Tween. It could act as an inducer, as its chemical nature is similar to some substrates of the enzyme, and as a surfactant. When *Trichosporon fermentans* was cultivated in a media containing surfactants such as Tween, Triton and Span, extracellular lipase activities reached levels of 2-3 times as much as that without surfactants (Chen *et al*., 1994). Nthangeni (2001) reported similar results for *B. licheniformis*, showing that although Tween 20/80 did not induce activity, the presence of either in the culture media significantly enhanced the extracellular lipolytic activity.

Long chain fatty acids, as end products of lipase activity, have an inhibitory effect on lipase production (Hegedus and Khachatourians, 1988). Contrary to this, oleic acid was found to be better than olive oil in the induction of lipase production by *Candida rugosa* (Del Rio *et al*., 1990). Knoesen (2004) showed that this was also true for *G. thermoleovorans* GE-7, with the difference that stearic acid was used for induction. Studies with different soluble short chain fatty acids have demonstrated that caprylic (C8) and capric (C10) acids could even be better than oleic acid as inducers of lipase production by *Candida rugosa* (Obradors *et al*., 1993). Addition

of a light divalent cation (Mg^{2+}), in culture media showed enhanced production of lipase activity (Hegedus and Khachatourians, 1988).

Stimulation of lipase production is not only limited to the addition of lipidic substances in the culture media as inducers. Investigations applying one-variable-at-a-time-optimisation procedures showed that extracellular lipase activity from different micro-organisms achieved maximal values when carbohydrates (Petrovic *et al*., 1990) were used as carbon sources and certain ammonium salts (Christakopoulos *et al*., 1992) served as nitrogenous sources. Sztajer and Maliszewska (1988) demonstrated that while starch induced maximal lipolytic activity in *Bacillus circulans*, *Streptomyces* sp., and *Pseudomonas fluorescens*, galactose and sucrose exerted an enhanced activity in *Bacillus* sp. The maximal intracellular lipolytic activity of *Nocardia asteroides* was observed in fructose-supplemented cultures (Nesbit and Gunasekaran, 1993). This was followed by cultures grown in glucose, maltose and sucrose; the least activity was observed in media containing starch and citrate. Although the extracellular lipase activity was much lower than the intracellular activity, in culture grown in monossacharides as the primary carbon source, it was significantly higher than that of cultures with maltose, sucrose and starch.

Lipase production in other micro-organisms is stimulated by the presence of alkanes in the culture media. The ability of *Acinetobacter woffi* strain to grow on pure alkanes was associated with the formation of cell-bound lipase (Breuil *et al*., 1978). Chen *et al*. (1994), reported that *Trichosporon fermentans* Wu-C12 could produce extracellular lipase with petroleum products as carbon sources. Kanwar and co-workers (2002) found the highest lipase production in *Pseudomonas* sp. G6 using hexadecane as sole carbon source, when compared with other lipid and non-lipid inducers. Using alkanes as a cosolvent, Wei *et a*l. (2005) and Maia *et al*. (2001) were able to show increased lipolytic activity in *Candida rugosa* and *Fusarium solani* during the fermentation process.

1.6 Molecular regulation of lipase biosynthesis

The studies described above have been conducted from the biotechnological point of view, with the aim of defining conditions for maximum lipase production. The molecular mechanisms regulating the expression of lipase genes have not yet been clearly elucidated. In general, the release of enzymatically active lipase into the culture medium requires the interaction of various cellular processes, starting with transcription of the lipase genes, proceeding with the translocation of respective mRNA's and subsequent secretion of the protein through the cell membranes. In bacterial lipases, most studies on the regulation of the biosynthesis of lipase genes have been on *Pseudomonas* (Rosenau and Jaeger, 2000) and *Staphylococcus* species (Rosenstein and Götz, 1998). This has probably been prompted by the difficulties experienced in the attempts to over-express *Pseudomonas* lipases in the heterologous hosts such as *Escherichia coli*, and their role as virulence factors in some *Pseudomonas* strains. *Staphylococcus* lipases have been implicated in the pathogenesis of human disease, and driven by the quest to understand the molecular basis of *Staphylococcus* pathogenesis, the molecular regulation of their lipase is becoming clear (Rosenstein and Götz, 2000; Rosenstein and Götz, 1998).

Studying the expression capabilities and transport of proteins into the extracellular media of Gram-positive bacteria such as *B. subtilis*, *B. amyliquefaciens* and *B. licheniformis* have gained use popularity as they are well known for their high capacity to secrete proteins into the extracellular environment, at gram per liter concentrations (van Dijl, 2002). Schalmey *et al*. (2004) reported levels of up to 20 - 25 g/l enzyme formed with *Bacillus* expression systems and hosts.

B. subtilis has been shown to produce two extracellular lipases, LipA and LipB (Eggert *et al*., 2002), but at present the exact regulation of the extracellular lipolytic enzymes is unknown (Eggert *et al*., 2003). With the isolation of the *lipA* gene, DNA sequence comparisons of the potential promotor region indicated a σ^A-dependant constitutive expression (Dartois *et al*., 1992). Initial experiments on the regulation of lipases in *B. subtilis* indicated a differential expression of the genes but the

regulatory signal remained elusive. Eggert and co-workers (2003) illustrated, using *B. subtilis* mutant strains that the pH of the medium had an influence on LipA activity. When the pH was approximately 5, there was expression of inactive lipase, when compared to growth at pH 7. With the help of transcriptional fusions with the *lacZ* reported gene, the lipases expression was shown to be regulated by amino acid supply. The l*ipA* expression was repressed by high amino acid concentrations, while no *lipB* gene expression was detected when grown in minimal media, something that could be reversed with the addition of casamino acids.

Secretion of the LipA from *B. subtilis* has been shown to use the Sec pathway for export (Jongbloed *et al*., 2002). The Sec pathway comprises of the Sec machinery, which is composed of a proteinaceous channel in the membrane and a translocator motor (SecA). The channel is formed by the SecY, SecE, SecG and SecDF-YrbF. SecY is the largest subunit of the membrane domain of the protein translocase and is encode by the *secY* gene and associates with SecE (a small integral membrane protein) preventing degradation of SecY by ATP-dependant proteinases. SecG is the third part of the protein translocase and was shown to stimulate SecYE-mediated protein translocation (van Wely *et al*., 2001). The last part consists of SecD and SecF, two large integral membrane proteins with an accessory function in protein translocation. These two form a complex with YrbF, another membrane protein. This heterodimeric SecDFYrbF complex loosely associates with SecYEG to form a supramolecular translocase complex. The Sec machinery is known to thread its substrates through the membrane in an unfolded state. (van Dijl *et al*., 2002).

Jongbloed and co-workers (2002) studied the signal peptides of the known *B. subtilis* proteins and identified 69 that could potentially use the Twin arginine translocation (Tat) pathway, as their signal peptides contained the RR- and RK-motifs recognise by the Tat machinery. The LipA signal peptide contained this motif and was thought to be translocated via this stringent Tat pathway. Studies using deletions in the Sec and Tat machinery, as well as sodium azide inhibition of the SecA, proved that the LipA makes use of the Sec pathway for translocation into the extracellular environment. The signal peptide is consequently removed after

transport across the membrane. The exact expression of the *Bacillus* lipases still has to be studied.

Rosenau and Jaeger (2000) reviewed the regulation of *Pseudomonas* lipase gene expression and mechanism of secretion. The prototype lipase from *Pseudomonas aeruginosa* is encoded in a bicistronic operon, which is transcribed from two different promoters; one of which depends on the alternative sigma factor σ^{54} (Rosenau and Jaeger, 2000). The lipase is synthesized as a pro-enzyme with the N-terminal signal sequence, which channels the lipase protein into the Sec-dependant export system for the secretion into the periplasmic space. It is in the periplasm where the *Pseudomonas* lipase protein assumes its catalytically active structure with he assistance of specific intermolecular chaperone named lipase-specific foldase (LiF), encoded by the cognate lipase gene operon. The final secretion to the extracellular medium is mediated by type II secretion pathway formed by a complex of 12 Xcp proteins located across the membranes with one of the proteins, XcpQ forming a pore-like structure in the outer membrane through which the lipase proteins released.

The molecular physiology regarding the mechanisms of lipase secretion by *Staphylococcus* species have been studied (Rosenstein and Götz, 2000). All *Staphylococcus* lipases are translated as a pre-pro-enzyme with a leader signal peptide of 35 to 38 amino acids, followed by a pro-sequence (207 - 321 amino acids) and the mature form, which is the active lipase that appears in the supernatant of the producing *Staphylococcus* strain (383 - 396 amino acids). The function of the signal peptide is to direct the protein into the secretory pathways. The signal peptide is cleaved before the protein is secreted into the extracellular medium. The processing of the pro-peptide has been found to occur after the protein has been excreted into the extracellular medium, and is mediated by two extracellular proteases, ShpI and SphII (Götz *et al*., 1998). The pro-peptide region turned out to be essential as an intramolecular chaperone, required for efficient folding and secretion of the lipase (Götz *et al*., 1998).

It is evident from these studies that it is difficult to predict the effect that a given compound would have on different microorganisms with respect to lipase production. Although a number of compounds are known to repress lipase production, the mechanisms of such effects have not yet been explained. For instance, *Acinebacter calcoaceticus*, secretes a number of extracellular lipolytic enzymes including *lipA* which is repressed by the presence of fatty acids in the culture medium (Kok *et al*., 1995). This suggests the existence of an unidentified regulatory protein, which is believed to repress lipase transcription upon binding of a fatty acid.

In *Staphylococcus*, a transpositional insertion into the *agr* locus resulted in reduced levels of exoproteins, including lipases (Kornblum *et al*., 1990). The *agr* locus is believed to be a global regulator consisting of an operon encoding four proteins, AgrB, AgrD, AgrC and AgrA (Novick *et al*., 1995). ArgC shows homology to signal transducers, and AgrA shows homology to response regulators found in bacterial signalling systems. The two proteins have been implicated in an autocatalytic signal transduction system that responds to environmental stimuli such as glucose and pH (Novick *et al*., 1993; Regassa *et al*., 1992). The AgrD component of the operon has been suggested to encode a transcriptional activator, which upon activation enhances the transcription of the targeted exopotein genes, including the genes encoding lipase activities, with the *argB* encoding a putative processing enzyme that is require for AgrD activity (Ji *et al*., 1995).

The powerful tools of molecular biology have been brought to bear, more new lipase amino acid sequences and three-dimensional structures are appearing, and new approaches for handling lipase's complicated interfacial kinetics are being reported. In addition, more ways are being discovered and used to control lipase activity and therefore using their catalytic power for inefficient chemical processes.

1.7 Conclusions

Applications for lipases can now be developed in much more rational fashion, however this purpose will not be fully achieved unless careful attempts are made to

comprehensively clarify and then integrate all information about the lipase and their properties. Several applications of lipases have been developed throughout the last two decades; innovative uses of these enzymes for a wide variety of organic syntheses and modification of existing fats and oils have increased exponentially during the last five years. Reasons which may partly account for this trend are the increasing availability of lipases from microbial sources coupled to their special capacity to act as catalysts at hydrophobic-hydrophilic interfaces.

The literature cited in this study clearly demonstrated the huge interest in the lipase enzyme. It is also evident from this thesis review that lipases have a wide range of properties, and one can argue that with careful screening, one can find the lipase enzyme with any desired property (Burton *et al*., 2001). The reader should note that literature cited did not include much of the work done on thermophillic lipases, as this will be used throughout this dissertation to set a comparative view, on the experiments performed and how they compare with the lipase discussed in this study.

CHAPTER 2

Introduction into present study

In 2002 Jaeger and Eggert made the statement that “Lipases constitute the most important group of biocatalysts for biotechnological applications”, a statement supported by the fact that more than 1000 original articles are reportedly published in this field annually. But the question remains: What is it that makes lipases so attractive? Firstly, lipases often display exquisite chemoselectivity, regioselectivity and stereoselectivity. Secondly, they are readily available in large quantities because many of them can be produced in high yields from microbial sources. Thirdly, the crystal structures of many lipases have been solved, facilitating considerably the design of rational engineering strategies. Finally, they usually do not require cofactors and do not catalyse side reactions.

To date, the majority of lipases used for biotransformations, have, however been obtained from mesophillic organisms and, despite their many advantages, the application of these enzymes is restricted due to their limited stability to extreme conditions of temperature, pH, ionic strength, etc. (Hough and Danson, 1999). The discovery of life in seemingly prohibited environments, such as hot springs, volcanic areas, Antarctic biotopes, to name but a few, introduced us to “Extremophiles”, organisms capable of living and reproducing in harsh environments (Schiraldi and De Rosa, 2002). These organisms are classified according to their capabilities of growth at extreme temperature, pressure, salinity and pH (Table 2.1).

Two of the most widely used enzymes from extremophiles (Table 2.2) are Taq polymerase from *Thermus aquaticus*, the key ingredient in the polymerase chain reactions (PCR), and Cellulase 103 isolated from an alkalophile, used in the detergent industry to help keep cotton fabric “looking as new even after a thousand washes” (Schiraldi and De Rosa, 2002).

Table 2.1: Examples of extremphiles and the typical environments factors they can withstand (Adapted from Hough and Danson, 1999)

Phenotype	Environment	Typical genus
Thermophillic	55 - 80ºC	*Methaobacterium, Thermus, Geobacillus*
Hyperthermophillic	80 - 113ºC	*Pyrococcus, Thermoproteus, Sulfolobus*
Psychrophillic	-2 to 20ºC	*Alteromonas, Psychrobacter*
Halophillic	2-5 M NaCl	*Haloarcula, Halobacterium, Haloferax*
Acidophillic	pH<4	*Acidianus, Sulfolobus, Thiobacillus*
Alkalophillic	pH>9	*Natronobacterium, Natranococcus*

Table 2.2: Relevant stabilities of extremophilic enzymes with possible future industrial application. (Adapted from Demirjian *et al*.; 2001).

Extremophile	Habitat	Enzyme	Representative application
Thermophile	Moderate thermophiles	Xylanases	Paper bleaching
	Thermophiles (65 - 85ºC)	Lipases	Waste water treatments, Detergent formulations
		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
Alkalophile	High pH	Cellulases	Polymer degradation in detergents

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.,* 2005, submitted). The new isolate, *Geobacillus thermoleovorans* GE-7, is a novel obligate thermophilic bacterium that grows in the temperature range of 45 - 70ºC and has a reported optimum of 65ºC. It is an aerobic, rod-shaped, Gram positive, spore forming bacteria which showed lipase activity and a broad substrate specificity against triacylglycerides ranging from C4 to C18. This isolate was not only able to grow on olive oil as the sole carbon source, but also on a variety of other lipid substrates and even emulsifiers (De Flaun *et al.,* 2005, submitted).

Researchers have found that strains of *G. thermoleovorans* (Lee *et al*., 1999), *G. steraothermophilus* (Sinchaikul *et al*., 2001 a) and *G. thermocatenulatus* (Schmidt-Dannert *et al*., 1994) grown in the presence of lipid substrates produce lipases which are stable at elevated temperatures and can withstand harsh conditions. The lipases from these organisms are grouped into family 1.5 lipases (Chapter 1) with some of the chracteristics of the lipases including the size of these lipases (40 - 45 kDa) and the optimum temperatures ranging from 50 - 70ºC. Various researchers were successful in the cloning the lipase open reading frame from *B. thermoleovorans* (Lee *et al*., 2001), *B. stearothermophilus* (Sinchaikul *et al*., 2001; Kim *et al*., 1998) and *B. thermocatenulatus* (Rua *et al*., 1997) and overexpress it in the mesophillic host *Escherichia coli*. Reports have also been made of modifications made to the lipase gene to either enhance or change the characteristics of the lipase, aid in the purifiction of the lipase and expression of the lipase in various other hosts (Chapter 4). Although the presence of a second lipase has been reported for *B. thermoleovorans* (Lee *et al*., 2001) and *B. thermocatenulatus* (Schmidt-Dannert *et al*., 1994) the aims of this project included:

(1) Purification of the larger “native” lipase produced by *Geobacillus thermoleovorans* GE-7 when grown in the presence of a lipid inducer (Chapter 3)

(2) Cloning of the lipase gene for heterologous expression in *Escherichia coli* to yield higher levels of this lipase (Chapter 4)
(3) Modification of the lipase gene for fast and efiicient purification of the enzyme (Chapter 4)
(4) Study the characteristics of the native and recombinant lipases, including the effect of the modifications of the acitvity of these lipases using both bulk (Chapter 5) and monomolecular techniques (Chapter 6).

Experiments performed, as well as results obtained, for each aim will be discussed in the chapters indicated.

CHAPTER 3

Purification of the "Native" *Geobacillus thermoleovorans* lipase

3.1 Introduction

Literature suggests that every microorganism capable of the production and secretion of lipases requires a very distinct set of environmental conditions for optimal enzyme 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 have a notable effect on production. These influences of culture conditions and other factors on lipase production have been studied extensively (Nesbit *et al*., 1993; Dharmsthiti and Luchai, 1999, Ghanem *et al*., 2000) and discussed in Chapter 1.

An article published by Schmidt-Dannert *et al*. (1994) illustrated the effect culture supernatant can elicit on lipase production by *B. thermocatenulatus*, when the lipase yields were doubled with the halving of the nutrient broth concentration utilised. The effect of additional carbon sources where reported by Ghanem *et al*. (2000) working on *B. alcalophilus* whose lipase production was increased by the addition of maltose, but inhibited by the addition of glucose. 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.

Not only do different lipase responses difer with different types of lipid substrates and even fatty acid chain lengths, but the type of lipid inducer also has an effect on the amount of lipase produced. In two closely related papers reporting on the

lipase production of two thermophilic *Bacillus* strains (*Bacillus* sp. THL027 and *B. thermoleovorans* ID-1) it was shown that induction by a variety of different lipid substrates demonstrated 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 and Luchai 1999).

Furthermore, *B. 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 however found when Triolein was used as the inducer, with Tween 80 proving to be the least effective. Tween 20 was the second most effective inducer, closely followed by soybean oil, mineral oil and olive oil (Lee *et al*., 1999). Lipase production in *G. thermoleovorans* GE-7 could be induced using varying concentrations of stearic acid, not only yielding higher lipase activity but the induction time could be shortened to 20 hours vs. the normal 60 hours with olive oil as inducer (Knoesen, 2004).

An observation made by Chartrain *et al*. (1993), was the sensitivity of lipase production to dissolved oxygen tension. During fed-batch cultivation of *Pseudomonas aeruginosa* it was found that cycling of oxygen tension achieved increased lipase production. Similar observations were made by Schmidt-Dannert and co-workers (1994) who illustrated increased lipase production when *B. thermocatenulatus* was grown in Erlenmeyer flasks with baffles, thus enhancing the aeration of the media. An interesting observation was made by Knoesen (2004) while growing *G. thermoleovorans* GE-7 in bioreactors. He reported the occurrence of a drop in dissolved oxygen tension preceding lipase production, and suggested that this phenomenon could be used as an indicator for the commencement of lipase production.

The purification of enzymes, including lipases, is not always necessary since most commercial applications do not require homogenous preparations. A certain degree of purity is however required to enable efficient and successful characterization of the new enzyme properties (Deutscher, 1990). For the

determination of their primary amino acid sequence and more recently their three-dimensional structure (Saxena *et al*., 2003), purified lipase preparations are however needed as well in industries employing the enzymes for the biocatalytic production of fine chemicals, pharmaceuticals and cosmetics.

In order to stay competitive, industry is constantly looking for faster, inexpensive processes that offer a high enzyme yield suitable for large-scale operations. Traditionally the purification strategies are time consuming and deliver low yields of enzymes. Alternative new techniques are being developed for rapid protein purification. Several excellent reviews on microbial lipase purification have been published (Saxena *et al*., 2003; Sharma *et al*., 2002; Palekar *et al*., 2000) dealing with conventional and newer technologies available for lipase purification. In general most of the microbial lipase purification strategies firstly deal with the removal of cells from the culture media (most of the lipases are extracellular) by either centrifugation or filtration followed by a concentration step utilising ultrafiltration, ammonium sulphate precipitation or organic solvent extraction. Ammonium sulphate precipitation on average offers a higher yield (~87 %) when compared to other concentration techniques (~60 - 70 %) (Aires-Barros *et al*., 1994).

In most cases a single chromatographic step is insufficient for total purification of lipases (Table 3.1), and is usually combined with a wide range of chromatographic steps. Ion exchage chromatography is the most common chromatographic method, with the diethylaminoethyl (DEAE) anion exchange and carboxymethyl cation exchange proving to be the most popular resins (Saxena *et al*., 2003). Stronger ion exchangers based on the triethylaminoethyl groups are becoming more popular for lipase purification. Gel filtration is the second most frequently employed method for lipase purification, with the affinity resins and hydrophobic resins (such as octyl, butyl and phenyl resins) used at varying stages of purification.

Table 3.1: Purification strategies for the “native” lipases from *Geobacillus* (formerly *Bacillus*) reported in literature

Organism	Purification strategy	Yield (Total activity)	Lipase size	Reference
B .stearothermophilus MC7	Ultracentrifugation of supernatant; Sephadex G-200; DEAE Servacell	10.2 % / 167 U	62.5 kDa	Kambourova *et al*., 2003
Bacillus sp. RSJ-1	Ultrafiltration of supernatant; ammonium sulphate precipitation; Q-Sepharose; Sephacryl S-200	19.7 % / 1006 U	37 kDa	Sharma *et al*., 2002
B. thermoleovorans ID-1 BTID-A	Ammonium sulphate precipitation; DEAE-Sepharose CL6B; Superdex 200; Resource PHE; Mono-Q	0.16 % / 0.12 U	18 kDa	Lee *et al*., 2001
B. thermoleovorans ID-1 BTID-B	Heat precipitation; DEAE-Sepharose CL6B; Sephacryl S-200	3.2 % / 73.1	43 kDa	Lee *et al*., 2001
B. alcalophilus	Ammonium sulphate precipitation; Sephadex G-100	5 % / 4500 U		Ghanem *et al*., 2000
B. thermoleovorans ID-1	Ammonium sulphate precipitation; DEAE-sephacel; Sephacryl S-200	19 %	34 kDa	Lee *et al*., 1999
B. stearothermophilus P1	Ultrafiltration of supernatant; Q HyperD column	71 % / 61650 U	43 kDa	Sinchaikul *et al*., 2001 a
Bacillus sp. THL027	Ultrafiltration of supernatnat; Sephadex G-100	27 % / 82.3 U	69 kDa	Dharmsthiti and Luchai, 1999
B. stearothermophilus SB-1	Partitioning of two lipase in aqueous two-phase system			Bradoo *et al*., 1999
B. thermocatenulatus BTL-1	Concentrate supernatant; Hexane extraction of calcium soap, methanol precipitation; Q-Sepharose	11 % / 75 U	16 kDa	Schmidt-Dannert *et al*., 1994
B. thermocatenulatus BTL-2	Butyl-Sepharose; Ultrafiltration; TSK-300 gelfiltration	32 % / 296391 U	40 kDa	Rua *et al*., 1997

Immunopurification, using either monoclonal or affinity purified polyclonal antibodies, is one of the novel purification technologies employed. This process is however percieved as an expensive method, but it is one of the most sensitive affinity purification procedures that can be employed. Bandmann *et al*. (2000) purified the modified recombinant cutinase variants produced in *E. coli* by IgG-affinity chromatography, employing an introduced N-terminal IgG-binding ZZ affinity fusion partner present in all variants.

Researchers working on *Geobacillus* (formerly *Bacillus*) lipases have not only utilised the accepted purification strategies (Table 3.1), but also employed new and exciting concepts for one step chromatographic purification protocols. Palomo *et al*. (2004 c) utilised the aggregative tendancies of *P. fluorescens* lipase in the purification of an *E. coli* expressed *B. thermocatenulatus* lipase via lipase-lipase interactions with the immobilised *P. fluorescens* lipase. Palomo *et al*. (2004 a and b) utilised the phenomenon of “interfacial activation”, i.e. opening of the lid covering the active site, on a hydrophobic support to purify lipases from *B. thermocatenulatus*, *Thermus thermophilus* and *T. aquaticus*. They were not only able to purify the lipases using octadecyl-Sepabeads, but the immobilsed lipase showed increased stabillity, being stable at 70ºC for up to 60 hours without any loss of actvity.

Another method employed for separation is phase separation, a general phenomenon that occurs when two water-soluble polymers are mixed. Instead of using polymers, a polymer and a salt solution can be used to form an aqueous two-phase system. Bradoo *et al*. (1999) used this method with varying PEG/phosphate/NaCl/pH combinations to purify neutral and acidic lipases from *B. stearothermohilus* SB-1.

Review articles on lipase purification procedures shows that no conclusions can be drawn regarding an optimal sequence of chromatographic method that maximizes recovery yields and purification fold. Based on the nature of the lipase produced by the organism, one has to design the protocol for purification involving precipitation and chromatographic steps. However, this will also be guided by the purity of the

enzyme required for its usage, availabillity of resins and chemicals and economic limitations.

3.2 *Materials and Methods*

3.2.1 Chemicals

Unless otherwise stated, all chemicals were obtained from commercial sources, were of analytical reagent grade or better and were used without further purification.

3.2.2 Bacterial strains and media

3.2.2.1 Bacterial strains

Geobacillus thermoleovorans, formally *Bacillus* GE-7, isolated from a Wes Driefontein mine, was kindly supplied by Dr. Mary de Flaun. Confirmation of the strain identity was done using 16S rDNA sequencing (Section 3.2.2.3).

3.2.2.2 Growth of the bacterial strains

G. thermoleovorans was grown and maintained on R_2A plates containing 0.5 g/l yeast extract; 0.5 g/l peptone; 0.5 g/l casamino acids; 0.5 g/l glucose; 0.5 g/l starch; 0.3 g/l sodium pyruvate; 0.3g/l potassium phosphate and 0.05 g/l magnesium phosphate; 16 g/l bacteriological agar, pH 7.5 at 55ºC.

Lipase production was performed in media defined by Knoesen (2004), termed Lipase production media (LPM). The media consisted of 5 g/l glucose; 2.5 g/l protease peptone; 0.25 g/l citrate; 0.4 g/l potassium phosphate; 0.2 g/l magnesium sulfate and 0.01 M calcium chloride, pH 6.5. To this 0.5 ml/l mineral solution consisting of 50 ml concentrated hydrochloric acid; 35 g/l $FeSO_4.7H_2O$; 11.48 g/l $MnSO_4.7H_2O$; 11 g/l $ZnSO_4.7H_2O$; 5 g/l $CuSO_4.5H_2O$; 2 g/l $CoCl_2.5H_2O$; 1.3 g/l

$Na_2MoO_4.2H_2O$; 2 g/l H_3BO_3; 0.35 g/l KI and 0.5 g/l $Al_2(SO_4)_3$ was added. Lipase activity was induced with 10 g/l olive oil unless stated otherwise.

Plate screening for lipases was performed using tributyrin plates prepared by mixing 450 ml LB plate mixture, consisting of 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl and 16 g/l agar, pH 7.0, mixed with 50 ml of a 1 g/l gum arabic and 10 g/l tributyrin mixture which were sonicated 3 times using 1 min sonication and 1 min resting cycles with a 50 % duty cycle.

3.2.2.3 Confirmation of bacterial strain identity

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 (Kieft *et al*., 1999).

The reaction mixture contained 5 μl of 10 X 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 hot-start, 2.6 U Expand High fidelity (Roche Diagnostics) enzyme was added, yielding a final reaction volume of 50 μl.

Thermal cycling was performed using an Eppendorf Mastercycler Temperature Gradient Personal thermal cycler 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 72ºC was added to ensure complete elongation of the amplified product.

The PCR product was visualized on a 1% (w/v) agarose gel containing 0.5 μg/ml (final concentration) ethidium bromide. The agarose gels were prepared in TAE buffer containing 100 mM Tris (2-amino-2(hydroxymethyl)-1,3propanediol)-HCl (pH

8.0), 50 mM EDTA (disodium ethylenediaminetetraacetic acid) and 100 mM glacial acetic acid. Electrophoresis was performed at 5.6 V/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 (Promega) as described in Section 4.2.5.3. The transformation of competent *E. coli* strain JM109 cells was performed according to the method described by Sambrook *et al.* (1989). The transformation mixture was then plated out on LB (Luria-Bertani)-media plates supplemented with ampicillin (60 mg/l), isopropylthio-β-D-galactoside (IPTG) (10 mg/l) and 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (40 mg/l). Plates were incubated at 37ºC for 20 hours. Blue-white colony selection was performed. White colonies were inoculated into tubes containing 5 ml 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 Sambrook *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 (Amersham Biosciences) and eluted into 50 μl elution buffer (10 mM 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 (Patterton and Graves, 1999).

3.2.3 Assays

3.2.3.1 Protein assay

Protein concentrations were estimated either by absorbance at 280 nm or by using the micro bicinchoninic acid (BCA) method (Smith *et al*., 1985), which was supplied as a kit by Pierce (Rockford, IL, USA).

The BCA protein assay reagent is a highly sensitive reagent for the spectrophotometric determination of protein concentration. The methods used were those supplied with the commercially available kits. A set of protein standards were prepared with bovine serum albumin (BSA), provided by Pierce as part of the kit, in the range of 25 - 200 µg/ml (Figure 3.1). Standards, as well as protein samples, were prepared in a 96 well flat-bottomed microtitre plate and read at a wavelength of 540 nm using a microtitre plate reader.

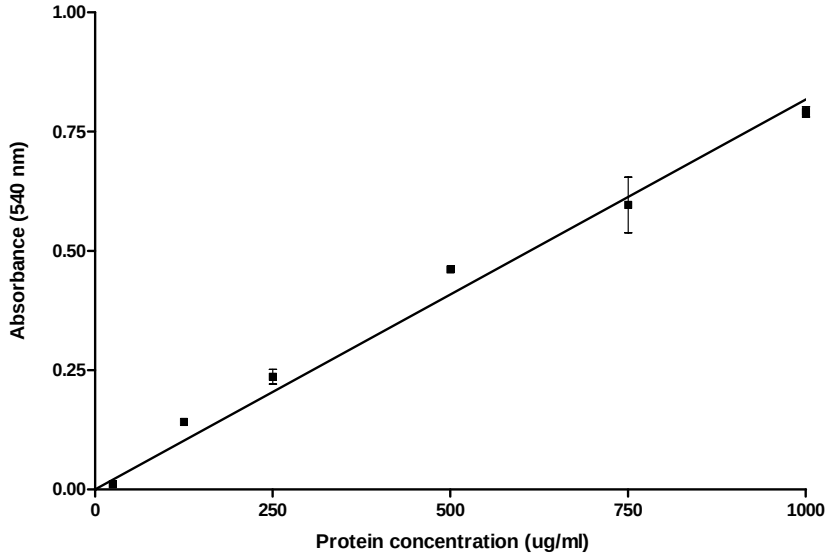


Figure 3.1: Standard curve for the BCA protein assay with BSA as protein standard.

3.2.3.2 Lipase assays

The assays were done according to standard methods described in literature, namely the olive oil assay and *p*-Nitrophenyl ester assay. Activity assays were always performed in duplicate and the appropriate blanks included.

3.2.3.2.1 Olive oil assay

The colorimetric method of Duncombe (1963) utilizes the formation of a Cu^{2+} soap with the fatty acids liberated by lipase, which is then extracted into an organic phase. The extracted Cu^{2+} is determined by reaction with diethyldithiocarbamate.

The copper reagent was prepared by separately dissolving 16.125 g $Cu(NO_3)_2.3H_2O$ and 32.5 ml triethanolamine in 200 ml volumes of distilled water. After mixing, the pH was adjusted to 7.5 and the volume made up to 500 ml in a volumetric flask. The sodium diethyldithiocarbamate reagent (0.25 % w/v) was freshly prepared in 2-butanol.

A standard curve was prepared by diluting a stearic acid stock solution to yield a range of 0 – 0.4 µmole fatty acid per tube. 0.5 ml H_2O and 2.5 ml copper reagent were added to each tube which was vortexed, 5 ml chloroform was added and again the tubes were vortexed for 30 seconds. It was then centrifuged for 2 min to separate the phases and the aqueous phase was removed by aspiration with a Pasteur pipette. An aliquot (2 ml) of the chloroform solution was pipetted into a clean dry tube, care being taken that the pipette did not touch the inner wall of either tube as traces of copper-containing aqueous phase might be transferred to the reagent. Diethyldithiocarbamate reagent (0.5 ml) was added to the chloroform solution and after the solutions had been mixed, the absorbance at 440 nm was measured against a blank solution that had been subjected to the same procedure. The calibration curve obtained is given in Figure 3.2.

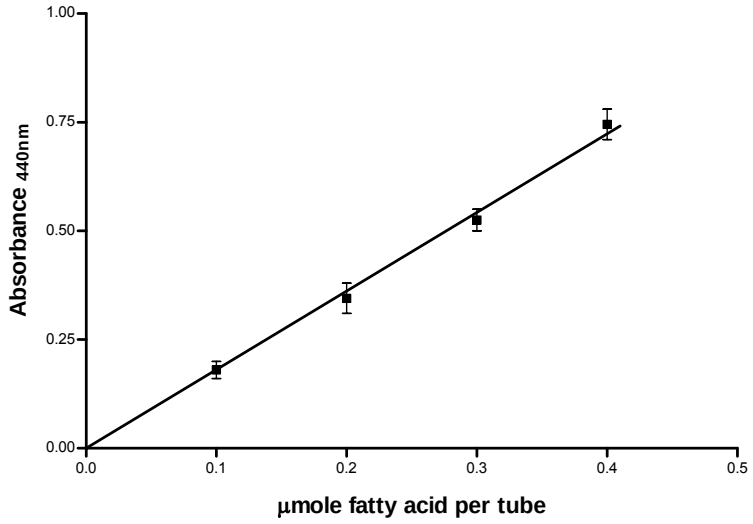


Figure 3.2: Standard curve for assay of fatty acids released with the olive oil assay using stearic acid as standard.

The olive oil emulsion used in the assay consisted of 10 % (v/v) olive oil and 1 % (w/v) gum Arabic in distilled water, emulsified by mixing for 2 min with a Branson Cell disrupter B-30. The assay buffer consisted of 1 mM Tris-HCl buffer, 0.1 M NaCl and 5 mM calcium chloride, pH 8.0.

The 0.5 ml assay buffer was mixed with the olive oil emulsion (0.5 ml), whereafter an appropriate enzyme dilution was added and incubated at 55ºC in a shaking water bath. The reaction was stopped by the addition of the copper reagent (2.5 ml) and chloroform (5 ml). The rest of the procedure was the same as for the standard curve.

Enzyme activity, U/ml (µmole fatty acid released min^{-1} ml^{-1} enzyme), was calculated using the following formula:

$$U/ml = \frac{V}{v \times E \times d} \times \frac{A_{440}}{t}$$

V = Final volume of chloroform analysed (ml)

v = Volume of enzyme assayed (ml)

E = Gradient of standard curve

t = Incubation time of assay (min)

d = Light path of cuvette (1 cm)

A_{440} = Absorbance at 440 nm

One unit (U) of lipase activity is defined as the amount of enzyme that releases 1 µmole of fatty acid per minute.

3.2.3.2.2 p-NPP assay

The assay method is based on the method used by Winkler and Stuckmann (1979) and determines both lipase and esterase activity. This substrtae is not hydrolised by all lipases, example the pancreatic lipases. An artificial substrate was used to assay lipase activity, namely *p*-nitrophenyl ester, *p*-nitophenylpalmitate (*p*-NPP, Figure 3.3). This assay measures the release of the *p*-nitropenyl (*p*-NP) from *p*-NPP. The *p*-NPP was prepared from two solutions. Solution 1 contained 30 mg *p*-NPP dissolved in 10 ml 2-propanol. Solution 2 contained 2 g sodium deoxycholate and 0.5 g gum arabic acid dissolved in 450 ml 50 mM Tris-HCl, pH 8.0. The assay mixture was prepared by the dropwise addition of 1 ml of solution 1 to 9 ml of solution 2 with continuous stirring to obtain a stable emulsion. 50 µl of the enzyme solution was then added to 600 µl of the assay mixture in a 2 ml plastic cuvette. The liberated *p*-NP was photometrically measured at a wavelength of 410 nm and a temperature of 60ºC using a Beckman DU 650 spectrophotometer fitted with a circulating water heated, Auto 6-sampler connected to a Haake D1-L heating bath/circulator. Rates were calculated using the kinetic software package supplied with the instrument.

Figure 3.3 The structure of *p*-nitrophenyl palmitate.

Enzyme activity, U/ml (µmole fatty acid released min^{-1} ml^{-1} enzyme), was calculated using the following formula:

$$U / ml = \frac{V}{v \times \varepsilon \times d} \times \frac{A_{440}}{t}$$

V = Substrate volume (ml)

v = Enzyme volume (ml)

ε = Extinction coefficient of *p*-NP at 410 nm (= 15 ml µmole^{-1} cm^{-1}) (Vorderwülbecke *et al*., 1992)

t = Incubation time of assay (min)

d = Light path of cuvette (1 cm)

A_{440} = Absorbance at 440 nm

3.2.4 Electrophoresis

3.2.4.1 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to monitor the purification process and to determine the relative molecular mass (M_r) of the lipase proteins by comparing its electrophoretic mobility with those of standard proteins with known molecular masses, using a 10 % resolving gel and 4 % stacking gel.

SDS-PAGE was performed using the “Mighty Small” miniature slab gel electrophoresis unit, SE 200 from Hoefer Scientific Instruments. Electrophoresis was performed on approximately 10 µg protein. The protocol was used as described by Laemmli (1970). The proteins were visualized by silver staining (Rabillot, 1989) or Coomassie Brilliant blue G250 staining done according to the Hoefer instruction manual. The protein standards used were obtained form BIO-RAD and sizes of the standards will be specified with each figure.

3.2.4.2 Isoelectric focusing (IEF)

Isoelectric focusing was carried out to determine the pI values as well as to confirm purity for the purified protein. The method described by Robertson *et al*. (1987) was used. Electrophoresis was performed using Pharmalyte carrier ampholytes (Pharmacia Fine Chemicals) pH 3-10 and loading approximately 20 µg protein sample. The gel was pre-focused at 150 V for 30 min and after loading the sample it was focused for 1½ h at 200 V and 1½ h at 400 V. The catholyte was 25 mM sodium hydroxide solution and the anolyte was 20 mM acetic acid. Protein standards with known pI's were obtained from BIO-RAD and were used as markers to determine the relative pI of the protein in the samples. Gels were fixed with 10 % (w/v) trichloroacteic acid for 30 min, followed by three rinses with 1 % (w/v) trichloroacteic acid overnight to effectively remove the ampholytes. The proteins were visualised using the Crocein S staining method (Pal *et al*., 2004).

3.2.4.3 Preparation of electrophoretic gels for zymograms

Initial activity stains using proteins separated in SDS-PAGE gels were unsuccessful. It was decided to use the method described by Tena and Renaudin (1998) where the proteins separated in the gels are first thoroughly denatured followed by renaturation of the proteins.

Following electrophoresis (Section 3.4.2.1), all traces of SDS was removed by rinsing the gels 2 times, each for 1 hour at room temperature, in washing buffer 1 (20 % (v/v) 2-propanol, 5 mM β-mercaptoethanol and 50 mM Tris-HCl, pH 8.0) and then for 1 h in washing buffer 2 (5 mM β-mercaptoethanol and 50 mM Tris-HCl, pH 8.0). The proteins were further denatured by rinsing the gels 2 times, each for 30 min at room temperature, in denaturing buffer (8 M urea, 5 mM β-mercaptoethanol and 50 mM Tris-HCl, pH 8.0). The gel-bound lipases were allowed to renature overnight at room temperature with 3 changes of the renaturing buffer (0.05 % (v/v) Tween-20, 5 mM β-mercaptoethanol and 50 mM Tris-HCl, pH

8.0). The gels were then incubated for 30 min in assay buffer (1 mM Tris-HCl, pH 8.0, 0.1 M NaCl and 5 mM $CaCl_2$) before doing the zymogram.

IEF gels were fixed for 30 min in 10 % (w/v) trichloroacteic acid, before the gel was rinsed overnight with three washes in 1 % (w/v) trichloroacetic acid. The gel was further rinsed in 50 mM Tris-HCl, pH 8.0 for 15 min followed by the zymogram.

3.2.4.4 Lipase Zymogram

Various zymogram methods were tested using fluorogenic substrates, chromogenic substrates and the use of triacylglycerides set in agarose gel. The fluorogenic zymogram was adapted from the Rhodamine-Tricglyceride-agarose asay described by Jette and Ziomek (1994) for the detecton of lipase activity. The assay “mixture” consisted of 1 % (w/v) agarose (final concentration) mixed with 0.02 % (v/v) Rhodamine 6B (final concentration) and triolein. Activity was monitored in the same fashion as for Rhodamine olive oil plates (Kouker and Jaeger, 1987). Methyl red or Nile blue was utilised as the chromogenic indicator in a procedure the same as previously desribed with the exception that Tween 20 was used as substrate (Samail *et al*., 1989). The principle used was that of a pH change in the support media upon the release of free fatty acids. The third method used was reported by Snellman (2002) using emulsified tributyrin (2.5 % v/v) mixed with 2 % (w/v) agarose. A positive assay was shown by the formation of a clearing zone, the same principle applied for tributyrin plates.

The method followed depended on the use of the overlay gel prepared by first making a suspension of 2.5 g/l triclyceride in 1 g/l gum arabic. This was emulsified by sonification and mixed with 2 % (w/v) melted agarose solution prepared in 50 mM Tris-HCl, pH 8.0. If needed the chromogenic- or fluorogenic substrates were added and allowed to set and the SDS-PAGE gel, which was equilibrated in 50 mM Tris-HCl, pH 8.0 for 30 min, placed on top and left at 55ºC overnight. Activity was visualized as described.

3.2.5 Purification of G. thermoleovorans lipase

3.2.5.1 Screening for lipase activity

Plate screening for lipase was done using tributyrin plates (Fryer *et al*., 1966) at 55ºC. Lipase screening in culture media were done in 500 ml Erlenmeyer flasks with 60 ml R_2A media (Section 3.2.2.2) containing 2.5 g/l of the different inducers. To this, a 5 ml aliquot of *G. thermoleovorans* GE-7, growing in mid-exponential phase (~60 k Klett units) was added and the flask left at 55ºC on a rotary shaker shaking at 200 rpm. Samples were withdrawn aseptically every 5 hours to determine lipase production. Lipase activity was monitored using a fast colorimetric assay with *p*-nitrophenyl palmitate as substrate (Section 3.2.3.2.2) or a discontinuous lipase assay with emulsified olive oil as substrate (Section 3.2.3.2.1). The resultant activity profiles for the two substrates correlated well (Figure 3.7), and the fast *p*-nitrophenyl palmitate assay was used in all further induction and growth experiments.

3.2.5.2 Production of lipase

Initial studies were performed with olive oil as the inducer for lipase activity. Studies by Knoesen (2004) showed that higher yields of enzyme could be obtained when stearic acid was used as the inducer. Thus stearic acid was used as the inducer in all subsequent experiments, which also solved the problem of the excess oil left in the media after growth when using olive oil.

Induction with olive oil (Protocol A-C) was done in LPM supplemented with 2.5 g/l olive oil. Stearic acid induction (Protocol D-E) was performed by the addition of stearic acid (dissolved in chloroform) to a final concentration of 1.6 mM in the media. This not only led to higher yields of the enzyme, but the enzyme was produced after only 17-20 hours (versus the normal 50 – 60 hours with olive oil) indicating the free fatty acid might be the real secretagogue.

3.2.5.3 Purification of lipase

All chromatographic steps were performed at 4ºC, with the exception of the experiments perfomed during Protocol D and E. These experiments were performed at room temperature.

3.2.5.3.1 Purification protocol A

Induction studies showed that the lipase was secreted into the culture medium, thus for all subsequent experiments the culture medium supernatant was used after the cells were removed by centrifugation at 10 000 xg for 10 min. The supernantant was applied to various chromatography resins, including DEAE-Toyopearl, Super-Q, Phenyl-Toyopearl and CM-Toyopearl. Initial small scale testing indicated that the lipase interacted well with anion exchange resins, such as DEAE-Toyopearl and Super-Q; therefore, the anion exchanger (DEAE-Toyopearl) was used as a first purification step. The sample was applied to a column (2.5 cm x 10 cm) pre-equilibrated with 50 mM Tris-HCl buffer, pH 8.0, at a flow rate of 1 ml/min and fractions were collected every 2.5 min. The column was washed with 2 column volumes 50 mM Tris-HCl buffer, pH 8.0, following the application of the sample and the bound proteins were eluted with 4 column volumes of a 0 – 0.5 M NaCl gradient in 50 mM Tris-HCl buffer, pH 8.0. Activity determinations were performed using the *p*NPP assay (Section 3.2.3.2.2) and the A_{280} readings used to measure protein concentration for construction of appropriate elution profiles.

The activity peak was concentrated with the Amicon Ultrafiltration unit fitted with a 10 kDa molecular weight cut-off membrane, and the sample applied to either Sephadex S-200-HR or Sephadex S-100-HR columns (1.5cm x 60cm). The columns were pre-equilibrated with 50 mM Tris-HCl buffer, pH 8.0, with 300 mM NaCl and run at a constant speed of 12 ml/h and 1.5 ml fractions were collected, assayed (Section 3.2.3.2.2) and protein concentrations determined (A_{280}). Fraction purity was assessed using SDS-PAGE (Section 3.2.4.1) to estimate the efficiency of each purification step.

3.2.5.3.2 Purification protocol B

The first step for this protocol was the concentration of the supernatant proteins by ammonium sulphate precipitation. Precipitation with $(NH_4)_2SO_4$ was performed to 50 % saturation at 4ºC followed by centrifugation for 30 min at 9 500 xg at 4ºC to separate the protein pellet from the supernatant.

The 50 % $(NH_4)_2SO_4$ pellet was dialyzed overnight against 50 mM Tris-HCl buffer, pH 8.0, at 4ºC, mixed with Triton X-100 (to reduce hydrophbic interactions) to a final concentration of 1 % (v/v) and applied to a pre-equilibrated Super-Q column (Nishio *et al*., 1987). The procedure followed was the same as previously described (Section 3.2.5.2.1) except for the addition of 1 % (v/v) Triton X-100 to the buffers. Several attempts were made to remove the Triton X-100 from the proteins (Deutscher, 1990) using various precipitation methods. The first method involved precipitating the protein with the slow addition of ice-cold acetone with 1 % (v/v) HCl, while the sample was stirred at 0ºC. This resulted in the precipitation of the proteins, but with no recovery of activity. Secondly, trichloroacetic acid (TCA) precipitation was tested by mixing the sample with TCA to a final concentration of 40 % (w/v), follow by centrifugation at 12 000 xg for 10 min. The pellet was rinsed with 2-propanol to remove the unbound Triton X-100, before the sample was resuspended in 50 mM Tris-HCl buffer, pH 8.0. The same results were obtained, with no detectable activity recovered. In a third attempt the sample was mixed with ice cold acetone in a 4:1 ratio (Acetone:sample), and the pellet collected by centrifugation as explained. No activity was obtained (Nishio *et al*., 1987).

The use of gel filtration with deoxycholate in the mobile phase has been reported for the removal of Triton X-100 from protein samples (Deutscher, 1990). The Sephadex S-200/100-HR media were prepared by the same procedure as in Section 3.2.5.2.1 with the addition of 1 % (w/v) deoxycholate to the mobile phase.

3.2.5.3.3 Purification protocol C

The initial part of the purification procedure was repeated as decribed (Section 3.2.5.2.2). Before attempting the gel filtration step the sample was applied to the Solvent-Detergent removal resin obtained from Sigma-Aldrich. The column (1.5 cm x 12 cm) was allowed to run with gravity and 2 ml fractions collected. The mobile phase used was 50 mM Tris-HCl buffer, pH8.0. Samples were analysed for protein concentration (A_{280}) and assayed for activity.

3.2.5.3.4 Purification Protocol D

Following $(NH_4)_2SO_4$ precipitation (Section 3.2.5.3.2) the precipitate was dissolved in a minimum amount of buffer and applied to the Phenyl Toyopearl column. All subsequent procedures were performed at room temperature. The flow rate for the column was approximately 1 ml/min and 2 ml fractions were collected. A_{280} readings were taken and *p*NPP-assays (Section 3.2.3.2.2) done to monitor activity.

After application of the protein sample onto the column (1.6cm x 5cm) it was washed with 2 column volumes of 50 mM Tris-HCl, pH 8.0 with 2 M $(NH_4)_2SO_4$. The proteins were then eluted with a linear gradient of 2 M – 0 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl, pH 8.0 followed by a washing step of 2 column volumes of 50 mM Tris-HCl, pH 8.0. This was followed by a 0 % - 25 % (v/v) ethanol gradient in 50 mM Tris-HCl, pH 8.0. The peak containg lipase activity was collected, concentrated using the Amicon stirred cell ultrafitration system with a 10 kDa cut off and applied to a gel filtration column (Toyopearl HW 50F). The column (1cm x 180 cm) was run at a speed of 12 ml/h and 1 ml fractions were collected. The column was equilibrated in 10 % (v/v) ethanol in 50 mM Tris-HCl, pH 8.0 and this buffer was used as running buffer. Samples were assayed for activity and aliquots taken were subjected to SDS-PAGE (Section 3.2.4.1) to evaluate the purification steps.

3.2.5.3.5 Purification protocol E

The culture supernatant was concentrated as described earlier (Section 3.2.5.3.2) with the difference that the protein precipitate was left to air-dry overnight. This was followed by a modification of the delipidification protocol reported by Verger *et al*. (1969). Briefly, the pellet was rinsed three times with 20 ml chloroform:*n*-butanol (9:1) mixture, followed by three rinses with 20 ml chloroform:*n*-butanol (4:1) mixture, two rinses with 10 ml acetone and lastly two rinses with 10 ml diethylether. The pellet was left to dry overnight and resuspended in a minimal amount of 50 mM Tris-HCl buffer, pH 8.0. The sample was applied to a pre-equilibrated Phenyl-Toyopearl column and the protocol followed as described previously.

The peak containing activity was applied to Toyopearl HW50F resin as described previously. Samples taken were assayed for activity and subjected to SDS-PAGE (Section 3.2.4.1) to evaluate the purification attempts. Zymograms (Sections 3.2.4.3 and 3.2.4.4) were done on the SDS-PAGE gel and samples which were subjected to isoelectric focusing (Section 3.2.4.2).

3.3 Results

3.3.1 Confirmation of the G. thermoleovorans (GE-7) strain identity

The PCR amplification was done according to section 3.2.2.3. Figure 3.3 depicts the 16S rDNA PCR amplification product obtained from *G. thermoleovorans* GE-7 cells. Lanes 1 and 3 show the isolated 16S rDNA approximately 1500 bp in size. The 16S rDNA product was cloned into pGEM®T-easy cloning vector and competent *Escherichia coli* (Strain JM 109) were transformed. Minipreps were then performed to isolate the plasmid.

Restriction digestion with *Eco*R1 enzyme produced two bands (approx. 900 bp and 600 bp indicated by A & B) and the pGem®T-easy vector (Figure 3.4, 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 564 bp marker bands are not visible due to low marker DNA concentrations). Lane 2 shows the untransformed pGem®T-easy plasmid digested with *Eco*R1.

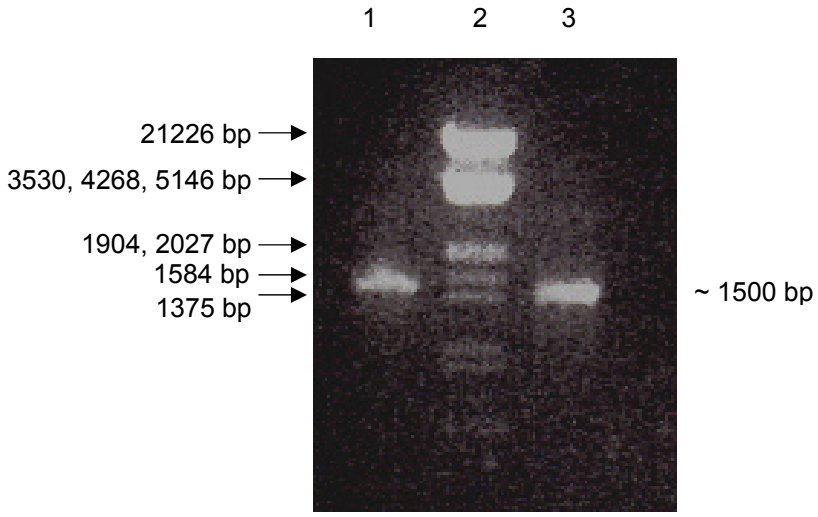


Figure 3.3 Gel electrophoresis of PCR amplification product of 16S rDNA of GE-7 strain. Lanes 1 and 3 are duplicate experiments and lane 2 was the λIII size marker set. Adjacent, to the left are the band sizes of the λIII marker set.

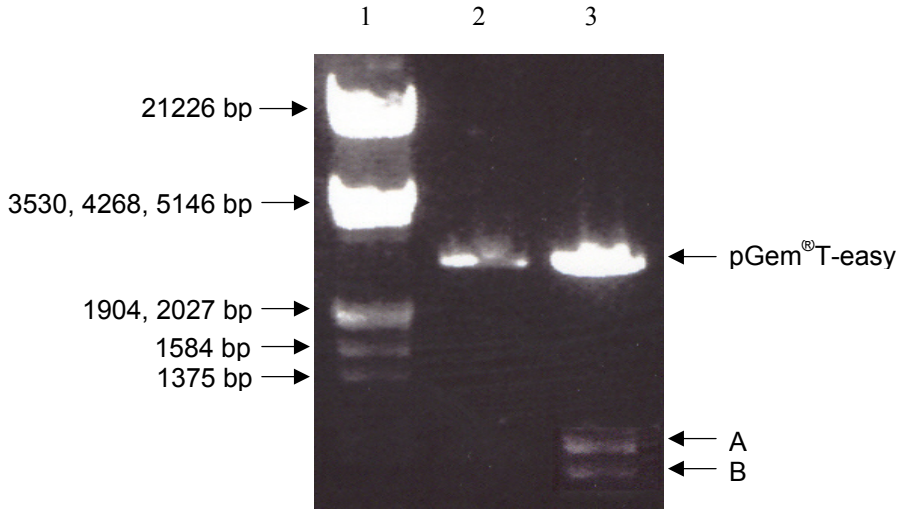


Figure 3.4 Gel electrophoreses of *Eco*R1 digest of 16S rDNA insert from the pGEM®T-easy vector (Lane 3). Lane 1 shows the λIII marker and lane 2 the *Eco*R1 digest of untransformed pGem®T-easy vector. Adjacent to the left are the band sizes of the λIII marker set.

A

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Ge7t7  1    CGGACTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGGATTAGAGTTTGATCA 65
GtT80  1                                                      TAGAGTTTGATCC 13

Ge7t7  66   TGGCTCAGGCACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCAAATCGGAGCT 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 3.5 Sequence alignments of *Geobacillus thermoleovorans* 16S rDNA partial sequences obtained by using T7 (forward primer) (A) and SP6 (reverse primer) with the 16S rDNA sequence for *G. thermoleovorans* T80. (B). Alignments were performed with DNAssist ver. 2.1 (Patterton and Graves, 1999)

Sequence analysis of the 1522 bp 16S rDNA insert ligated into a pGEM®T-easy cloning vector gave a 413 bp (forward sequence) and 490 bp (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 (Figure 3.5). 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 was sufficient evidence to confirm the identity of this isolate.

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 (Patterton and Graves, 1999). The exact position of the restriction site divided the 1522 bp 16S rDNA fragment into a 678- and 844 bp fragments respectively. This correlates well with the band sizes obtained during our *Eco*R1 restriction analysis (Figure 3.4). The strain's authenticity was confirmed with 16S sequencing during every series of experiments requiring growth of the bacterium.

3.3.2 Screening for lipase 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* GE-7 colonies indicated the presence of lipase activity (Figure 3.6).

It was clear that both olive oil and Tween 80 were excellent candidates as inducers, with olive oil giving slightly higher enzyme yields (Figure 3.7). The use of olive oil also led to the appearance of two lipase peaks, an occurrence also found during growth of the organism in LPM, the relevance of which will be discussed later in this chapter. Similar results were obtained when the olive oil- and *p*NPP assay (Figure 3.8) were compared, and it was decided to use the faster *p*NPP assay for the purification experiments.

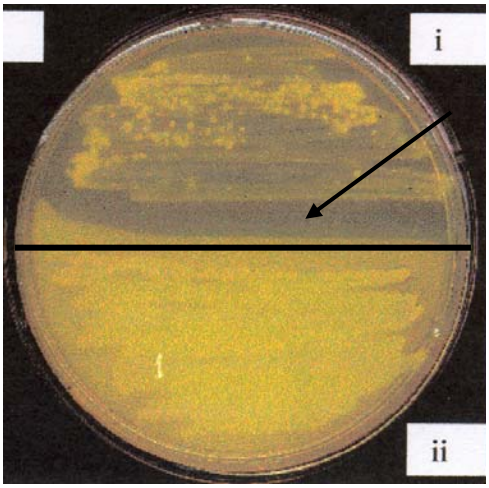


Figure 3.6 Glycerol tributyrate agar with *G. thermoleovorans* (i) and a known non-producer of lipase (*E. coli* JM109) (ii). The arrow indicates the clearance zone where the tributyrin has been hydrolysed, the opaque zone on the other side of the line is where no lipase activity is evident.

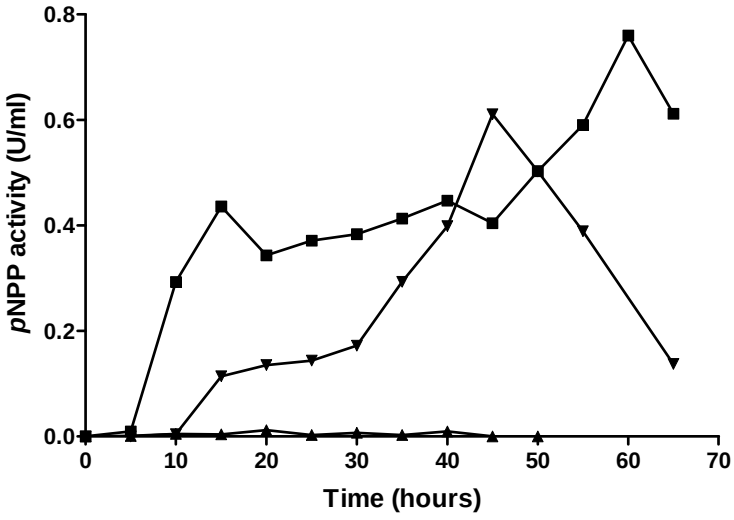


Figure 3.7: Lipase induction for *G. thermoleovorans* GE-7 grown in R_2A media in the presence of olive oil (■),Tween 80 (▼) and tributyrin (▲). The values shown are the means of two independent experiments.

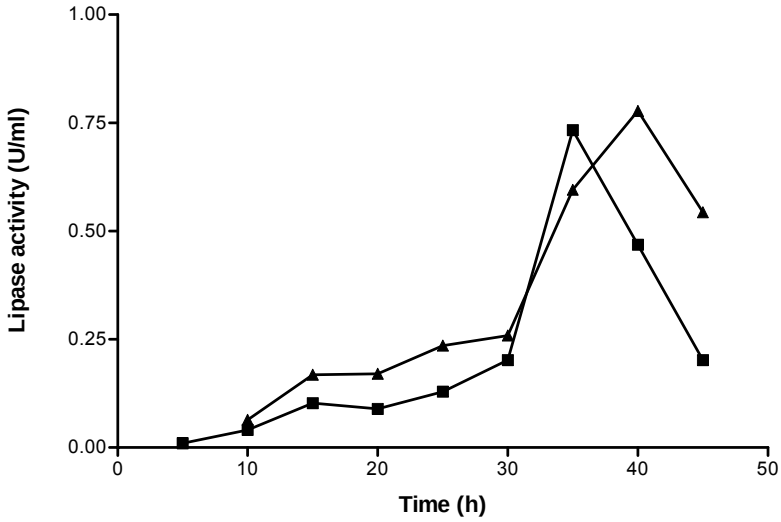


Figure 3.8 Activity obtained using the *p*-Nitrophenyl-palmitate (■) and olive oil activity assays (▲). Cultivation was performed in lipase production media with 2.5g/l olive oil as inducer.

3.3.3 Growth and lipase production

Correlating growth and lipase production was always done in the following manner: the strain would be streaked out onto R_2A plates and grown overnight at 55ºC. This would be used as source from which the pre-inocula (100 ml LPM in a 500 ml Erlenmeyer flask) was inoculated. The pre-inoculum was allowed to grow on a rotary shaker at 200 rpm at 55ºC until a Klett reading of 60 was obtained. 5 ml of this pre-inoculum was used to inoculate the lipase production flasks (500 ml Erlenmeyer flasks) containing 60 ml LPM with the inducer to be used for that experiment. The cells were again grown on the rotary shaker at 200 rpm at 55ºC until the flasks were harvested. Lipase activity was monitored using the p-NPP assay (Section 3.2.3.2.2) for reasons explained above.

Addition of the olive oil was done before sterilization of the media and was added to each flask to obtain a final concentration of 2.5 g/l. With the use of stearic acid as the inducer it was dissolved in chloroform and added to the flasks minutes before inoculation to a final concentration of 40 mM.

3.3.4 Purification of "native" lipase

3.3.4.1 Purification protocol A

Cells were separated from the culture supernatant by centrifugation at 10 000 xg for 10 min at 4ºC. This procedure was followed for all purification protocols. What was notable, especially when olive oil was used as inducer, were the formation of an emulsion in the culture and the presence of a "milky" substance at the top of the supernatant after centrifugation. The milky layer was removed by aspiration before experiments were started as it showed only low levels of lipase activity.

Preceding the purification attempts, culture supernatant was added to 5 ml of different chromatographic resins (anionic, cationic and hydrophobic) to evaluate the suitability of each and to decide on the best resin for the initial purification step. Following application of the sample to the resins, non-bound proteins were removed by rinsing with appropriate buffer and the bound proteins eluted using stepwise increases in the NaCl concentrations (anionic and cationic resins) and decreasing ammonium sulphate concentrations (hydrophobic resins). Lipase activity could be recovered from the anion exchange resins (DEAE-Toyopearl and Super-Q) but very low yields were obtained for cation exchange resins (CM-Sepharose and CM-Sephadex) and no activity was recovered from the hydrophobic resins (Phenyl-Toyopearl and Butyl-Toyopearl). The data pointed to the use of either DEAE-Toyopearl or Super-Q anionic resins as the first purification step.

Different combinations of DEAE-Toyopearl or Super-Q resin coupled to Sephacryl S-200-HR and Sephacryl S-100-HR combinations were tested as the first part of our purification strategy, as described in Section 3.2.5.3.1. A_{280} readings and *p*-NPP assays was performed and the typical elution profile obtained when either DEAE-Toyopearl or Super-Q resin was used is represented in Figure 3.9 a. The best "activity peak" (example fractions 90 - 130, Figure 3.9 a) was pooled after the ion exchange column runs and concentrated with the Amicon stirred cell ultrafiltration unit with a 10 kDa cut-off membrane to 5 % of the gel filtration column

volume before application to the different type of gel filtration resin used (Figure 3.9 b) in the presence of 300 mM NaCl.

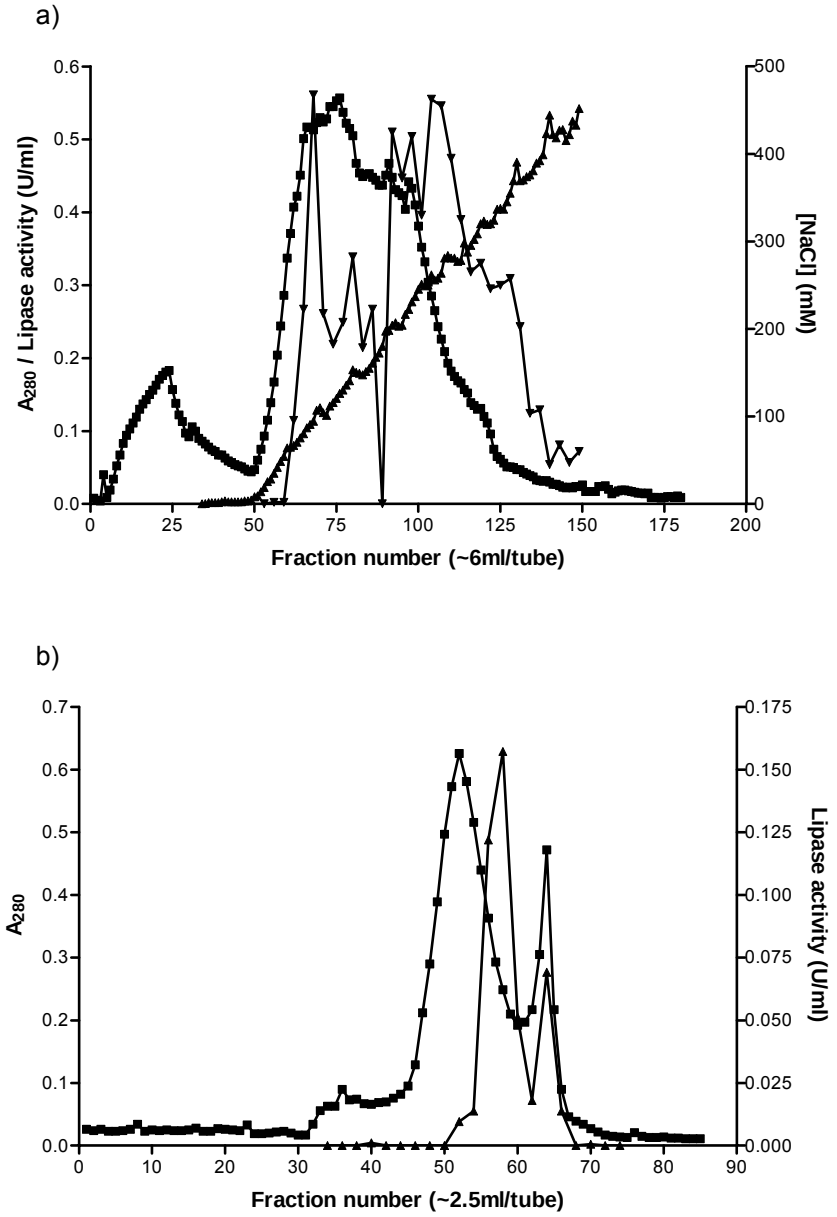


Figure 3.9: a) Typical elution profile obtained for the Super-Q and DEAE-Toyopearl resins showing the A_{280} readings (■), *p*NPP acivity (▼) and NaCl concentrations (▲). b) Typical elution profiles obtained for the Sephacryl S-200-HR and Sephacryl S-100-HR columns showing A_{280} readings (■) and the lipase activity was measured using the *p*NPP assay (▼).

It is clear from the data presented in Table 3.2 that attempts to separate the lipase fraction from the rest of the proteins present in the culture supernatant were successful, no matter what combinations of ion exchanger and gel filtration resins was used. Coupled to this the normal lipase yield after these steps varied from 4 - 25 % and the specific activity could only be increased to a maximum of 24 U/mg, with almost no effect on the protein purity. The best was a a specific activity of 539 U/mg and a yield of 0.4 %, with the lipase still not in a homogenous state. This was the exception and the activity yield was too low to succesfully attempt any other purification steps after this.

Table 3.2 Typical purification table for experiments done following Purification protocol A

Purification step	Total activity U	Specific activity U/mg	Purification fold	Yield %
Culture supernatant	1531	2.6	1	100
Dialysis	1600	4.2	1.6	104
DEAE-Toyopearl	1108	12.7	4.9	72
Gel filtration	383	24.3	9.4	25

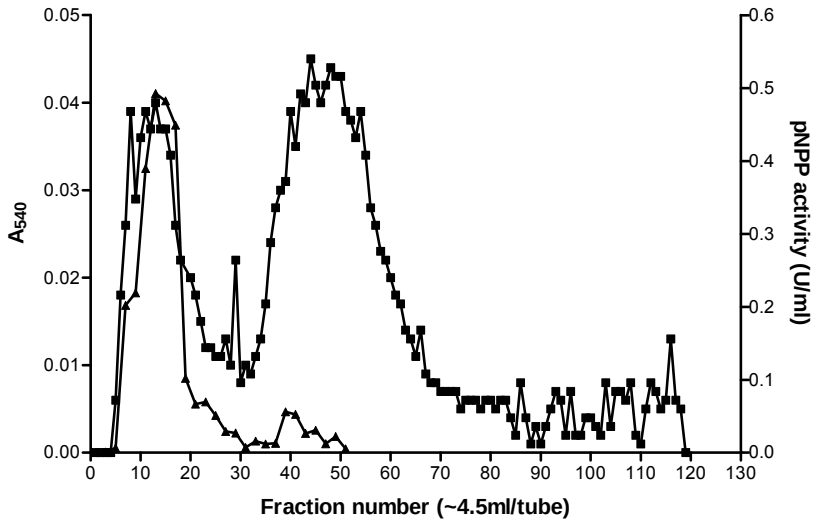
3.3.4.2 Purification protocol B

The culture supernatant was concentrated using ammonium sulphate to 50 % saturation, not only to concentrate the sample but to serve as a possible purification step. Initial tests showed that this level of saturation resulted in the best ratio of recovered lipase versus total protein content. The typical values obtained were an 80 % activity yield and removal of up to 75 % (with an average of 50 %) of the contaminating proteins.

The sample was first applied to the DEAE-Toyopearl and Super-Q columns after dialysis without the addition of any detergents, but the same elution profiles were obtained as in Figure 3.9 a. The decision was made to add Triton X-100 to the sample and column buffers to attempt better separation of the proteins. Upon the addition of the detergent the lipase eluted in the non-binding fraction (Figure 3.10

a). Although this was not the preferred elution choice, the step did apparently separate the lipase from an approximately 70 % of the contaminating proteins. A_{280} readings were taken after the spectrophotometer was blanked with 50 mM Tris-HCl buffer, pH 8.0 with the 1 % (v/v) Triton X-100.

a)



b)

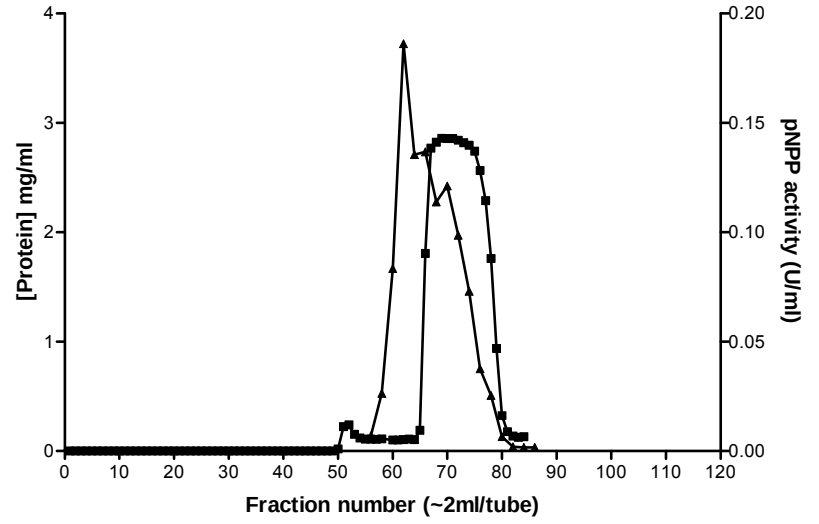


Figure 3.10: a) Elution profile obtained for the lipase fraction run on the Super-Q resin run in the presence of 1 % (v/v) Triton X-100. The graph shows the A_{280} readings (■) and *p*NPP acivity (▲). b) Elution profile obtained for the Triton X-100 sample run on the Sephadex S-200-HR resin in the presence of 2 % deoxycholate. Protein concentration (■) and *p*NPP acivity (▲) are shown on the graph.

In order to remove the Triton X-100 the activity peak obtained from the Super-Q resin was concentrated using the Amicon stirred cell ultra filtration system and applied to the Sephacryl S-200-HR column pre-equilibrated with 2 % (w/v) deoxycholate. Figure 3.10 b shows the elution profile obtained for the Sephacryl S-200-HR column. It can be seen that the lipase activity emerges from the column followed closely by the Triton X-100 and deoxycholate, probably indicating the presence of lipase aggregates. One of the problems encountered with this strategy was that the lipase activity yield was lower after ultra filtration (Table 3.3). In addition, the interaction between the Triton X-100 and deoxyxcholate is extremely pH and temperature sensitive and the buffer would form a gel when it was warmed to room temperature. Samples from this column run was analysed by SDS-PAGE and showed an excellent separation of the lipase from the larger proteins (Figure 3.11).

Table 3.3 Typical purification table for experiments done following Purification protocol B

Purification step	Total activity U	Specific activity U/mg	Purification fold	Yield %
Culture supernatant	767.7	1.2	1	100
Ammonium sulphate precipitation	648.6	1.81	1.5	84.5
Super-Q column with Triton X-100	430	3.07	2.56	56
Ultrafiltration	50.8	3.15	2.62	6.6

Unfortunately, due to the problems experienced with the buffer and the detergent combinations, the protocol was abandoned.

3.3.4.3 Purification protocol C

The protocol was the same as that used in the previous section, with the exception that it was decided to first attempt the removal of the excess Triton X-100 using the "Solvent-detergent removal resin" obtained from Sigma-Aldrich, a resin guaranteed to remove up to 95 % of free Triton X-100. The sample was applied according to the manufacturer's instructions and allowed to elute with gravity with 2 ml samples taken. Unfortunately both the Triton X-100 and the lipase stayed bound to the

resin, as no activity could be recovered. The decision was made to abandon this approach

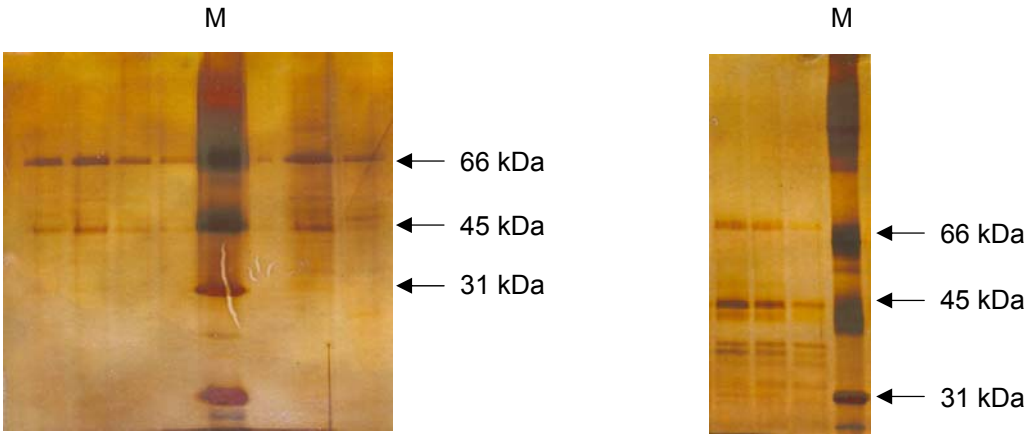


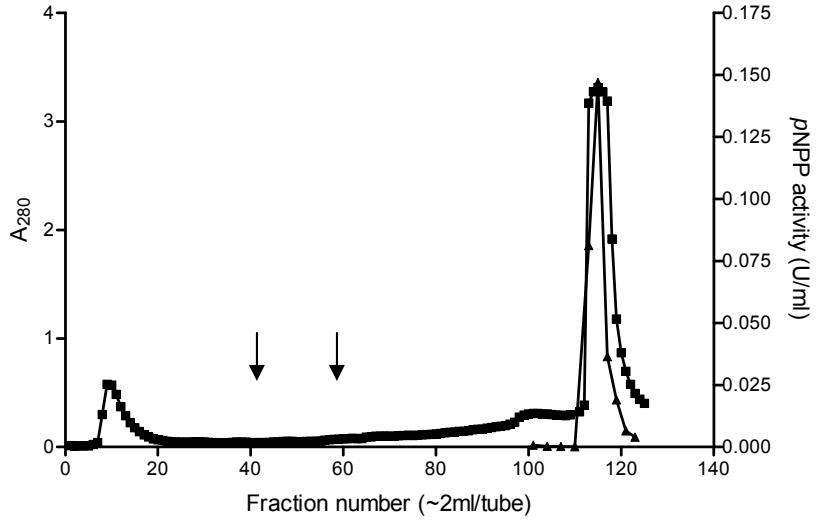
Figure 3.11 SDS-PAGE of fractions obtained from attempts made for the removal of Triton X-100 from the protein fractions using deoxycholate and Sephadex S-200-HR column chromatography. M indicates the molecular weight markers used.

3.3.4.4 Purification protocol D

With the initial testing of resins that might be used, hydrophobic resins were tested but not used on larger scale, due to the fact that little if any lipase activity could be recovered. It was decided to retry these resins in an attempt to purify the lipase. After concentration of the activity by ammonium sulphate precipitation, the pellet was dissolved in the minimum amount of buffer needed, and the sample applied to the Phenyl Toyopearl resin. Following the washing and elution steps with decreasing amounts of ammonium sulphate, no activity could be detected. According to the supplier the resin could be cleaned with up to 50 % (v/v) ethanol and in an attempt to at least recover the protein, a decision was made to use normal cleaning solutions to elute the proteins. The stability of the lipase sample was tested in the presence of varying concentrations of ethanol and it was found that the activity was not lost in ethanol concentrations up to 30 % (v/v). The column was subsequently washed with a linear gradient of 0 - 25 % (v/v) ethanol and the lipase activity was recovered (Figure 3.12 a). Unfortunately a large amount of contaminating proteins was still associated with the lipase sample (Table 3.4).

The fractions containing most of the activity (Fractions 112 - 120, Figure 3.12 a) were pooled together and concentrated with the Amicon stirred cell ultrafiltration system, and applied to a Toyopearl HW-50F gel filtration column.

a)



b)

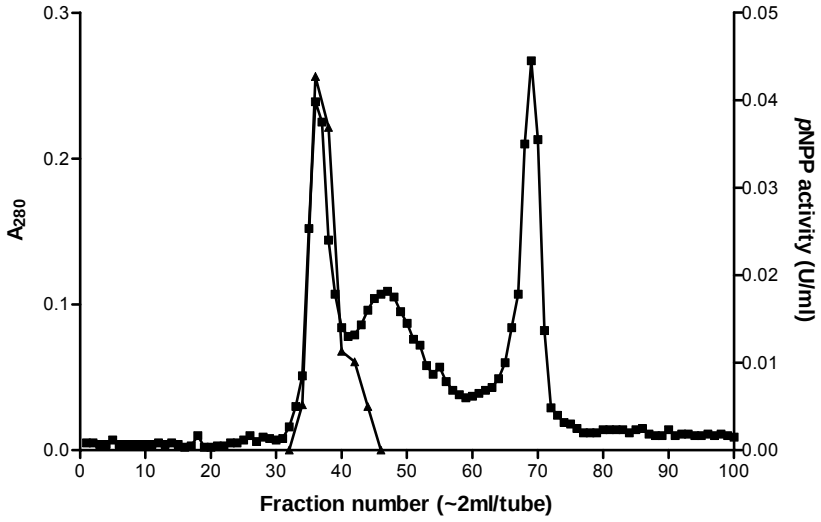


Figure 3.12: a) Elution profile obtained for the lipase fraction run on the Phenyl-Toyopearl resin The first gradient (ammonium sulphate) was applied from tubes 2-40, followed by a wash step with 50 mM Tris-HCl, pH8.0 (first arrow). The ethanol gradient was applied after this wash step (second arrow). The graph shows the A_{280} readings (■) and *p*NPP activity (▲). b) Elution profile obtained for the Phenyl-Toyopearl sample run on the Toypearl-HW50F resin in the presence of 10 % (v/v) ethanol. Protein concentration (■) and *p*NPP acivity (▲) is shown on the graph.

The column was run in the presence of 10 % (v/v) ethanol to try and limit the hydrophobic interactions between the different proteins in the sample (Figure 3.12 b). Although the elution profile indicated that a better separation had been achieved, factors that had to be considered were the low yield of activity after the last step, along with the fact that the sample was still not homogeneous according to SDS-PAGE analysis of fractions obtained from the column.

Table 3.4 Typical purification table for experiments done following Purification protocol D

Purification step	Total activity U	Specific activity U/mg	Purification fold	Yield %
Culture supernatant	1045.5	0.43	1	100
Ammonium sulphate precipitation	745.9	23.1	53.9	71.35
Dialysis	560.6	15.3	35.6	53.62
Super-Q column	20.7	2.2	5.15	1.98
Ultrafiltration	18.5	3.9	9.13	1.77
Toyopearl HW-50F	10.9	4.95	11.56	1.04

3.3.4.5 Purification protocol E

The lipase from hog pancreas was reportedly “delipidified” before purification (Verger *et al*., 1969) leading to the separation of what was termed a fast and slow lipase on gel filtration. The fast lipase was associated with the contaminating proteins by interactions involving free fatty acids and other lipid substances present in the sample. The removal of water is however essential to guarantee the enzyme stabillity during this delipidification process, and the authors freeze-dried the tissue sample before treatment with the organic solvents.

We were unable to dry the culture medium supernatant using lyophilization, and instead used ammonium sulphate precipitation to concentrate and pellet the lipase activity. This was allowed to air-dry before the organic solvent extraction was performed. Due to the small size of the pellet, any loss of activity could not be monitored during the delipidation steps. After the organic solvent extraction, or delipidation process, the sample was allowed to dissolve in a minimum amount of

50 mM Tris-HCl buffer, pH 8.0, before it was applied to the Phenyl-Toyopearl column. Upon elution of the lipase it was found that when compared to Figure 3.12 a, there was the appearance of a second activity peak (Figure 3.13) with the ethanol wash.

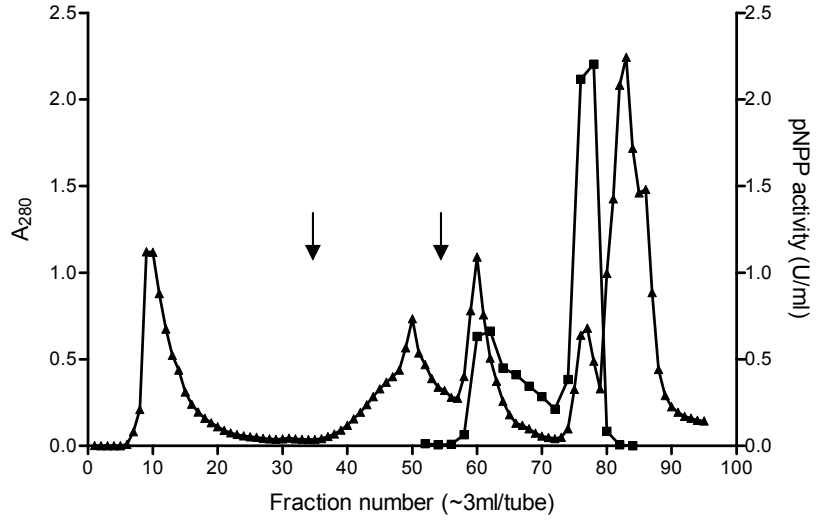
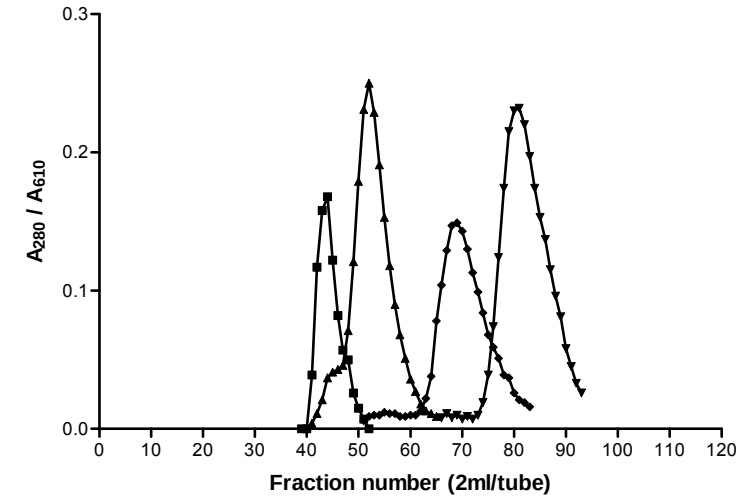


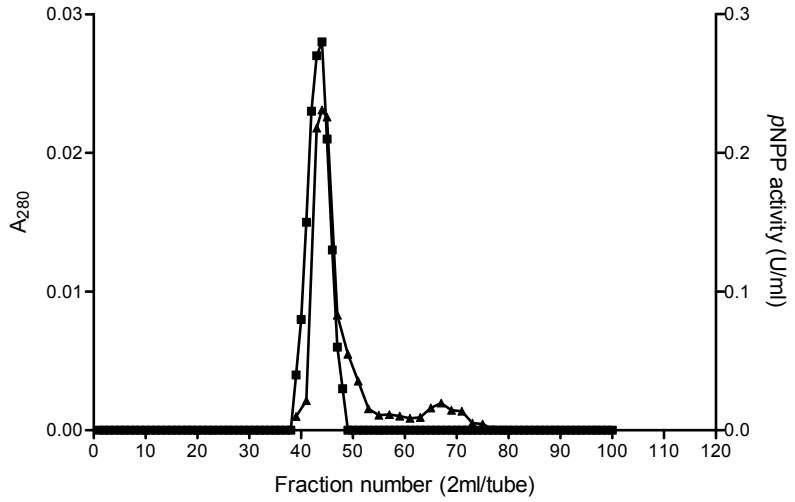
Figure 3.13: Elution profile obtained for the lipase fraction run on the Phenyl-Toyopearl resin The first gradient (ammonium sulphate) was applied from tubes 2-35, followed by a wash step with 50 mM Tris-HCl, pH8.0 (first arrow). The ethanol gradient was applied after this wash step (second arrow). The graph shows the A_{280} readings (▲) and *p*NPP acivity (■).

The two activity peaks obtained were separately applied to a pre-equilibrated and calibrated gel filtration column to estimate the sizes of the lipase/s found in the hope that a slow and fast lipase could be identified (Verger *et al*., 1969). As can be seen from Figure 3.14 b and c, both the lipase fractions eluted with all the proteins in the void volume, very likely due to aggregation behaviour of the hydrophobic protein. The lipase activity was extremely stable during the delipidation and a yield of 82 % (Table 3.5) was obtained after the ammonium sulphate precipitation and organic solvent extraction. Interestingly when the sample applied to the Phenyl-Toyopearl column was run on a SDS-PAGE gel (i.e. before loading the sample), the zymogram showed two activity bands (Figure 3.15), with estimated sizes of ~37 kDa and ~29 kDa.

a)



b)



c)

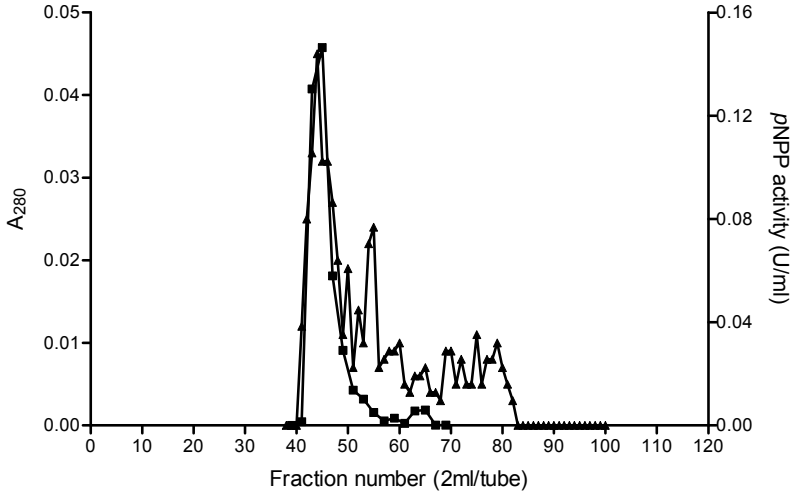


Figure 3.14 a) The calibration of the Sephacryl S-100-HR column with dextran blue (■), ovalbumin (▲), cytochrome C (▼) and aprotinin (●). Dextran blue indicates the void volume for the column and was followed using A_{610}. b) The first activity peak run on a Sephacryl S-100-HR column and c) the second activity peak run on the Sephacryl S-100. The graphs show the A_{280} readings (▲) and *p*NPP acivity (■) as used for following the elution of protein.

Table 3.5 Typical purification table for experiments done following Purification protocol D

Purification step	Total activity U	Specific activity U/mg	Purification fold	Yield %
Culture supernatant	190	0.43	1	100
Ammonium sulphate precipitation	152.2	23.1	53.9	80
Phenyl-Toyopearl column Peak1	7.8	1.62	3.8	4.1
Phenyl-Toyopearl column Peak2	7.9	3.38	7.8	4.2
Toyopearl HW-50 F peak 1	0.266	8.11	18.9	0.14
Toyopearl HW-50 F peak 2	1.46	6.17	14.3	0.768

Only one band could be seen on the zymogram after the samples obtained from the Phenyl-Toyopearl column (Figure 3.13) were further seperated on the gelfiltration columns (Figure 3.14) and visualised on SDS-PAGE gels. Using the partially pure lipase sample we were able to determine aa isoelectric point (pI) of approximately 7.0 for the lipase band after gel filtration (Figure 3.16).

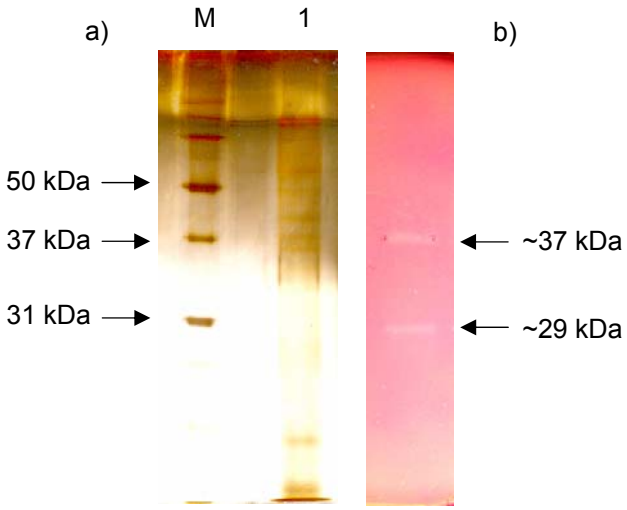


Figure 3.15 SDS-PAGE gel of the sample applied to the Phenyl-Toyopearl column in lane 1 (a) and the corresponding olive oil zymogram showing the two activity bands (b).

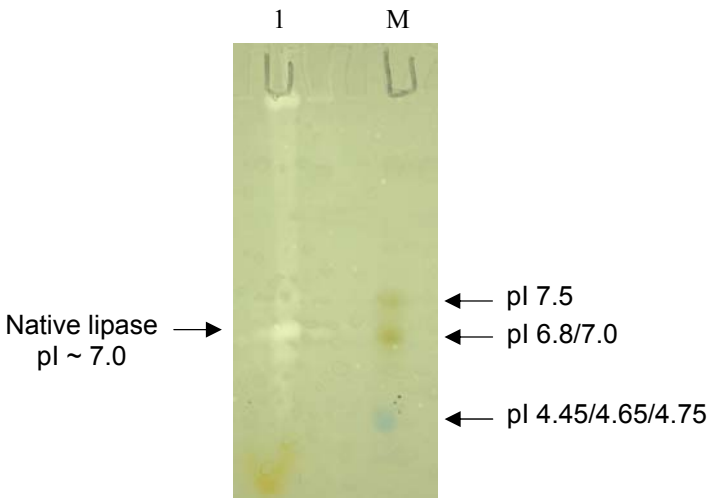


Figure 3.16 IEF gel overlain with the tributyrin zymogram obtained for the native lipase (lane 1) run with pI standards (M).

3.4 Discussion

Confirming the identity of the bacterium, is essential to ensure the results obtained are truly associated with that specific bacterium. It also forms part of the quality assurance process during the development of the isolation procedures to ensure that the culture is not contaminated. 16S rDNA sequencing offers a quick, easy and reproducable method for monitoring the authentisity of the bacterial strain used for experiments. The method can easily be shortened by direct sequencing of the PCR product obtained from whole cells, coupled with BLAST searches. For the purpose of this study the full process of PCR, cloning, subcloning and sequencing were performed. Although the entire gene was not sequenced, approximately 900 bases of the 1500 bp in the gene were obtained and the presence of the *Eco*R1 restriction site together with the high degree of identity with the *G. thermoleovorans* T80 strain (99 %) was taken as more than satisfactory evidence for confirming the culture identity.

The bacterium showed high lipolytic activity when grown on tributyrin plates, results that contradicted those obtained when tributyrin was used as the inducer in liquid cultures. Induction studies in liquid cultures lead to lipase production in the presence of olive oil and Tween 80. Similar results were reported for induction with

olive oil in *B. thermocatenulatus* (Schmidt-Dannert *et al*., 1994), *B. thermoleovorans* ID-1 (Lee *et al*., 1999) and *B. stearothermophilus* MC7 (Kambourova *et al*., 2003). The *B. thermoleovorans* ID-1 (Lee *et al*., 1999) strain showed high levels of lipase expression in the presence of triolein and Tween 20 and the *Bacillus* sp. RSJ-1 with Tween 80 (Sharma *et al*., 2002), although Schmidt-Dannert and co-workers found no induction of activity when Tween 80 was used. Researchers reported on the use of cottonseed oil (Sharma *et al*., 2002), soybean oil (Ghanem *et al*., 2000) and ricebran oil (Dharmstiti and Luchai, 1999) as good inducers.We were unable to test these substrates, as they were not available at the time.

In experiments where the bacterium was grown and lipase activity monitored, the volume of media in the flask was always kept to approximately 10 % of the flask volume to ensure proper aeration of the media. Knoesen (2004) found this volume yielded the best results in terms of lipase activity, confirming observations made by Schmidt-Dannert *et al.* (1994) that proper aeration is essential for lipase production. The use of stearic acid as inducer for the *Geobacillus* was proposed by Knoesen (2004) due to the fact that the lipase activity increased together with an increase in fatty acid content of the media. Lipase activity appears in a shorter time and at higher levels using stearic acid. In experiments done by the author with olive oil as inducer, two distinct peaks were obtained. The same was observed by Knoesen (2004) who followed the availibility of free fatty acids during growth of the bacterium in a bioreactor. What Knoesen noticed was that the first activity peak was present from the beginning of growth and that the second peak would only appear as the free fatty acid concentration increased in the culture supernatant. Knoesen hypothesised the presence of a constitutively expressed and an inducible lipase, results supported by researchers working with *B. thermoleovorans* ID-1 (Lee *et al*., 1999) and *B. thermocatenulatus* (Rua *et al*., 1997; Schmidt-Dannert *et al*., 1994).

Development of an isolation protocol was not successful using 5 different protocols. The problems encountered were highlighted in this section. When looking at the structure of the *Geobacillus* lipases (Chapter 5), it is reported that the

active site is surrounded by high quantities of hydrophobic amino acids, thus increasing the hydrophobicity of the enzyme, a factor that apparently strongly influenced the purification steps. Results obtained in section 3.3.4.1 found that the lipase showed association behaviour with the rest of the protein mixture, making it impossible to perform experiments with reproducible results. This was contrary to reports by Kambourova *et al*. (2003) and Lee *et al*. (1999) who used similar steps to purify the lipases from *B. stearothermphilus* MC7 and *B. thermoleovorans* ID-1 lipase BTID-B. The lipase BTID-B was however expressed in *E. coli* and heat precipitation was utilised as the initial concentration step, a step that could not be used in our experiments.

The addition of the non-ionic detergent, Triton X-100, to the protein sample was used in an attempt to minimize the interactions between the different proteins in the mixture. It appeared to work with the separation of the protein from the rest of the bound proteins on anion exchange resins, but it had other disadvantages. The major one was the loss of activity when the sample was concentrated for gel filtration (Table 3.3), with ~6.6 % activity left for further experiments. The next being the inabillity to remove the Triton X-100 from the enzyme, using precipitation reactions, competition for binding with milder detergents during gel filtration and the Solvent-Detergent Removal resin.

Examination of the SDS-PAGE gels from the Triton X-100 experiments clearly indicated that although the enzyme could be purified to almost 50 % homogeneity in the one experiment, the results for the next experiment differed (Figure 3.11). Using the results obtained for the last purification attempts we could show by using zymogram staining that the protein band of approximately 40 kDa on the SDS-PAGE gel was the inducible lipase as reported in nature. The presence of the smaller activity band most probably correlates to the reported constituteviley expressed smaller lipase.

The true hydrophobic nature of the lipase from *G. thermoleovorans* GE-7 became more apparent with the ensuing experiments, where ethanol had to be used for the elution of the lipase from the Phenyl-Toyopearl columns. None of the other papers

on the purification of native lipases from *Geobacillus* (formely *Bacillus*) had to use this type of elution method (Table 3.1). Coupling this to gel filtration columns could not significantly enhance the purity of the lipase allthough the lipase seemed to be stable in the 10 % (v/v) ethanol added to the mobile phase. The delipidation experiments done did lead to the separation of two lipase activity peaks on Phenyl-Toyopearl resin (Figure 3.13), but once more the following gel filtration steps did not aid in the purification steps, with both the lipase fractions eluting with the other proteins in the void volume of the column (Figure 3.14). The yields obtained from these experiments, 0.14 % for the first column and 0.78 % for the second column, was very discouraging rendering us unable to attempt more purification steps. Similar problems were reported by Rua *et al*. (1994) who investigated the high aggregation tendencies of the lipase from *B. thermocatenulatus*. They reported the aggregative manner of this lipase in gel filtration columns where the mobile phase was supplemented with various concentrations of cholic acid, salts and isopropanol. The researchers found that in the presence of 30 % (v/v) isopropanol a more accurate elution position and esimated size could be obtained that correlated well with sizes obtained via SDS-PAGE gel elctrophoresis. The possibillity exist that such high concentrations of the alcohol could have the same effect on the lipase from *G. thermoleovorans* GE-7, but in our case the addition of the isopropanol was accompanied by losses of activity when the sample was mixed with higher concentrations of isopropanol.

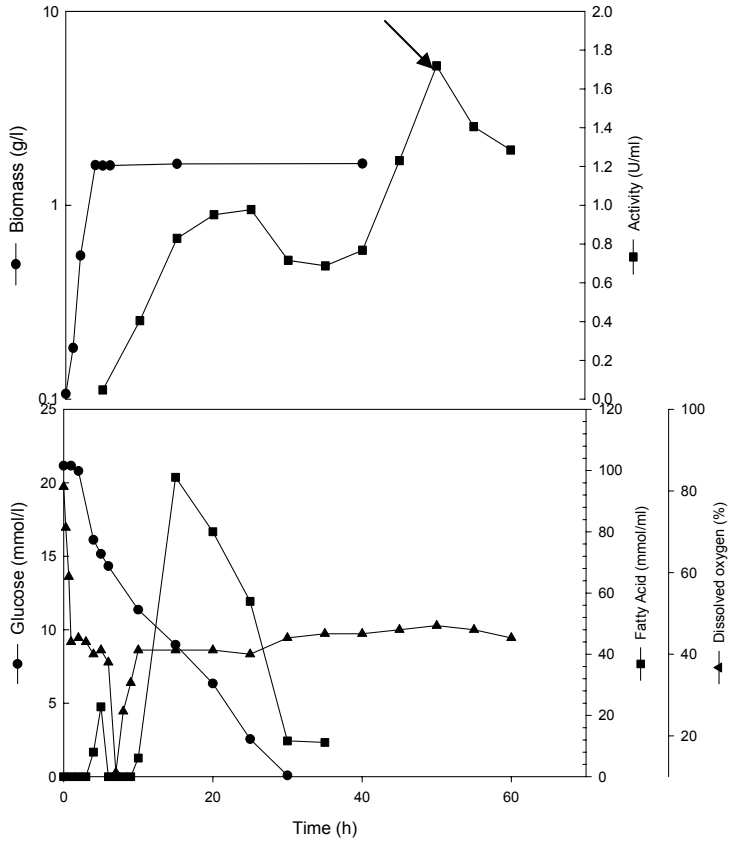
Nthangeni *et al.* (2001) noted that purification of *Bacillus* lipases for biochemical characterization and crystallographic studies is difficult, mainly due to stringent regulatory mechanisms of their synthesis which often lead to low levels of production. Misset *et al*. (1994) reported that purification of about 100 mg of *B. subtilis* lipase requires the initial construction of a *Bacillus* strain overpoducing the lipase followed by cultivation in a 30 l fermentor. Most of the reported purification strategies used 10 – 20 l fermentors for the growth of the organisms (Rua *et al*., 1997), leading to much higher activity yields, and making it possible to employ steps where loss of significant activity could be accommodated. In our laboratory the organism could only be grown to a final volume of 1 l due to the fact that small volumes had to be used in the Erlenmeyer flask to optimize the

dissolved oxygen ratio. Using upscaled volumes as described by Knoesen (2004) in our laboratories would not help since the purification protocol is too difficult.

Probably the most usefull tool we had to our disposal was the zymograms. This enabled us to identify with certainty the lipase band on the SDS-PAGE gels. Initial experiments with this protocol were problematic because the zymogram could only be used with native gels, a problem we circumvented by using a protocol used for the study of MAP kinases in plant species (Tena and Renaudin, 1998). The method is similar to that used for the regeneration of proteins isolated from inclusion bodies in *E. coli* (de Bernardez Clark, 2001). Basically the protein is completely denatured with the use of urea and then allowed to renature in the presence of a detergent and reducing agent, to assist with the correct refolding of the protein. With this technique we could use the zymogram directly on SDS-PAGE gels. Testing of the zymograms described in section 3.2.4.4, it was found that the zymogram using only the tributyrin as the inducer gave the best results in the shortest amount of time.

Considering the appearance of both lipase peaks during induction studies and when a sample preceding the Phenyl–Toypearl after delipification was analyzed (Figure 3.15), lends itself to some interesting hypothesis. The presence of two lipases have been reported for both *B. thermocatenulatus* (Schmidt-Dannert *et al*, 1994) and *B. thermoleovorans* ID-1 (Lee *et al*., 2001). In the first case the authors reported the presence of a smaller lipase (~16 kDa) as the inducible lipase and Lee *et. al* (2001) found a constitutively expressed lipase with an estimated size of 18 kDa. Both authors obtained the larger lipase by screening genomic DNA libraries. Our hyphothesis is that in the case of *G. thermoleovorans* GE-7 the smaller lipase is constitutevely produced as a “scout” enzyme by the organism. The larger of the lipases is then produced when such substrates are detected and broken down releasing free fatty acids responsible for, or leading to, the induction of the second lipase. The hypothesis is supported by results obtained by Knoesen (2004) who showed that when the organism was grown in the presence of stearic acid the second lipase peaked after 20 hours versus the normal 60 hours (Figure 3.17) with olive oil induction. This might also be used to explain why the second

a)



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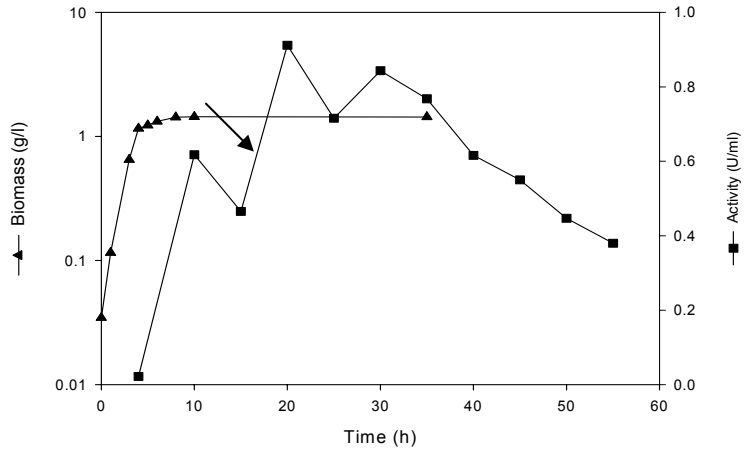


Figure 3.17 Comparison of the olive oil (a) and stearic acid induced (b) lipase production by *G. thermoleovorans* GE-7 showing the decrease in production time of the second lipase peak (shown by arrow). Note the decrease in free fatty acid before the onset of the second lipase peak. (taken from Knoesen, 2004)

lipase was not detected during the initial purification step where induction was done using olive oil. Schmidt-Dannert *et al*. (1994) reported that this lipase mostly associates with the left-over olive oil and $CaCl_2$ soaps formed when the supernatant is left to stand overnight. They extracted this lipase with various organic solvents extractions. As soon as stearic acid was used as inducer, the possibility exists that both lipases are formed similtaneously, and thus we were able to detect it using the tributyrin zymogram. It was also reported that the smaller of the lipases is expressed at much lower levels (Schmidt-Dannert *et al*., 1994). This phenomenon still needs to be further investigated (this did not fall into the scope of this project), as not much is known about the expression of the lipase genes, nor the export of the lipase proteins for the *Geobacillus* species.

The difficulties associated with the development of a purification protocol meant that another approach had to be followed, because the only way to characterize a specific protein and its features is to study it without the interaction of other proteins as well as without the interactions of lipids and/or detergents, which was the case with the native lipase. We therefore decided to obtain the pure lipase by cloning of the gene and expressing the protein using a suitable vector in *E. coli.*

CHAPTER 4

Cloning and expression of the *Geobacillus thermoleovorans* lipase

4.1 Introduction

In 1915 the first industrial enzyme was used in detergent applications, introducing the use of enzymes as biocatalysts (Fujiwara, 2002). Lipases, in particular, have received a lot of attention due the variety of reactions they can catalyse, their specificities and their resistance to various agents, such as organic solvents (Chapter 1). Since microbial lipases are usually more thermostable than animal or plant lipases, they received much more attention for their potential use in industries and diagnostics (Sugihara *et al*., 1991). A major requirement for a commercial enzyme is thermal stability, because thermal denaturation is a common cause of enzyme inactivation (Leow *et al*., 2004). In addition, increasing thermostability would allow enzymatic reactions to be done at higher temperatures, leading to increased conversion rates, substrate solubility, the reduced possibility of microbial growth and lower viscosity of the medium (Mozhaev *et al*., 1994). Thermophiles make good candidates for producing these thermostable enzymes, but are impractical due to the low yields and the need for specialised equipment for high temperature fermentation (Sonnleitner and Fiechter, 1983).

The use of molecular biological techniques not only makes it possible for the over expression of these genes, but also allows us to clone genes encoding novel enzymes and to modify these genes for future applications. Methods for the isolation and identification of novel genes have improved rapidly in the last few years and newer technologies have been successfully utilised for this purpose. One of the most interesting approaches is the screening of soil metagenome libraries (Sanchez *et al*., 2002; Daniel, 2004). Taking into consideration that only

approximately 1 % of the total microbial community in soil can be cultivated using known microbiological techniques (Amman *et al*., 1995), it is apparent that methods are needed for an culture-independent way to screen the rest of the 99 % of the organisms for enzymes produced. Metagenomic libraries are constructed by the isolation of total DNA from the environmental samples, restriction enzyme digestion and cloning of the DNA followed by expression of the genes, usually in *E. coli* (Daniel, 2004). These libraries can then be screened using conventional screening methods, i.e. plate screening, or by PCR screening for targeted enzymes. Bell *et al*. (2002) reported the use of PCR for the screening of novel lipases by using primers designed specifically for conserved regions in the lipase genes. A third approach would be the use of novel cloning plasmids, used for genome walking and sequencing, and analysing the sequences for possible novel enzymes (Nthangeni *et al*., 2005).

The cloning of the lipase genes from thermophilic bacteria have received great interest, and the lipase genes from *B. stearothermophilus* (Kim *et al*., 1998; 2000), *B. thermocatenulatus* (Schlieben *et al*., 2004; Rua *et al*., 1997), *B. thermoleovorans* ID-1 (Lee *et al.*, 2001) and unclassified *Geobacillus* species (Leow *et al*., 2004) have been reported. The authors were able to not only clone the gene, but also over-express the gene in *E. coli* and purify and characterise the proteins. All the authors found an open reading frame of 1254 bp coding for the lipase signal peptide of 29 amino acids and the mature lipase of 388 amino acids. This served as the basis for the experiments performed in this study.

In order to assist the purification protocols, researchers have begun to modify the genes to express active enzymes fused to a “tag” to rapidly purify the recombinant protein. One of the most popular techniques is the inclusion of a 6 X histidine tag at either the N-terminal or C-terminal end of the enzyme. Schlieben *et al*. (2004) were successful in modifying the lipase from *B. thermocatenulatus* to include a histidine tag offering a one-step purification of the lipase. Lipases from *B. licheniformus* (Nthangeni *et al*., 2001) and *S. hyicus* (Simons *et al.,* 1996) have also successfully been purified using histidine tags.

Fusion of the lipases to certain domains of other proteins has been used with great success for the expression and immobilization of these enzymes in order to display the lipolytic enzymes on the cell-surface of the host. Becker and co-workers (2005) fused the lipases from *B. subtilis*, *Serratia marcescens* and the cutinase from *Fusarium solani pisi* to an inactive EstA variant, an outer-membrane anchored esterase from *P. aeruginosa*, and display active enzymes on the cell surface of *E*. c*oli*. Matsumoto *et al*. (2002) displayed the *Rhizopus oryzae* lipase on the cell surface of *Candida tropicalis* using the cell wall anchor region of the Flo1p flocculation functional domain. In 1999, Tsuchiya and co-workers localized the *B*. *subtilis* LipB lipase on the cell surface of *B. subtilis* by fusing the protein to the cell wall-binding domain of the major cell wall hydrolase, autolysin, leading to expression of active lipase.

The last important consideration to be made is the expression host to be utilised. *E. coli* is the host of favour as it facilitates protein expression by its relative simplicity, its inexpensive and fast high-density cultivation, the well known genetics and the large number of compatible tools available for biotechnology (Sørenson and Mortenson, 2005). A major advantage is the variety of available plasmids, recombinant fusion partners and mutant strains, advancing the possibilities of *E. coli*. Obstacles are however encountered with the expression of heterologous proteins lacking relevant interaction partners in *E. coli*. Researchers are investigating other expression hosts such as the bacteria *B. subtilis*, (Sanchez *et al.,* 2002), *Xanthomonas camprestis* (Leza *et al*., 1996) and *Lactobacillus curvatus* Lc-2c (Vogel *et al*., 1990) and the yeasts *Saccharomyces cerevisiae* (Sanchez *et al*., 2002; Ahn *et al*., 2004) and *Pichia pastoris* (Quyen *et al*., 2003). In all instances the authors were able to heterologously express active lipases. The use of eukaryotic hosts for expression of especially thermophilic lipase is gaining more attention since they generally lead to higher expression than their prokaryotic counterparts. Ahn *et al*. (2004) could increase *B. stearothermophilus* L1 lipase in *S. cerevisiae* to levels of 98 000 U/l or 1.3 g/l of the protein. Quyen and co-workers (2003) showed that using *Pichia pastoris* they could produce *B. thermocatenulatus* BTL2 lipase at levels of 309 000 U/l, in both cases much higher than produced in *E. coli*.

The ideal expression host would however be a thermophile itself, and research is currently being done on the construction of shuttle vectors between *E. coli* and *Thermus thermophilus*. Moreno *and* co-workers (2003) constructed a vector with both the *lacZ* promotor for *E. coli* expression and the strong nitrate reductase promotor for expression in *T. thermophilus*. The genes are only transcribed during the late stationary phase of *T. thermophilus* growth due to onset of micro-anaerobic conditions, thus switching on the reductase promotor. They found superior overexpression of several *Thermus* proteins in *T. thermophilus* when compared to expression in *E. coli*. The reason might be due to better folding, post-translational modification and transport of the recombinant proteins in the thermophile when compared to mesophilic expression.

It is thus clear that molecular biology offers powerful tools for studying thermophilic enzymes and will be used for the cloning and modification of the *G. thermoleovorans* GE-7 lipase gene, over-expression of the gene in a mesophilic host and purification of the modified lipases for characterisation.

4.2 Materials and Methods

Unless otherwise stated, all chemicals were obtained from commercial sources, were of analytical reagent grade or better and were used without further purification.

4.2.1 Bacterial strains and plasmids used

4.2.1.1 Bacterial strains

Bacterial strains used for this section are described in Table 4.1.

4.2.1.2 Plasmids used

Plasmids used for this section are described in Table 4.2.

Table 4.1: Bacterial strains used in molecular characterization with some of their functions and properties.

Bacterial strain	Functions / Properties
Escherichia coli JM109 [a]	Competent *E. coli* strain used for transformation experiments with the pET 28a expression and pGEM®T-easy cloning plasmids
Escherichia coli JM109(DE3) [a]	Competent *E. coli* strain used for transformation experiments with the pET 28a expression plasmid; contains the T7 RNA polymerase gene for induction of the genes used
Geobacillus thermoleovorans GE-7[b]	Used for genomic DNA isolation and isolation of the lipase gene

[a] Strains purchased from Promega

[b] Kindly supplied by Dr. M. de Flaun of GeoSyntec Consultants

Table 4.2: Plasmids used in molecular characterization with some of their functions and properties.

Plasmid	Function / Properties
pET 28a expression vector[a]	• Expression vector used for the incorporation of a thrombin removable 6 X Histidine tag. • Allows expression of cloned genes under transcriptional control of a bacteriophage T7 promotor; expression initiated in bacterial strains containing a chromosomal copy of the T7 RNA polymerase gene. • Contains the gene coding for aminoglycoside phosphotransferase leading to kanamycin resistance.
pGEM®T-easy cloning vector[b]	• Linear plasmid containing T-overhangs for ligation of PCR products. • Contains the gene coding for ß-lactamase leading to ampicillin resistance.

[a] Purchased from Novagen

[b] Purchased from Promega

4.2.2 Growth and induction of lipase production

4.2.2.1 Media and growth conditions

E .coli was grown and maintained on LB plates (10 g/l peptone; 5 g/l yeast extract; 10 g/l sodium chloride; 16 g/l agar; pH 7.0) and grown at 37°C. Bacteria transformed with the pGEM®T-easy vector were grown on LB plates supplemented with ampicillin (60 µg/ml), IPTG (9.6 µg/ml) and X-Gal (40 µg/ml). Bacteria transformed with the pET 28a vector were grown on LB plates supplemented with kanamycin (60 µg/ml), IPTG (9.6 µg/ml) and X-Gal (40 µg/ml).

Plate screening for lipase activity was performed using tributyrin plates prepared by mixing 450 ml LB plate mixture mixed with 50 ml of a Gum Arabic (1 g/l) and tributyrin (10 g/l) mixture which was sonicated 3 times (Branson Cell Disrupter B-30; SmithKline company) using 1 min sonication and 1 min resting cycles with a 50 % duty cycle. Antibiotics were added as needed to ensure selective pressure. *G. thermoleovorans* GE-7 was grown as described in section 3.2.2.2.

4.2.2.2 Induction studies

Production of recombinant lipases was performed by inoculating 100 ml LB media supplemented with the appropriate antibiotic (10 mg/ml) with 1 ml of an overnight culture. Unless stated otherwise the cultures were grown at 200 rpm on an orbital shaker at 37°C. Growth times and induction procedure will be stated for each experiment.

4.2.3 Bacterial transformations

4.2.3.1 Preparation of the bacterial cells for transformation

Competent cells were prepared according to the method described by Tang *et al.* (1994). Briefly, the desired *E. coli* strain was cultured overnight at 37°C in 5 ml LB-

broth (Section 4.2.2.1), and 1 ml of the culture was transferred to 100 ml PSI-broth (5 g/l Bacto yeast extract; 20 g/l Bacto tryptone; 5 g/l magnesium sulphate; pH 7.6 adjusted with potassium hydroxide) and allowed to grow to an optical density (OD_{550}) of 0.48. The cells were harvested by centrifugation at 4 000 xg for 5 min at 4°C and the pellet resuspended in 0.4 X of the original volume TfB1 (30 mM potassium acetate; 100 mM rubidium chloride; 10 mM calcium chloride; 50 mM manganese chloride; 15 % v/v glycerol; pH 5.8 adjusted with diluted acetic acid). The mixture was left on ice for 15 min and centrifuged at 4 000 xg for 5 min at 4°C. The pellet finally resuspended in 0.04 X of the original volume Tfb2 (10 mM MOPS; 75 mM calcium chloride; 10 mM rubidium chloride; 15 % v/v glycerol; pH 6.5 adjusted with diluted sodium hydroxide). The suspensions were dispensed into 80 μl volumes, snap-frozen using liquid nitrogen and stored at -70°C.

4.2.3.2 Bacterial transformation

Plasmids (1 μg) containing the desired inserts were added to competent *E. coli* cells, left on ice for 30 min then incubated at 42°C for 90 sec and placed on ice for 4 min. LB-media (800 μl) containing 40 % (w/v) glucose was added to the suspension and incubated for 1 hour at 37°C. Cells were harvested by centrifugation at 4 000 xg for 2 min, resuspended in LB-media, plated out onto LB plates containing IPTG, X-Gal and ampicillin (Section 4.2.2.1).

4.2.4 Isolation of DNA

4.2.4.1 Genomic DNA isolation

Genomic DNA was isolated according to the method described by Towner (1991) using SDS/Proteinase K treatment. Cells were harvested and the pellet resuspended in TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) in a ratio of 0.5 g cells in 40 ml buffer. After centrifugation at 3000 xg for 5 min at 4ºC the pellet was resuspended in 50 mM Tris-HCl buffer, pH 8.0 containing sucrose (0.7 mM). Lysozyme (20 mg/ml) was added in a ratio of 0.6 ml to 0.5 g cells and left on ice for 5 min. This was followed by the addition of EDTA (0.5 M; pH 8.0) in the same ratio

as the lysozyme and SDS (10 % w/v) at a ratio of 0.5 ml to 0.5 g cells, and left on ice for 5 min. Digestion buffer (50 mM Tris-HCl, 1 % w/v, 0.1 M EDTA, 0.2 M NaCl, 0.5 mg/ml Proteinase K, pH 9.0) was added (10 ml for every 0.5 g cells) and the mixture incubated at 55 ºC for 16 h with mild agitation.

After incubation, one volume of pH-calibrated biophenol was added and gently mixed for 3 h at 25ºC followed by centrifugation at 4000 xg for 10 min. The supernatant was mixed with a phenol:chloroform mixture (pH calibrated biophenol, 25 % v/v; chloroform:isoamylalcohol (24:1), 25 % v/v; TE-buffer, 50 % v/v; pH 8.0), centrifuged at 3000 xg for 10 min at 25ºC and the supernatant placed on ice for 10 min. Sodium acetate (5 M; pH 5.7) was added (one tenth of the initial volume) along with ice-cold ethanol (99.9 %, 10 ml). Precipitated DNA was collected and centrifuged for 1 min at 4ºC, the pellet washed with ethanol (70 % v/v), left to air dry and resuspended in sufficient volume TE-buffer supplemented with RNase (50 µg/ml). The pellet was left at 30ºC until the genomic DNA was dissolved and a sample evaluated on an agarose gel (Section 4.2.5.4) to verify that the DNA was intact.

4.2.4.2 Plasmid isolations

Single white colonies were inoculated into 5 ml LB-media containing ampicillin (30 µg/ml) and incubated at 37°C for 16 h. The cells were harvested by centrifugation for 1 min. The pellets were resuspended in STET buffer (8 % (w/v) sucrose; 5 % (v/v) Triton X-100; 50 mM EDTA; 50 mM Tris-HCl, pH8.0), lysozyme (50 mg/ml) was added and immediately placed in a boiling water bath for 1 min. The samples were placed on ice for 10 min and centrifuged at 10 000 xg for 15 min at 4°C. The pellet was removed using a sterile toothpick; isopropanol was added to the supernatant and incubated for 10 min at -20°C. The mixture was centrifuged for 10 min at 10 000 xg, the supernatant aspirated and the sample dried in a Speedvac Concentrator and resuspended in TE-buffer containing RNase (50 µg/ml).

4.2.5 Manipulation of DNA

4.2.5.1 Polymerase chain reaction (PCR)

PCR reactions were performed in a Thermal Cycler (Perkin-Elmer, USA) in a total volume of 50 μl. Each reaction consisted of 10 X Taq buffer; 10 μg DNA; 1 mM dNTP's; 50 pmol forward and reverse primers (Table 4.3) and 5 U Taq polymerase. The reactions were subjected to a pre-denaturing step at 94°C for 5 min followed by 25-30 cycles denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 2 min. This procedure was followed for all the experiments except were stated differently.

4.2.5.2 Restriction enzyme digestions

Restriction enzyme digestions, to verify fragment inserts, were performed according to the supplier's (Roche Diagnostic) recommendations. Usually DNA (approximately 5 μg) was digested with 1 U restriction enzyme in a reaction containing 10 X enzyme buffer at the appropriate optimum temperature for the individual enzymes for 1-2 hours. Products were separated on an agarose gel (Section 4.2.5.4) for evaluation.

4.2.5.3 Ligation of DNA

For ligation, fragments obtained from genomic DNA digestions (Section 4.2.5.2) were purified from the gel (Section 4.2.5.5) and the desired plasmid and DNA combinations made. Ligations were performed in a total volume of 23 μl containing 10 X Ligation buffer and DNA ligase (1 U, New England Biosystems) and incubated at 16°C overnight. PCR products were ligated into pGEM®T-easy vector (Promega) at 4°C according to the manufacturer’s specifications

Table 4.3 Primers used for PCR and sequencing reactions. Restriction sites and additions are shown underlined and in bold for each insertion and corresponding sequence.

Primer	Primer sequence	Function of primer
LIP1F	5'-GAK ARS ATG ATG AAA KGC TG-3'	• Amplification of the lipase open reading frame (Signal peptide and mature lipase)
BtLR	5'-TTA AGG CCC GAA GCT CG-3'	• Amplification of the lipase open reading frame (Signal peptide and mature lipase)
BtLHisF	5'-GCG GAT AGC **CAT ATG** ATG AAA KGC TGT CGG-3'	• Amplification of the mature lipase coding sequence. • Introduction of the ***Nde*I** restriction site at the 5' end of the coding sequence for cloning into pET 28a.
BtLHisR	5'-TGC **GGA TCC** TCA TTA **GTG GTG GTG GTG GTG GTG** AGG CCC GAA GCT CG-3'	• Amplification of the mature lipase coding sequence. • Introduction of the ***Hind*III** restriction site of the 3' end of the coding sequence for cloning into pET 28a. • Introduction of the coding sequence for **6 histidine** residues.
BtLHF	5'-GCC ATG GGC **CAT ATG** GCT TCR CGM GCC AAC GAT GCR CCS A-3'	• Amplification of the coding sequence for the mature lipase. • Introduction of the ***Nde*I** restriction site at the 5' end of the coding sequence for cloning into pET 28a.
BtLHR	5'-CTG **GGA TCC** TCA TTA AGG CYS SAA RCT CGC CAA CTG CTC-3'	• Amplification of the complete lipase open reading frame. • Introduction of the ***Hind*III** restriction site at the 5' end of the coding sequence for cloning into pET 28a.
GtL2F	5'-ATC CAT ATC ATC GCC CAC AGC CAA G-3'	• Internal forward primer used for sequencing of the lipase open reading frame.
Gtl2R	5'-CGT CCG TTC TGT GGC AAA GCT CAA A-3'	• Internal reverse primer used for sequencing of the lipase open reasing frame.
T7	5'-GAT ATA CGA CTC AGA TA-3'	• Forward primer used for sequencing of inserts in the pET 28a and pGEM®T-easy vector.
SP6	5'-TAC CAT TTA GGT GAC ACT ATA G-3'	• Reverse primers used for sequencing of inserts in the pGEM®T-easy and pET 28a vector.

4.2.5.4 Electrophoresis of DNA

DNA was analyzed on a horizontal agarose slab gel [1 % (w/v)] with ethidium bromide (0.5 μg/ml) in TAE buffer (40 mM Tris acetate; 2 mM EDTA, pH 8.3). Electrophoresis was done for 1-2 hours in electric field strength of 5.9 $V.cm^{-1}$ gel and the DNA was visualized with UV light (Spectroline Transilluminator, USA).

The relative sizes of the DNA fragments were estimated by comparing their electrophoretic mobility with that of the standards run with the samples on each gel. Either 1kB Plus marker (Life Technologies) or *Hind*III/*Eco*R1 digested λ DNA (Promega) were used as standards.

4.2.5.5 Purification of PCR products from agarose gels

Purification was achieved using the "High pure PCR product purification kit" (Roche Applied Sciences) according to the manufacturer's instructions.

4.2.6 Sequencing

Sequencing reactions were performed using the DYEnamic™ Terminator Cycle Sequencing Premix kit (Amersham Pharmacia Biotech, Inc.) and the ABI Prism® Big Dye® Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems). Sequencing reactions were done using the various manufacturers' instructions using the universal T7 and SP6 primers. Sequencing was done on an ABI Prism 377 DNA Sequencer (PE Biosystems). The data was analyzed using Sequencing analysis V 3.3 software. Sequences were reverse complemented and compared using Sequence Navigator V 1.0.1 and assembled using AutoAssembler V 1.4.0 and DNAssist V 1.02 (Patterton and Grave, 1999). Analyzed sequences were used to search the Genbank Database (http://www.ncbi.nlm.nih.gov/).

4.2.7 Cloning and modification of the lipase genes

4.2.7.1 Amplification of the lipase open reading frame

Genomic DNA from *G. thermoleovorans* GE-7 was obtained by growing the organism as described in section 3.2.2 and isolating the total genomic DNA using the Proteinase K/SDS protocol (Section 4.2.4.1). DNA was visualized on a 1 % agarose gel (Section 4.2.5.4) with ethidium bromide.

The degenerate primers, Lip1F and BtlR (Table 4.3), were designed from multiple alignments of lipases from various *G. thermoleovorans* and *G. stearothermophilus* strains (Figure 4.2) with conserved regions used as the template.

Genomic DNA was used as the template for the amplification of the lipase open reading frame using the PCR procedure explained in section 4.2.5.1 with the Lip1F and BtlR primers. The annealing temperature used was 50°C. The PCR product was visualized on an agarose gel and cloned into the pGEM®T-easy vector as described in section 4.2.5.3. The plasmid, LipA, was transformed (Section 4.2.3.2) into and maintained in *E.coli* JM109 cells.

Plates showing bacterial growth after 12-16 hours were replica plated onto tributyrin plates (Section 4.2.2.1) supplemented with ampicillin and left at 37°C until zones of clearance were observed. Clones showing lipase activity were grown in LB media and plasmids isolated (Section 4.2.4.2), the DNA was digested with *Eco*RI (Section 4.2.5.2) to excise the insert and visualized on an agarose gel. The selected clone was subjected to sequencing using the T7 and SP6 primers as well as internal primers GtL2F and GtL2R to achieve sufficient overlapping regions.

Measuring expression levels and localization of the LipA lipase was done by inoculating 100 ml LB media with 2 ml of an overnight culture. Samples (600 µl) were taken every 40 min after an optical density reading (OD_{600}) of ~0.7 was reached. Cells were separated from the supernatant, washed 2 X in 50 mM Tris-

HCl buffer, pH 8.0, before being resuspended in 50 mM Tris-HCl buffer, pH 8.0, (600 µl) supplemented with 1 mg/ml lysozyme and left on ice for 20 min. This was followed by sonfication of the cells using a Branson Cell disruptor B30 (SmithKline Company) at 60 % work output for 4 repeats of 10 sec sonication and 10 sec resting periods. The cell debris and membrane fraction were separated from the periplasmic- and cytoplasmic proteins by centrifugation of the sample at 14 000 xg for 10 min at room temperature. Appropriate dilutions were prepared and assayed using the *p*NPP assay (Section 3.2.3.2.2) and a graph of lipase concentration with time was constructed (data not shown). It could clearly be seen that most activity was localized intracellularly and that the cells had to be harvested after approximately 240 min.

Using the data obtained, the experiments were scaled up to produce larger quantities of the cells by harvesting the cells 2 hours after an OD_{600} of 0.7 was reached. Cells were harvested by centrifugation at 4°C for 10 min at 6 000 xg, washed three times with 50 mM Tris-HCl buffer, pH 8.0, and finally resuspended in 50 mM Tris-HCl buffer, pH 8.0, containing 1 mg/ml lysozyme (3 ml/g cells). This was incubated for 20 min at 4°C followed by breaking of the cells using sonification at 60 % work output with 8 repeats of 10 sec sonication with 10 sec resting periods. The fractions was separated by centrifugation at 9 500 xg for 30 min at 4°C.

The supernatant was subjected to differential precipitation with ammonium sulphate up to 60% saturation levels. The precipitate was separated by centrifugation at 9 500 xg for 30 min at 4°C. The pellet was dissolved in the minimum volume of 50 mM Tris-HCL buffer, pH 8.0, and applied to a Phenyl-Toyopearl resin (10 cm x 2.5 cm) equilibrated with1 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl buffer, pH 8.0.

The activity was adsorbed onto the resin by mixing it with the resin for 30 min on an orbital shaker. The column was packed, washed and proteins eluted using a flow rate of 60 ml/hour and 2 ml fractions were collected. The column was washed using three column volumes of the $(NH_4)_2SO_4$/Tris-HCl buffer before the first

gradient was applied. Proteins were eluted using a 2 column volume $(NH_4)_2SO_4$ gradient (1 M – 0 M) followed by washing of the resin with 2 column volumes 50 mM Tris-HCl, pH 8.0. To elute the enzyme, a 2 column volumes ethanol gradient (0 % – 25 % (v/v) in 50 mM Tris-HCl buffer, pH 8.0, was applied followed by washing of the resin with 25 % (v/v) ethanol in 50 mM Tris-HCl buffer, pH 8.0. Elution of the proteins was monitored using with A_{280} readings and by doing the *p*NPP assay (Section 3.2.3.2.2). Samples containing the lipase activity was pooled, concentrated and applied to a 12 % SDS-PAGE gel (Section 3.2.4.1).

The extracellular LipA was purified using a similar procedure as described above. The supernatant was precipitated with $(NH_4)_2SO_4$ to a 50 % saturation level. The precipitate was collected by centrifugation, allowed to bind to the Phenyl-Toyopearl resin, the column run as described and the active fractions applied to a 12 % SDS-PAGE gel.

Part of the gel was subjected to the in gel denaturation/renaturation protocol (Section 3.2.4.3) and used for the zymogram (Section 3.2.4.4) to estimate the position of the lipase protein in the gel. This was followed by blotting of the enzyme onto a PVDF-Plus membrane (Osmonics Inc.) using the TE 62X Transphor II electrophoresis Unit (Hoefer) using the method supplied by the manufacturer. This membrane was sent to the Molecular Diagnostics Unit (KwaZulu Natal University) for amino acid sequencing using the Edman-degradation method (Hewick *et al*., 1981)).

4.2.7.2 C-terminal Histidine tagged lipase

The incorporation of a C-terminal His_6 tag did not require the deletion of the N-terminal signal peptide as this would potentially be cleaved off by the *E. coli* signal peptidase. The primer set BtlHisF and BtlHisR (Table 4.3) was used for the modification of the open reading frame using PCR (Section 4.2.5.1) as this allowed the incorporation of the 5' *Nde*I- and 3' *Hind*III restriction sites. The reverse primer was also used ot incorporate the six histidines at the C-terminal of the protein. The PCR product was subjected to restriction enzyme digestions with *Nde*I and *Hind*III

(Section 4.2.5.2), run on an agarose gel (Section 4.2.5.4), the product cleaned from the agarose gel (Section 4.2.5.5), ligated into the pET 28a vector (Section 4.2.5.3), transformed into competent *E. coli* JM109 cells (Section 4.2.3.2) and plated onto LB-plates supplemented with kanamycin (Section 4.2.2.1). White colonies were grown in LB media and plasmids isolated (Section 4.2.4.2), the insert size confirmed by restriction enzyme digestion with *Nde*I and *Hind*III and plasmids showing the correct insert were transformed into competent *E. coli* JM109(DE3) cells. Plates showing bacterial growth after 12-16 hours were replica plated onto tributyrin plates (Section 4.2.2.1) supplemented with kanamycin and left at 37°C until zones of clearance was observed. The clone showing the best activity on the tributyrin plates were used for the subsequent induction studies.

Initial induction studies were performed to monitor lipase production. Briefly, 100 ml LB media containing kanamycin (10 mg) was inoculated with 1 ml of an overnight culture and the growth followed using OD_{600} readings. When the OD_{600} of 0.6 was reached the lipase production was induced with 1 mM IPTG. Samples (600 µl) were taken every 30 min, the cells pelleted by centrifugation at 10 000 xg for 1 min, washed 2 X with 50 mM Tris-HCl buffer, pH 8.0, and resuspended in 600 µl 50 mM Tris-HCl buffer, pH 8.0, supplemented with 1 mg/ml lysozyme. This suspension was left on ice for 10 min, followed by the purification method described in section 4.2.7.1. Appropriate dilutions were prepared and assayed using the *p*NPP assay (Section 3.2.3.2.2) and the enzyme induction curve constructed.

Experiments were scaled up and the lipase extracted from the cells as described in section 4.2.7.1 to produce the cleared lysate, with the exception that the extraction buffer (lysis buffer) was supplemented with 300 mM NaCl and 10 mM imidazole.

The cleared lysate was mixed with Ni-NTA resin previously equilibrated with lysis buffer. The C-tagged lipase was allowed to bind to the nickel resin for 30 min with mild agitation on an orbital shaker. The adsorption liquor was assayed for lipase activity using the *p*NPP assay and resin added at 30 min intervals until approximately 95 % of the lipase was bound. The resin was separated from the non-binding fraction by centrifugation at 5 000 xg for 5 min and the supernatant

removed. The resin was washed 3 times with 6 X lysis buffer (batch wise) before it was packed into a column and washed with lysis buffer until a constant, low A_{280} reading was observed. The bound lipase was eluted using 250 mM imidazole and the elution followed using the *p*NPP assay. The active fractions were applied to SDS-PAGE gel electrophoresis and the samples with the lipase band were pooled, and dialysed against 50 mM Tris-HCl buffer, pH 8.0, at 4°C with 3 buffer changes over 2 days. Samples were used for the determination of the pI (Section 3.2.4.2) and to perform the zymogram (Section 3.2.4.4). Liquid samples were sent to the Molecular Diagnostics Unit (KwaZulu Natal University) for amino acid sequencing.

4.2.7.3 N-terminal Histidine tag

In order to obtain a functional N-terminally labelled His_6 tagged lipase, the signal peptide had to be removed and only the sequence for the mature lipase used. This was achieved by using the BtLHF and BtLHR primers (Table 4.3) in the PCR reaction (Section 4.2.5.1). The primers simultaneously incorporated a 5' *Nde*I- and 3' *Hind*III restriction site for cloning of the lipase gene. The PCR reaction was performed using an annealing temperature of 50°C. The rest of the procedures were followed as described in the previous section. Liquid samples were sent to the Molecular Diagnostics Unit (KwaZulu Natal University) for amino acid sequencing.

4.2.7.4 Removal of N-terminal Histidine tag

Incorporated into the plasmid is a thrombin cleavage site for effective removal of the N-terminal histidine tag. Removal of the tag was achieved by mixing the N-tagged lipase with thrombin in a 1:500 ratio (mg thrombin:mg lipase) and incubating overnight at 25°C. This sample was mixed with Ni-NTA resin and left to bind as previously noted. Instead of batch washing, the resin was packed into in the column and the Detagged lipase allowed to wash out. Samples were analysed by SDS-PAGE (Section 3.2.4.1) and the pI was determined (Section 3.2.4.2). Liquid samples were sent to the Molecular Diagnostics Unit (KwaZulu Natal University) for amino acid sequencing.

4.3 Results

4.3.1 Amplification of the lipase open reading frame

Alignment of the lipase genes from *B. stearothermophilus* and *B. thermoleovorans* showed a high degree of identity (~94%) between the lipase genes (Figure 4.2). Using the primers we were able to successfully amplify the lipase open reading frame of the approximately 1254 bp from *G. thermoleovorans* GE-7 genomic DNA, and clone the gene into the pGEM®T-easy vector (Figure 4.1). Transformation of the *E. coli* JM109 cells with the new construct, pGEMGtherm-LipA (Figure 4.3), and screening of the positive clones for lipase activity (Figure 4.4), showed strong over expression of the lipase gene, although only the antibiotic, and not IPTG, was included into the plates.

The clone showing the largest zone of clearance was selected as the clone to be used for further experiments. The first step was to monitor the production and location of the lipase. Using the induction methods described, we were able to construct the curve shown in Figure 4.5, clearly illustrating that the majority of the produced lipase, termed LipA, was located intracellularly. The decision was made to attempt purification of both the intra- and extracellular lipases.

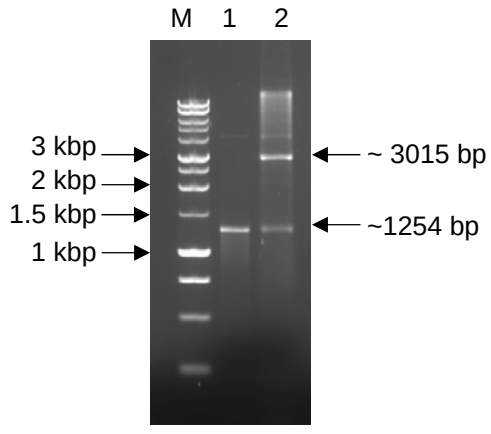


Figure 4.1 Agarose gel showing the amplified PCR product (lane 1) and the gene cloned into pGEM®T-easy, digested with *Eco*R1 (lane 2). M indicates the marker used.

```
gs     1    ATGATGAAATGCTGTCGGCGTGTTGCCCTTGTGTTGCTCGGATTATGGTTTGTGTTCTGCATATC 65
lipa   1        ATGAAAGGCTGCCGG---GTTATGTTTGTGTTGCTCGGATTATGGCTTGTATTCGGCCTATC 59
ihi91  1        ATGAAATGCTGTCGG---GTTATGTTTGTGTTGCTCGGATTATGGCTTGTGTTCGGCCTATC 59

gs     66   TGTTCTGGGAGGGCGAGCTGAAGCGGCGGCTTCGCGAGCCAACGATGCGCCGATTGTACTTCTCC 130
lipa   60   GGTCCCGGGAGGGCGGGCTGAAGCGGCAACTTCACGCGCCAACGATGCACCCATCGTGCTTCTCC 124
ihi91  60   GGTCTCGGGAGGACGGGCTGAAGCGGCAGCTTCACGCGCCAACGATGCGCCGATTGTACTTCTCC 124

gs     131  ACGGGTTTACCGGCTGGGGAAGAGAAGAAATGTTTGGGTTCAAGTACTGGGGCGGCGTGCGCGGT 195
lipa   125  ATGGTTTTACCGGCTGGGGAAGAGAAGAAATGTTTGGGTTCAAGTACTGGGGCGGCGTGCGCGGC 189
ihi91  125  ATGGCTTTACTGGCTGGGGAAGAGAAGAAATGTTTGGGTTCAAGTACTGGGGCGGCGTGCGCGGC 189

gs     196  GATATCGAACAATGGCTGAACGACAACGGTTATCGAACTTATACGCTGGCGGTCGGCCCGCTCTC 260
lipa   190  GATATCGAACAATGGCTGAACGACAACGGTTATCGAACTTATACGCTGGCGGTCGGACCGCTCTC 254
ihi91  190  GATATCGAACAATGGCTGAACGACAACGGTTATCGAACTTATACGCTGGCGGTCGGACCGCTCTC 254

gs     261  GAGCAACTGGGATCGGGCGTGCGAAGCGTATGCCCAGCTTGTCGGCGGGACGGTCGATTATGGGG 325
lipa   255  GAGCAACTGGGACCGGGCGTGTGAAGCGTATGCTCAGCTTGTCGGCGGGACGGTCGATTATGGGG 319
ihi91  255  GAGCAACTGGGACCGGGCGTGTGAAGCGTATGCTCAACTTGTCGGCGGGACGGTCGATTATGGGG 319

gs     326  CAGCCCATGCGGCAAAGCACGGCCATGCGCGGTTTGGCCGCACCTATCCCGGCCTGTTGCCGGAA 390
lipa   320  CAGCCCATGCGGCAAAGCACGGCCATGCGCGGTTTGGCCGCACTTATCCCGGCCTGTTGCCGGAA 384
ihi91  320  CAGCCCATGCGGCAAAGCACGGCCATGCGCGGTTTGGCCGCACTTATCCCGGCCTGTTGCCGGAA 384

gs     391  TTGAAAAGGGGCCGGTCGCATCCATATCATCGCCCACAGCCAAGGAGGGCAGACGGCCCGCATGCT 455
lipa   385  TTGAAAAGGGGTGGCCGCATCCATATCATCGCCCACAGCCAAGGGGGGCAGACGGCCCGCATGCT 449
ihi91  385  TTGAAAAGGGGTGGCCGCATCCATATCATCGCCCACAGCCAAGGGGGGCAGACGGCCCGCATGCT 449

gs     456  TGTATCGCTCCTAGAGAATGGAAGCCAAGAAGAGCGGGAGTACGCTAAGGCGCATAACGTGTCTT 520
lipa   450  TGTCTCGCTCCTAGAGAACGGAAGCCAAGAAGAGCGGGAGTACGCCAAGGCGCACAACGTGTCGT 514
ihi91  450  TGTCTCGCTCCTAGAGAACGGAAGCCAAGAAGAGCGGGAGTACGCCAAGGCGCACAACGTGTCGT 514

gs     521  TGTCGCCGTTGTTTGAAGGCGGACATCATTTTGTGTTGAGCGTGACAACCATTGCCACCCCTCAT 585
lipa   515  TGTCGCCGTTGTTTGAAGGTGGACATCATTTTGTGTTGAGTGTGACGACCATCGCCACTCCTCAT 579
ihi91  515  TGTCACCGTTGTTTGAAGGTGGACATCATTTTGTGTTGAGTGTGACGACCATCGCCACTCCTCAT 579

gs     586  GACGGGACGACGCTTGTCAACATGGTTGATTTCACCGATCGCTTTTTTGACTTGCAAAAAGCGGT 650
lipa   580  GACGGGACGACGCTTGTCAACATGGTTGATTTCACCGATCGCTTTTTTGACTTGCAAAAAGCGGT 644
ihi91  580  GACGGGACGACGCTTGTCAACATGGTTGATTTCACCGATCGCTTTTTTGACTTGCAAAAAGCGGT 644

gs     651  GTTGAAAGCAGCGGCTGTCGCCAGCAATGTGCCGTACACAAGTCAAGTATACGATTTTAAGCTCG 715
lipa   645  GTTGGAAGCGGCGGCTGTCGCCAGCAACGCGCCGTACACAAGCGAAATATACGATTTTAAGCTCG 709
ihi91  645  GTTGGAAGCGGCGGCTGTCGCCAGCAACGTGCCGTACACGAGTCAAGTATACGATTTTAAGCTTG 709

gs     716  ACCAATGGGGACTGCGCCGCCAGCCAGGTGAATCGTTTGACCAATATTTTGAACGGCTCAAACGG 780
lipa   710  ACCAATGGGGGCTGCGCCGCGAGCCAGGCGAATCGTTCGACCATTATTTTGAACGGCTCAAGCGC 774
ihi91  710  ACCAATGGGGACTGCGCCGCCAGCCGGGTGAATCGTTCGACCATTATTTTGAACGGCTCAAGCGC 774

gs     781  TCCCCTGTTTGGACGTCGACAGATACCGCTCGCTATGATTTATCCGTTCCCGGGGCTGAGAAGTT 845
lipa   775  TCCCCTGTCTGGACATCGACCGATACCGCCCGCTACGATTTATCCGTTCCCGGGGCTGAGACGTT 839
ihi91  775  TCCCCTGTTTGGACGTCCACAGATACCGCCCGCTACGATTTATCCGTTTCCGGAGCTGAGAAGTT 839

gs     846  GAATCAATGGGTGAAAGCAAGCCCGAATACGTATTATTTGAGCTTTGCCACAGAACGGACGTATC 910
lipa   840  GAATCGATGGGTGAAAGCCAGCCCGAATACGTATTATTTGAGCTTTCTACCGAACGGACGTATC 904
ihi91  840  GAATCAATGGGTGCAAGCAAGCCCGAATACGTATTATTTGAGCTTTGCCACAGAACGGACGTATC 904

gs     911  GCGGAGCGCTCACAGGCAACTATTATCCCGAACTCGGAATGAATGCATTCAGCGCCGTCGTATGC 975
lipa   905  GAGGAGCTCTGACAGGCAACTATTATCCCGAACTTGGAATGAACGCATTCAGCGCGATTGTCTGC 969
ihi91  905  GCGGAGCGCTCACAGGCAACTATTATCCCGAACTCGGAATGAATGCATTCAGCGCGGTCGTATGC 969

gs     976  GCCCCGTTTCTCGGTTCGTACCGCAATGCGACGCTCGGCATTGACGACCGCTGGCTTGAGAACGA 1040
lipa   970  GCCCCGTTTCTCGGCTCGTACCGCAATGCGGCGCTTGGCATTGACAGCCATTGGCTTGAGAACGA 1034
ihi91  970  GCTCCGTTTCTCGGTTCGTACCGCAATCCGACGCTCGGCATTGACGACCGCTGGCTTGAAAACGA 1034

gs     1041 TGGCATCGTCAATACGTTTTCGATGAACGGACCAAAGCGCGGATCGACCGATCGGATCGTGCCGT 1105
lipa   1035 CGGCATTGTCAATACCATTTCGATGAACGGTCCGAAGCGTGGATCAAGCGATCGGATCGTACCGT 1099
ihi91  1035 TGGCATTGTCAATACGGTTTCCATGAACGGTCCAAAGCGTGGATCAAGCGATCGGATCGTACCGT 1099

gs     1106 ATGACGGGACAATAAAAAAAGGGGTTTGGAATGATATGGGAACGTACAATGTCGATCATTTGGAA 1170
lipa   1100 ATGACGGGGCGTTGAAAAAAGGGGTTTGGAATGACATGGGAACGTACAATGTCGACCATTTGGAA 1164
ihi91  1100 ATGACGGGGCGTTGAAAAAAGGGGTTTGGAATGACATGGGAACGTACAATGTCGACCATTTGGAA 1164

gs     1171 GTCATCGGCGTTGACCCGAATCCGTTGTTTGATATCCGTGCCTTTTATTTGCGCCTTGCCGAGCA 1235
lipa   1165 ATCATCGGCGTTGACCCGAATCCGTCATTTGATATTCGCGCCTTTTATTTGCGGCTTGCCGAGCA 1229
ihi91  1165 ATCATCGGCGTTGACCCGAATCCGTCATTTGATATTCGCGCCTTTTATTTGCGACTTGCCGAGCA 1229

gs     1236 GTTGGCGAGCTTGCAGCCTTAAAACTAGTATTTTGCGAAAAAAGCCATCTCGATCGGATGGCGCT 1300
lipa   1230 GTTGGCGAGCTTCGGGCCTTAA                                            1251
ihi91  1230 GTTGGCGAGTTTGCGGCCTTAA                                            1251

gs     1301 TTTTTTCGTGAACATTC 1317
lipa   1252                  1251
ihi91  1252                  1251
```

Figure 4.2 Sequence alignment of the *B. stearothermophilus* (gs) and *B. thermoleovorans* lipase genes (lipa and ihi-91).

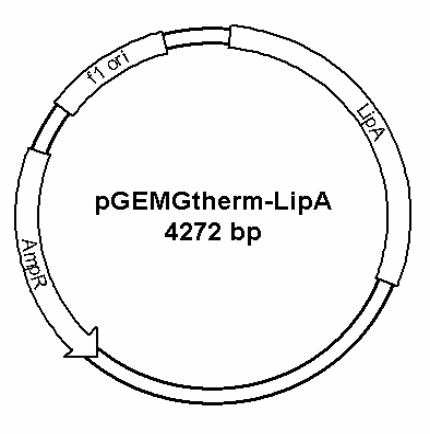


Figure 4.3 Plasmid map of the cloned LipA gene into pGEM®T-easy, showing the position and of the lipase gene and the ampicillin resistance gene (AmpR).

Figure 4.4 Tributyrin plate used for the screening of lipase clones. Positive clones show the formation of a clearance zone around the colonies.

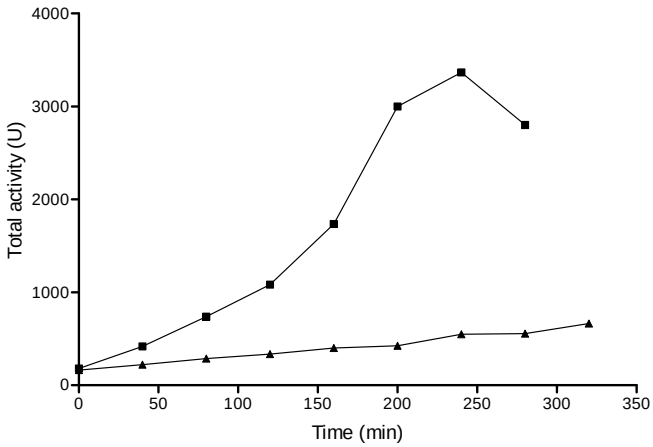
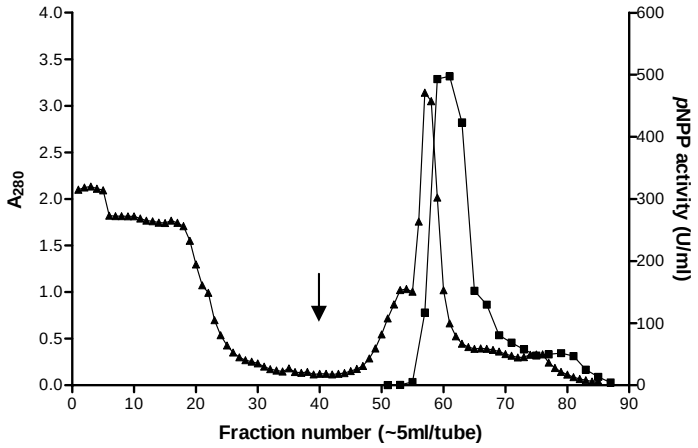


Figure 4.5 Lipase production of LipA in *E. coli* Jm109 cell showing the intracellular (■) and extracellular (▲) lipase activity.

The purification protocol used for the purification of the LipA proteins was similar to Purification protocol D used for the partial purification of the native lipase (Chapter 3). In both cases the ammonium sulphate precipitation was not as affective as for the native lipase (Table 4.4). Elution profiles obtained from the Phenyl-Toyopearl columns (Figure 4.6) were similar for the intra- and extracellular lipase, and correlated well with the results obtained for the native lipase, with the lipase only eluting after the addition of the ethanol gradient.

a)



b)

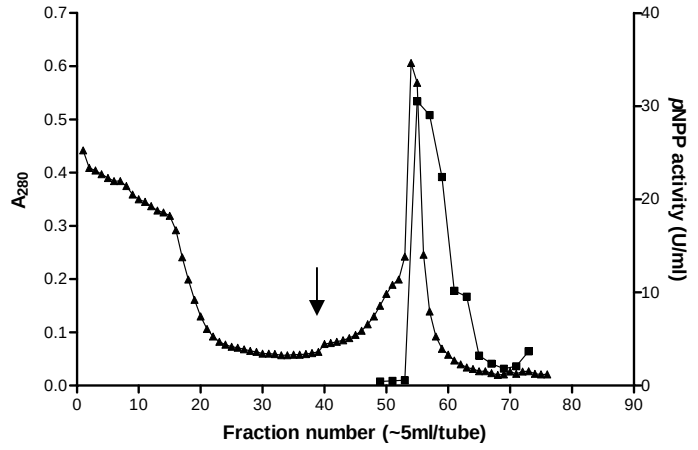


Figure 4.6 Elution profiles obtained form the Phenyl-Toyopearl columns for intacellular (a) and extracellular (b) lipases. The estimated protein concentrations (▲) and *p*NPP activity (■) is shown. In both cases the ethanol gradient was started at tube 40 (arrow).

The SDS-PAGE gels and the zymograms (Figure 4.7) showed that in both cases the lipases had the expected sizes of ~ 41 kDa and showed strong activity on the zymograms. Alignment of the deduced amino acid sequence of the LipA gene showed high similarity with other deduced *Geobacillus* lipase amino acid sequences (Figure 4.9). N-terminal amino acid sequencing data obtained showed that *E. coli* recognised the signal peptide sequence and was able to remove the signal peptide (Figure 4.8).

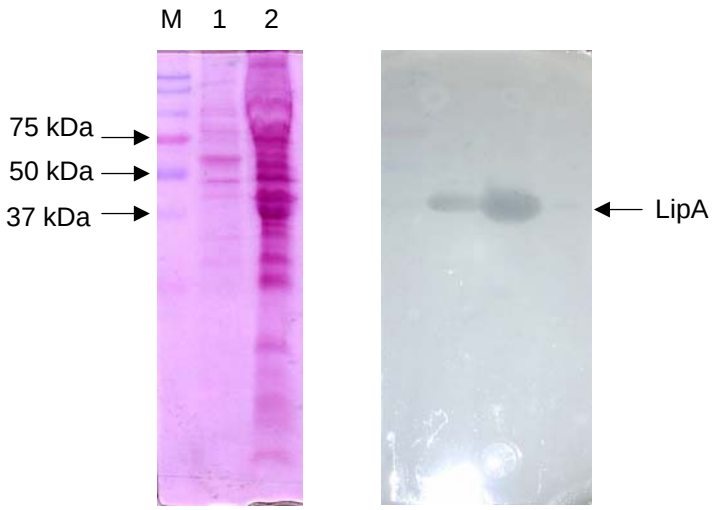


Figure 4.7 a) SDS-PAGE gel if the LipA intracellular (lane 1) and extracellular lipase (lane 2) produced in *E. coli* after the hydrophobic interaction chromatography. b) The corresponding zymogram for the SDS-PAGE gel. M indicates the marker used.

```
NSR                                          NASRYND
LipA     1   MMKCCRVMFVLLGLWLVFGLSVPGGRAEAAASRANDAPIVLLHGFTGWGRE    51
```

Figure 4.8 Amino acid alignments of the LipA sequence with the N-Terminal sequence obtained from KwaZulu Natal University. The signal peptidase cleavage site is underlined

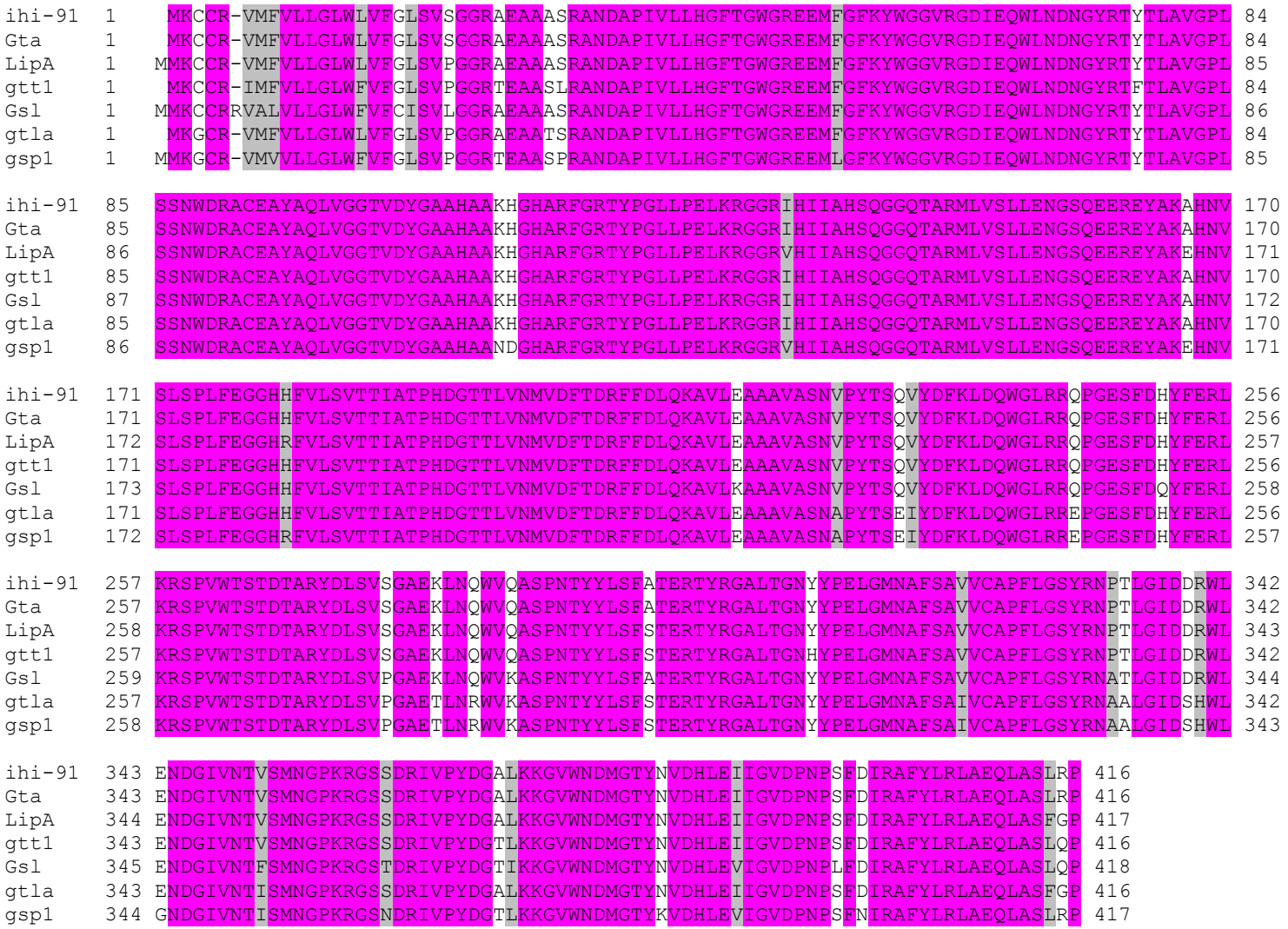


Figure 4.9 Multiple alignments of the translated *G. thermoleovorans* GE-7 lipase (LipA) gene with lipases from *B. stearothermophilus* P1 (gsp1) and L1 (gsl) and *B. thermoleovorans* IHI-91 (ihi-91), LipA (gtla) and ID-1 (gta) showing varying identity (96-99%) between the strains.

Table 4.4 Purification tables for the LipA intra- and extracellular lipases

Purification step	Total activity U	Total protein mg	Specific activity U/mg	Purification fold	Yield %
LipA intracellular					
Cell extract	17328	147.3	117.6	1	100
Ammonium sulphate precipitation	10708	66.3	161.6	1.37	62
Phenyl-Toyopearl	13197	46.5	283.8	2.4	76
Ultrafiltration	5984	7.3	820	7	34.5
LipA extracellular					
Culture medium	1.996	2.7	0.75	1	100
Ammonium sulphate precipitation	6.2	0.18	33.8	45	9.2
Phenyl-Toyopearl	4.6	0.09	53.2	71	6.8
Ultrafiltration	23	0.28	80.7	108	12.4

4.3.2 Addition of the C-terminal histidine tag

The PCR performed on the LipA gene with the BtlHisF and BtlHisR primers gave the expected product of approximately 1290 bp and could be successfully cloned into the pET 28a vector (Figure 4.10).

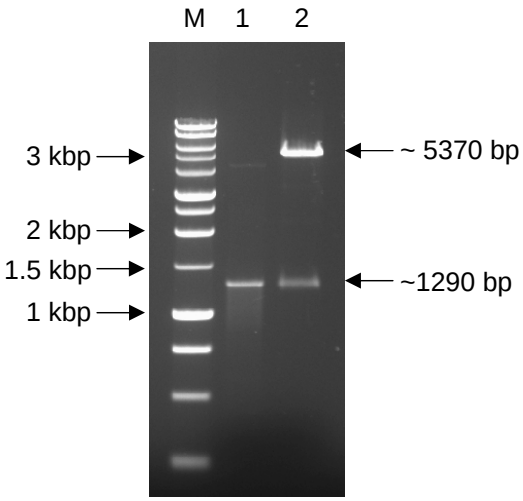


Figure 4.10 Agarose gel showing the amplified PCR product (lane 1) and the gene cloned into pET 28a vector, digested with *Nde*I and *Hind*III (lane 2). M indicates the marker used.

The resulting construct was termed pETGtherm-LipA(C) (Figure 4.11) and was transformed into both *E. coli* Jm109 and *E. coli* JM109(DE3) with the positive clones showing activity on tributyrin plates (Figure 4.4). Induction of lipase activity was initially done as described in 4.2.7.2 with 1 mM IPTG at 37ºC with both the intracellular and extracellular activities being determined.

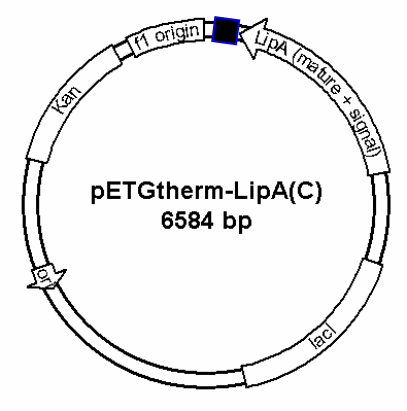
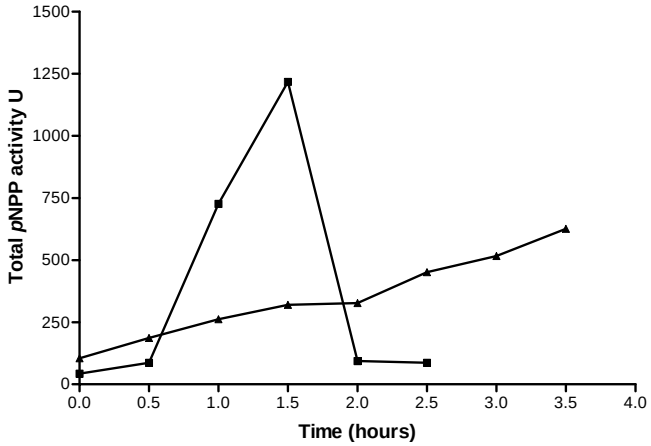


Figure 4.11 Plasmid map of the cloned C-tagged gene in pET 28a, showing the position of the lipase gene and the kanamycin resistance gene (Kan). The His_6 tag is indicated by the solid box.

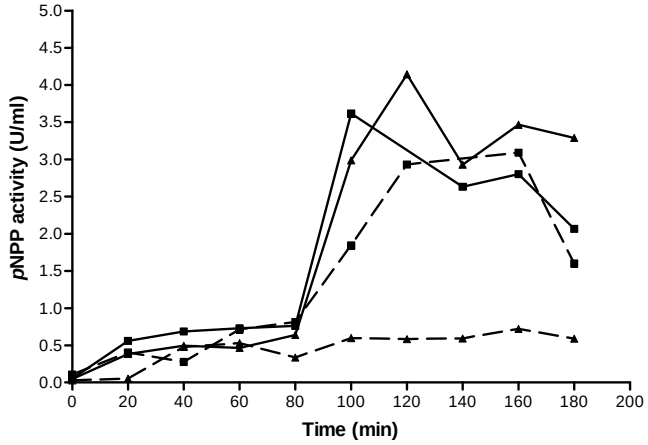
The activity however was shown to be produced intracellularly only for 90 min after which the lipases appeared to be exported to the extracellular environment (Figure 4.12 a). According to the manufacturer of the plasmid, the induction criteria could be modified to secure higher levels of expression and intracellular localization. The experiments were repeated at 25ºC and 37ºC with IPTG concentrations of 0.5 mM and 1 mM tested at each temperature. The results obtained showed the combination of growth at 25ºC and induction with 1 mM IPTG gave the best intracellular lipase yield after 2 hours (Figure 4.12 b and c), and was thus used for the rest of the experiments when C-tagged lipase was produced. Intracellular accumulation of recombinant proteins aids in the purification of these proteins.

Purification of the C-tagged lipase was done with the help of the Ni^{2+} resin, binding specifically the 6 X histidine tag. The cleared cell lysate produced was allowed to mix with the resin for 30 min, the resin allowed to settle and the supernatant assayed for remaining activity. This was repeated with the addition of more resin until less than 10 % activity could be detected in the supernatant.

a)



b)



c)

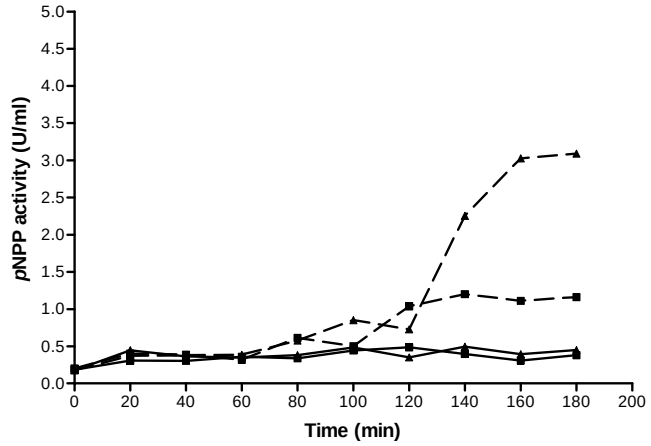


Figure 4.12 Induction profiles obtained for the C-tagged lipase. a) Initial induction experiments of the lipase with 1 mM IPTG at 37ºC showing both intra- (■) and extracellular activity (▲). b) The induction studies done at varying temperatures and [IPTG] showing the intracellular activity and c) extracellular activity obtained. In both cases a solid line indicated 25ºC and broken line 37ºC. ■ indicates the use of 0.5 mM IPTG and ▲ the use of 1 mM IPTG for induction.

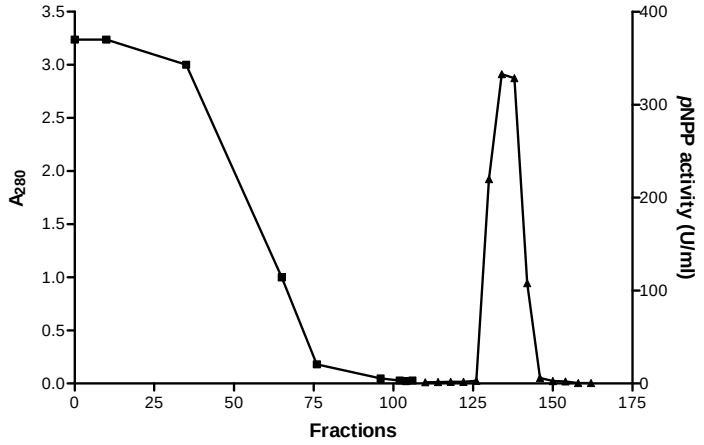


Figure 4.13 Elution profile of the C-tagged lipase on the Ni^{2+} resin showing the A_{280} (■) and *p*NPP activity (▲). Elution of the enzyme could not be followed using A_{280} readings due to interference of the imidazole in the buffer.

The use of the batchwise purification step proved to be an efficient way of removing most of the unbound enzyme. The elution profile obtained for the C-tagged enzyme is shown in Figure 4.13. The wash step before elution proved to be crucial to facilitate obtaining a pure enzyme fraction in one step and elution was started only after a constant and low A_{280} reading was obtained. Analyses of the fractions obtained from the nickel resin with SDS-PAGE and IEF gels showed that the enzyme was pure with respect to size and isoelectric point. Using the zymogram we were able to show that the band seen on the gels are in fact a lipase (Figure 4.15). Difficulties were encountered with the stability of the C-tagged lipase, as an extensive loss of activity was experienced with each purification step and during storage (Table 4.5). This was overcome by the addition of 1 mM $CaCl_2$ and 1 mM $ZnSO_4$, which was introduced to the enzyme by dialysis against buffers containing 1 mM of the cations. Nucleotide sequencing showed the correct addition of the six histidine residues to the C-terminal part of the protein (Figure 4.14).

Using SDS-PAGE and IEF gels the sizes were estimated at approximately 42 kDa with an estimated pI of 7.3. N-terminal sequencing for the rest of the samples were not finished at the time of completion of this study due to technical difficulties.

```
Ctag-end  1222  CCGAGCAACTGGCGAGCTTCGGGCCTCACCACCACCACCACCACTAA  1269
Ctag-end        A  E  Q  L  A  S  F  G  P  H  H  H  H  H  H  *
LipA      1222  CCGAGCAACTGGCGAGCTTCGGGCCTTAAAAT                1254
LipA            A  E  Q  L  A  S  F  G  P  *  *
```

Figure 4.14 Nucleotide and amino acid sequence alignment obtained for LipA and the C-tagged lipase showing the two stop codons (*, LipA) deleted with the insertions of six histidine residues.

Table 4.5 Purification tables obtained for the purification of the C-tagged lipase in the presence and absence of metal ions.

Purification step	Total activity U	Total protein mg	Specific activity U/mg	Purification fold	Yield %
C-tagged without metals					
Fractionation	1584	29.88	19.54	1	100
Ni-NTA column	214	1	204.84	10.8	13.5
C-tagged with metals					
Fractionation	216	113.7	1.9	1	100
Ni-NTA column	199	0.88	225.91	20	91.9

4.3.3 Addition of the N-terminal Histidine tag

The PCR performed with the BtlHF and BtLHR primers on the LipA gene to remove the signal peptide and introduce the *Nde*I and *Hind*III sites for cloning of the PCR product into pET 28a (Figure 4.16) was successful and yielded the expected product of approximately 1190 bp. The PCR product was cloned into pET 28a and the new construct, termed pETGtherm-LipA(N) (Figure 4.17) was transformed into both *E. coli* JM109 and *E. coli* JM109(DE3) cells. Clones were screened for activity (Figure 4.4) and the best clone selected for the induction studies

.

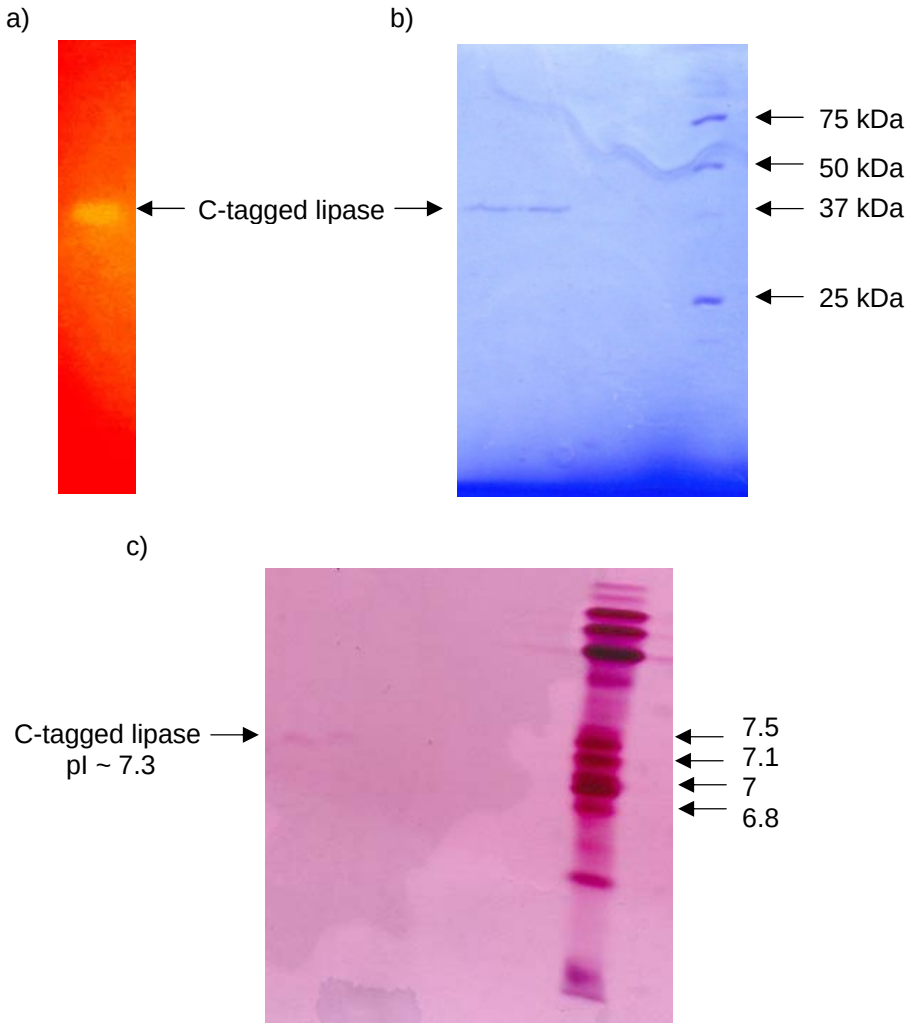


Figure 4.15 a) and b) shows the zymogram obtained using olive oil and Rhodamine 6B with the corresponding SDS-PAGE gel stained with Coomassie Brilliant blue showing the purified C-tagged lipase. The IEF gel, stained with Crocein S, of the C-tagged lipase is shown in c).

The mature lipase was cloned in frame into the pET 28a vector to use the vector for the addition of the six-histidine residues at the N-terminal part of the lipase gene. This plasmid also incorporates a thrombin specific cleavage site between the tag and the protein to effectively remove the histidine tag. The induction studies were performed at 37ºC with 1 mM IPTG and it was found that under these circumstances optimal intracellular lipase production occurred after 2 hours (Figure 4.18). For all further experiments, the cultures were harvested 2 hours after induction, and the cells used for purification of the lipase.

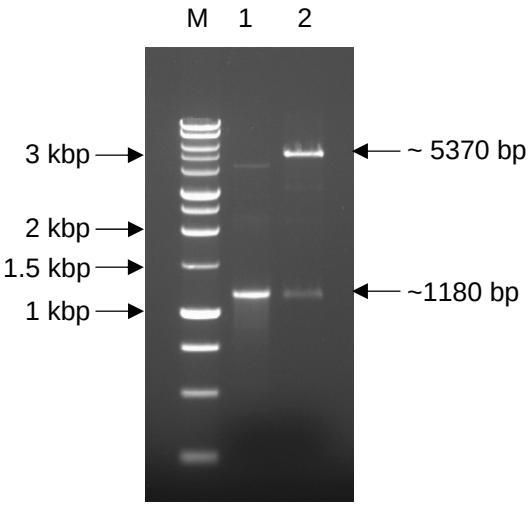


Figure 4.16 Agarose gel showing the amplified PCR product (lane 1) and the gene cloned into pET 28a vector, digested with *Nde*I and *Hind*III (lane 2). M indicates the marker used.

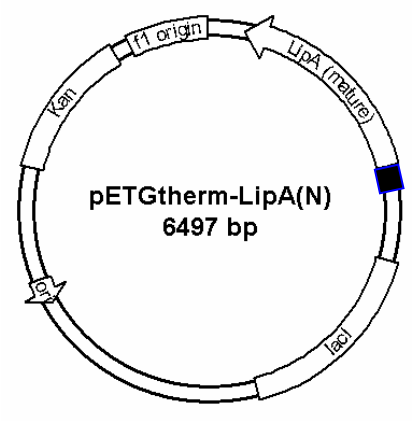


Figure 4.17 Plasmid map of the clone LipA mature lipase coding region gene into pET 28a, showing the position of the lipase gene and the kanamycin resistance gene (Kan). The His_6 tag is indicated by the solid box.

The cells were fractionated, the clarified lysate was applied to the resin and the column run as described in section 4.2.7.2. The elution profile obtained for the N-tagged lipase is given in Figure 4.19 and is similar to that obtained for the C-tagged lipase.

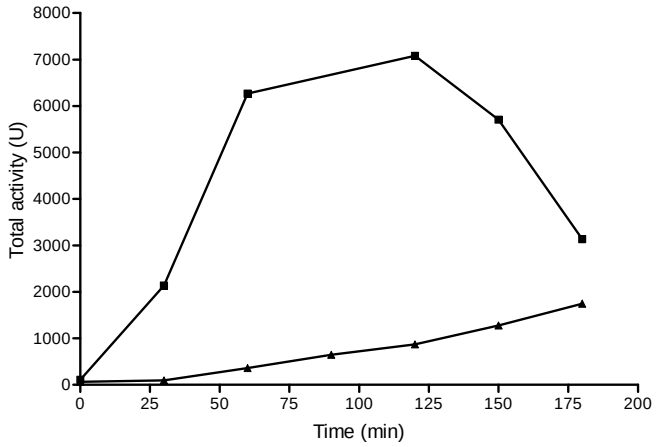


Figure 4.18 Induction profile obtained for the N-tagged lipase in *E. coli* JM109(DE3) showing the intracellular (■) and extracellular (▲) lipase activity.

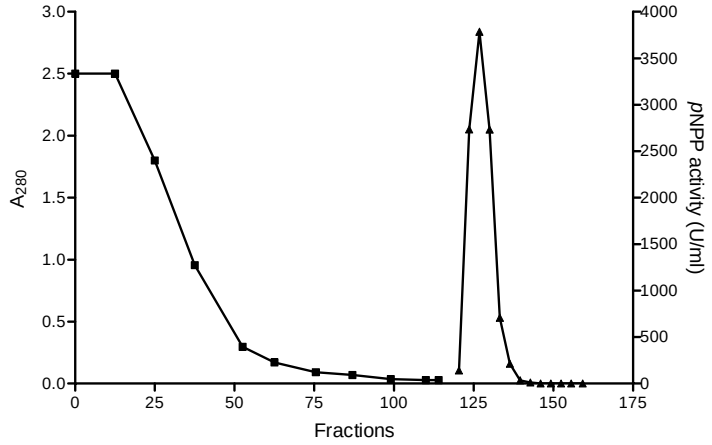


Figure 4.19 Elution profile of the N-tagged lipase on the Ni^{2+} resin showing the A_{280} (■) and *p*NPP activity (▲).

The N-tagged lipase activity was much more stable than the C-tagged lipase and no divalent metal had to be added to the N-tagged lipase to aid in the stability of the enzyme. The drop in enzyme yield shown in the purification table (Table 4.6) was a combined effect of unbound enzyme (~10 %) and the fact that column fractions were conservatively pooled to only include tubes with the highest amount of activity.

Table 4.6 Purification table obtained for the purification of the N-tagged lipase

Purification step	Total activity U	Total Protein mg	Specific activity U/mg	Purification fold	Yield %
Fractionation	23239	41.75	556.6	1	100
Ni-NTA column	11376	3.68	3090	5.55	49
Dialysis	13656	3.68	3706.8	6.66	59

Sequencing of the clone showed that the lipase was cloned into the correct reading frame, thus the histidine and the thrombin cleavage site is incorporated. This is seen when the amino acid sequence is deduced (Figure 4.20). The deduced size of the N-tagged lipase was 40.1 kDa and pI 7.1 (Figure 4.21).

```
Ntag   1  CATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGCTTCACGCGCCAACGAT   69
Ntag        H  H  H  H  H  H  S  S  G  L  V  P  R  G  S  H  M  A  S  R  A  N  D
LipA  90                                                    GCTTCACGCGCCAACGAT   111
LipA                                                        A  A  S  R  A  N  D
```

Figure 4.20 Nucleotide and amino acid sequence alignments obtained for LipA and the N-tagged lipase showing the insertions of six histidine residues and thrombin cleavage site (underlined).

4.3.4 Removal of N-terminal histidine tag

The histidine tag was removed from purified N-tagged lipase as described in section 4.2.7.4. After overnight cleavage, the mixture was allowed to mix with the Ni-NTA resin as described in the previous sections. The difference however was that the amount of lipase not bound to the resin was monitored, giving an idea of the effectiveness of the tag removal. The mixture was immediately packed into the column and the lipase eluted separating it from the histidine tag bound to the column. The normal removal efficiency was 92-95 % and using SDS-PAGE gels we could visualise the removal of the histidine tag (Figure 4.22).

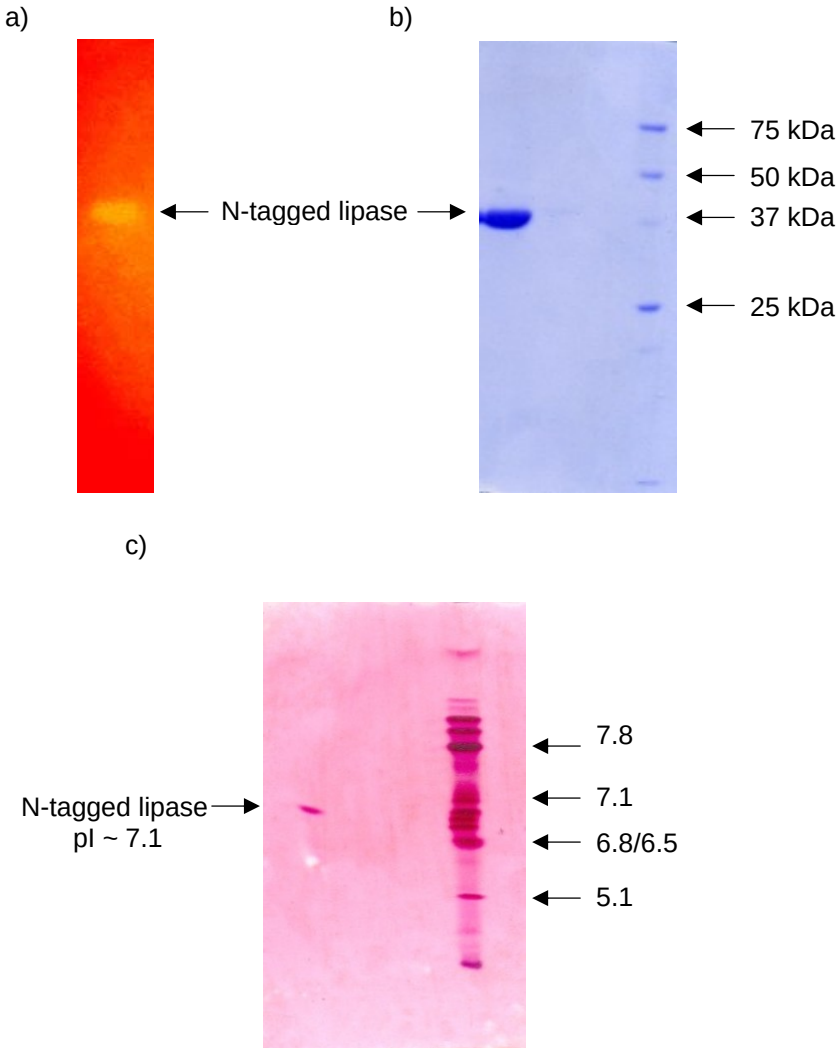


Figure 4.21 a) Shows the zymogram obtained using olive oil and Rhodamine 6B and b) the corresponding SDS-PAGE gel showing the purified N-tagged lipase. The IEF gel of the N-tagged lipase is shown in c).

The deduced size of the Detagged lipase was 39 kDa, corresponding to the size expected after removal of the histidine tag. It should be noted that the removal of the tag left behind three additional amino acids at the N-terminus of the Detagged lipase (Figure 4.23). The pI determined was 6.8.

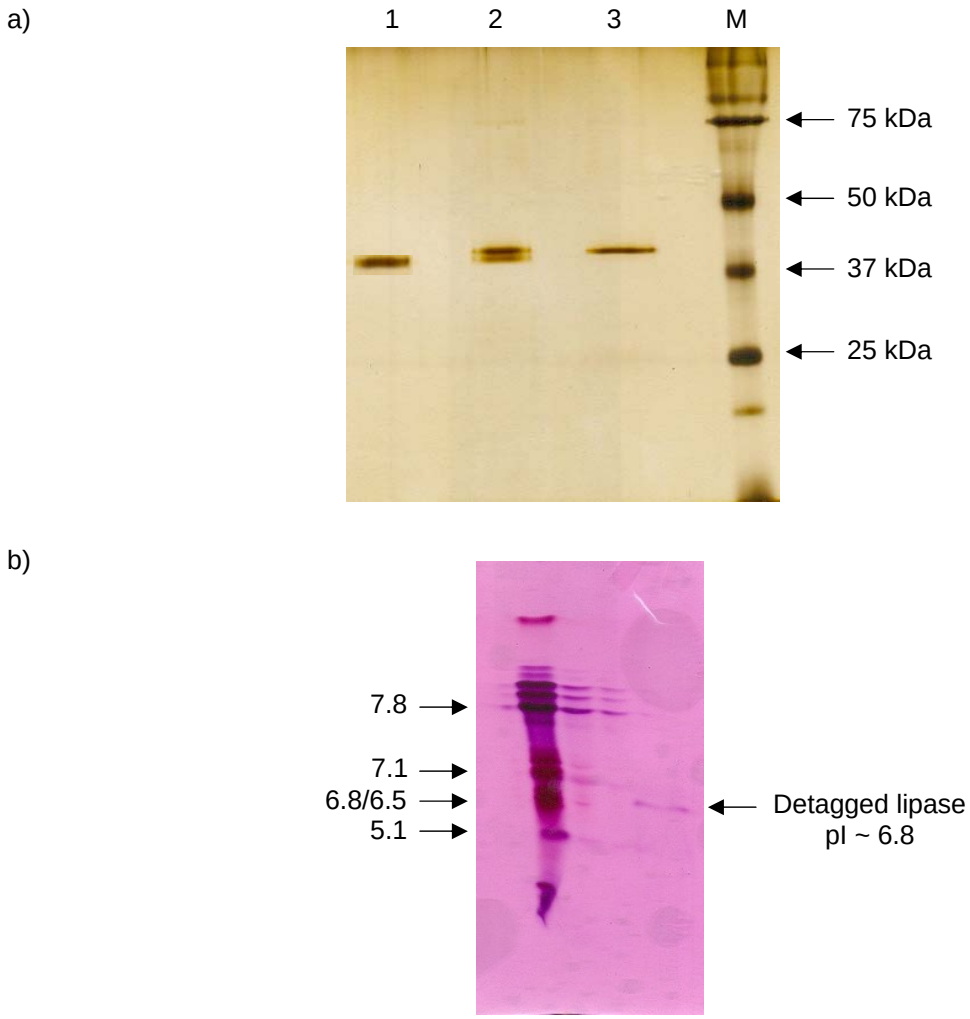


Figure 4.22 a) SDS-PAGE gel of the “Detagged” lipase (lane 1), a mixture of the Detagged and N-tagged lipase taken after16 hours during the thrombin digestion (lane 2) and the N-tagged lipase (lane 3). The marker is shown in lane M. b) The isoelectric focussing gel for the Detagged lipase.

```
Ntag        39    GGCAGCCATATGGCTTCACGCGCCAACGATGCACCCATC    78
Ntag              G  S  H  M  A  S  R  A  N  D  A  P  I
LipA        90                GCTTCACGCGCCAACGATGCACCCATC   120
LipA              A  A  S  R  A  N  D  A  P  I
```

Figure 4.23 Nucleotide and amino acid sequence alignments obtained for LipA and the Detagged lipase showing the three additional amino acids (underlined) left behind at the N-terminus of the Detagged lipase.

4.4 Discussion

The cloning of the lipase gene was successful and an expression vector was constructed which could be used to produce high titres of activity and facilitated the purification of enzymes containing a C-terminal His_6 tag, an N-terminal His_6 tag or the enzyme with the His_6 tag removed. Bell *et al*. (1999) used similar primers in the screening of pure cultures of *Bacillus* (now *Geobacillus*) and biomass from thermophilic environmental samples, and in most cases were able to detect lipase activity after the PCR product was cloned. An interesting finding was that the lipase could be expressed using the pGEM®T-easy vector; even though the PCR product was cloned in the wrong orientation, as found when sequencing was performed.

Leow *et al.* (2004) reported on the use of 4 different types of expression systems for the over expression of the *Geobacillus* sp. strain T1 lipase and found that in their experiments the use of the pET 22 vector, using the T7 *lac* promotor, gave the lowest levels of expression compared to the pBAD expression system (*araBAD* promotor), pRSET C expression system (T7 promotor) and the pGEX expression system (*tac* promotor). In this study, however the pET 28a vector (T7 *lac* promotor), gave high levels of expression in the case of the N-tagged lipase, reaching levels of 550 U/mg cell lysate, higher than the reported 20.02 U/mg cell lysate reported by Leow et al. (2004). The C-tagged lipase however showed much lower expression levels, only reaching on average 30 U/mg cell lysate, which is still higher than the reported by Leow and co-workers (2004). Kim *et al*. (2000) did similar experiments with the pET 22b expression vector using the *B. stearothermophilus* L1 lipase. They found expression levels of up to 448 000 U/g protein for the mature lipase cloned into this vector under optimised conditions. Kim *et al*. (2000) also constructed a plasmid containing the total open reading frame of the lipase and found reduced levels of expression.

It might thus be feasible to test the use of other expression systems for increased expression of the N-tagged lipase. The use of large scale fermenters in which the dissolved oxygen, temperature and pH could be monitored and controlled might lead ot increased expression. The experiments performed in our laboratory were always in 500 ml Erlenmeyer flasks with 100 ml media, which is not the

recommended 10 % of the total flask volume, and which might have had an influence on expression.

The purification of the recombinant LipA fractions gave similar results and problems as already discussed (Chapter 3), and it was decided to only partially purify the proteins. The histidine tagged enzymes were however much easier and faster to purify, with the enzyme purified after only one chromatographic step. An important factor that had to be considered for these experiments was the pH of the mobile phase used with the nickel resin. Schlieben *et al*. (2004) reported that for the separation of the His-tagged *B. thermocatenulatus* lipase, the pH had a significant effect on the separation of the proteins. At pH values above 8, they could not get significant purification of the lipase, but by lowering the pH to 7 or 7.5 they got two distinct protein peaks eluting, with the purified lipase eluting in the second peak. We did not experience similar difficulties with the purification of the N-tagged lipase, as unbound lipase was still found, however more than 55 % of the activity was recovered after selective pooling of the activity. Lowering of the pH might have an effect on the lipase binding, but this was not something investigated in this study.

The N-tagged lipase showed a highly aggregative manner (precipitation of the lipase) when it was concentrated by ultrafiltration, something that Schlieben and co-workers (2004) also experienced, and were able to overcome by changing the pH to 9 and adding 10 % (v/v) isopropanol. The C-tagged enzyme was found to be less stable than the N-tagged lipase, something that could be rectified by the addition of Ca^{2+}- and Zn^{2+}-ions. In literature it was reported that the *Geobacillus* lipases are more stable in the presence of Ca^{2+}, and structural analyses showed the presence of a Ca^{2+} and a novel Zn^{2+} binding domain (Chapter 5), leading to the decision to add both ions (Jeong *et al*., 2002). The detagging experiment proved to be a simple and effective manner for removal of the histidine tag, resulting in a lipase similar to the native lipase, which will be used in the subsequent experiments as a representative of the native *Geobacillus thermoleovorans* lipase. Results from the SDS-PAGE gels and IEF gels gave similar results to that

described in the literature, with the general size of the tagged lipase ~41 kDA and the Detagged lipase ~39 kDa (Table 4.1).

CHAPTER 5

Characterisation of the *Geobacillus thermoleovorans* lipase

5.1 Introduction

Structural determinations of various lipases have shown that all lipases contain the α/β-hydrolase fold motif, a structural motif common to a wide variety of hydrolases (Van Pouderoyen *et al*., 2001; Ollis *et al*., 1992). The lipase active site usually contains the catalytic triad Ser,His,Asp/Glu, similar to that of other serine hydrolases. With the exception of the catalytic serine residues, amino acid alignments of the lipases for which the structures were determined showed little homology (Sinchaikul *et al*., 2001), contrary to the similarities obtained with their 3D-structures. According to the lipase structures, the catalytic serine is embedded in a signature pentapeptide sequence, Gly-X-Ser-X-Gly, located at the C-terminal of a section of parallel strands of β-sheet, with the initial glycine of the pentapeptide replaced with an alanine in *Bacillus* lipases (Tyndall *et al*., 2002). The catalytic serine is further embedded in a tight bend between an α-helix and β-strand (Sinchaikul *et al*., 2001). The majority of the microbial lipases contain a helical segment, called the lid, covering the active site when the enzyme is in the so-called closed conformation, with the exception of the *B. subtilis* lipase (Van Pouderoyen *et al*., 2001) and the *Fusarium pisi* cutinase (Promper *et al*., 1999) that shows no lid region.

The lipase structures of two *B. stearothermophilus* lipases (L1 and P1) have been resolved and although they share some similarities to the microbial lipase structures reported there are a few prominent differences (Tyndall *et al*., 2002; Sinchaikul *et al*., 2002; Jeong *et al*., 2002). One of the characteristics for the classification of these Gram-positive bacterial lipases is their size of 40-45 kDa (Jeong *et al*., 2002), which can be attributed to the inclusion of various α-helices

and β-sheets surrounding a novel zinc binding site and included in an elongated lid region. Figure 5.1 shows a comparison of the secondary structures of *B. stearothermophilus* P1 lipase and the canonical lipase fold.

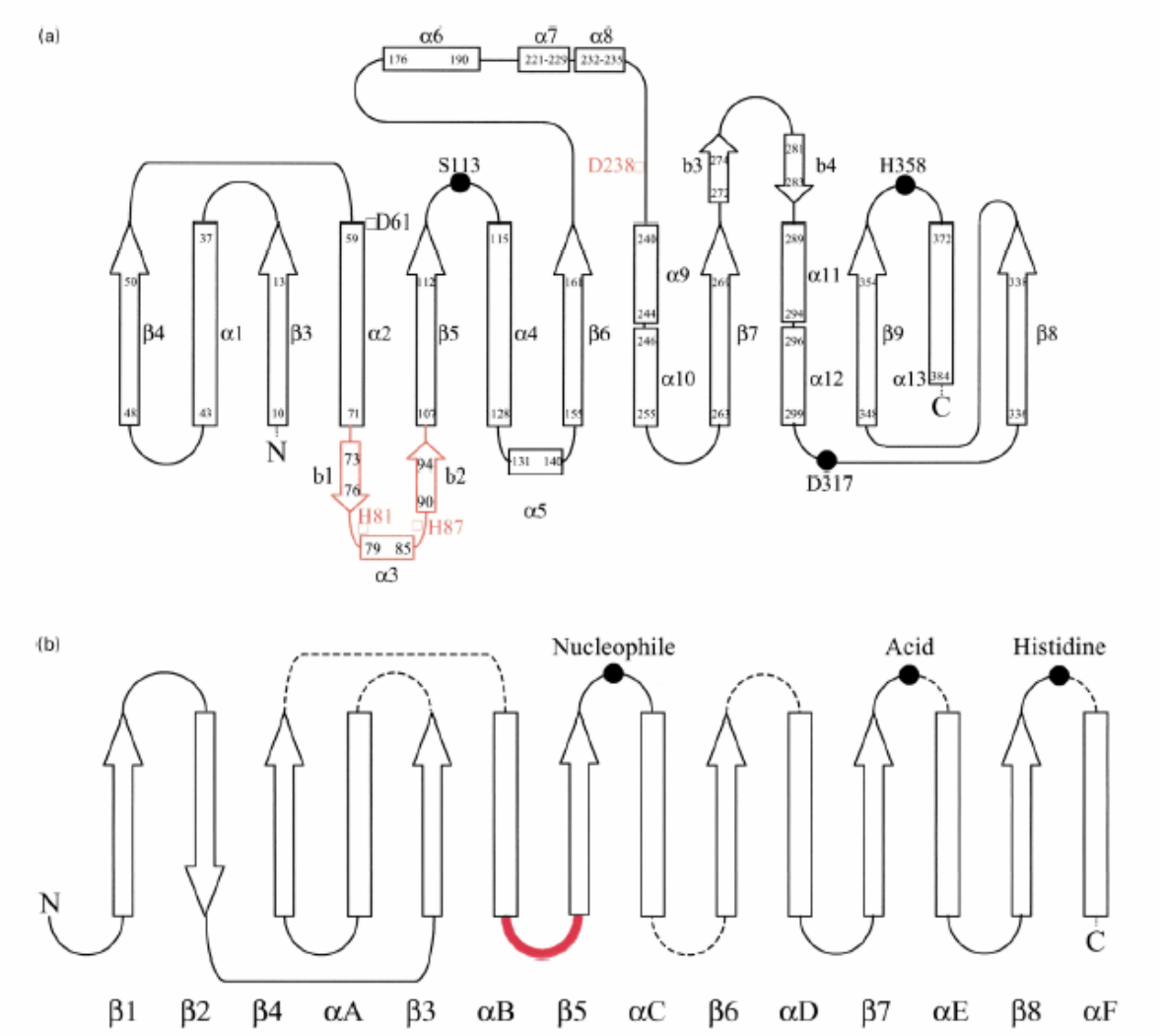


Figure 5.1 Comparison of the secondary structure of *B. stearothermophilus* P1 lipase (BSP) with the canonical lipase fold. a) Secondary structure topology of BSP showing the general α/β hydrolase fold including the catalytic triad and the zinc-binding structural elements and residues indicated in black and red, respectively. Rectangles and arrows represent α-helices and β-strands, respectively. New structural elements, strand b1 and helix α3 are shown in red. b) Secondary structure topology diagram of the canonical α/β hydrolase fold. Broken lines indicate possible sites of insertions. The heavy line depicts the position of the new deviation from the known fold. (Taken from Tyndall *et al*., 2002)

The lid region of the *Geobacillus* lipases is characterised by the insertion of a large region following helix α6 consisting of a large loop followed by helices α7, α8 and α9 (Tyndall *et al*., 2002). Helix α6 forms the lid with helices α7-9 believed to surround helix α6 to form a tightly packed structure above the active site (Jeong *et al*., 2002). The inner face of the lid helix is packed with hydrophobic residues and faces towards the active site, allowing for strong hydrophobic interactions with the active site, thus protecting the active site from the surroundings in the closed formation (Jeong *et al*., 2002).

The additional Zn-binding domain was the first example reported among all known lipases. The Zn^{2+} binding site stabilizes the extra domain consisting of helices α3 and strands b1 and b2, and the associated loops by forming coordination bonds with two histidines from the extra domains and two aspartic acids from the core domain of the protein (Jeong *et al*., 2002). The zinc-coordinated extra domain provides tight interaction with the core domain as well as the characteristic cluster of helices and loops around the lid helix (α6). The zinc-binding region also makes tight contacts with the loop extended from the lid helix, and the helix is thought to play a role in the opening of the lid (Tyndall *et al*., 2002).

The active site of the *Geobacillus* lipases contains the catalytic triad assigned to Ser113, His358 and Asp317 (Sinchaikul *et al*., 2001), with the catalytic serine situated in a nucleophilic elbow between strand β5 and helix α6 (Tyndall *et al*., 2002). The catalytic serine is part of the pentapeptide consisting of Ala-X-Ser-X-Gly, similar to that found in other *Bacillus* lipases. The active site and surrounding area is mainly made up by aromatic and hydrophobic amino acids, leading to a more rigid structure via tight side-chain packing (Jeong *et al*., 2002). The tight packing of the active site, additional hydrophobic cores in the lipase structure and the hydrophobic inner face of the lid region are speculated to contribute to the thermal activity profile and stability of these enzymes at elevated temperatures. The authors noted that at lower temperatures the rigidity in the catalytic triad region would inhibit enzymes activity, as the enzyme requires certain flexibility of the active site for efficient catalysis (Jeong *et al*., 2002).

Calcium binding domains found the *Geobacillus* lipases with the calcium ion coordinated to Glu360 and Asp365 carboxyl oxygens along with the carboxyl oxygens from the main chain Gly286 and Pro366 along with two water molecules. The calcium stabilises the active His358 loop but is thought to play a role in stability and not activity. The calcium ion of *Pseudomonas* group lipases are covered by helix α8, but this is not the case for *Geobacillus* lipases with this helix missing, exposing the calcium-binding site to the surrounding solvents (Jeong *et al*., 2002; Tyndall *et al*., 2002).The unique structural attributes of the *Geobacillus* lipases coupled to the associated effects they elicit on the enzyme, in terms of temperature, pH and organic solvents stability (Table 5.1a and b), is what makes these lipases such suitable candidates for use in industry. Some of these traits will be further explored in this chapter.

5.2 Material and Methods

5.2.1 Chemicals

Unless otherwise stated, all chemicals were obtained from commercial sources, were of analytical reagent grade or better and were used without further purification.

5.2.2 Enzymes used

The enzymes used for these experiments included the partially pure native *G. thermoleovorans* (Chapter 3, protocol E) and LipA recombinant lipase (Chapter 5), and the pure N-tagged, Detagged and C-tagged recombinant lipases (Chapter 5). All the enzymes were stored in 50 mM Tris-HCl buffer, pH 8.0, with the buffer supplemented with 1 mM $CaCl_2$ and 1 mM $ZnSO_4$ for the C-tagged lipase.

5.2.3 Assays used

5.2.3.1 Olive oil assay

The olive oil assay was used as described in Section 3.2.3.2.1.

Table 5.1a Characteristics of the lipases belonging to the family 1.5 lipases

Organism	Optimum pH / temperature	pH/ temperature stabillity	Substrate preference	Influence of metals on activity	Influence of other chemicals	Reference
Geobacillus sp. TW1 (cloned)	pH 7-8; 40ºC	pH 8.5-11 (40ºC); Activity at 90ºC	Not reported	Enhanced by Ca^{2+}, Mg^{2+}, Zn^{2+}; inhibited by Cu^{2+}, Mn^{2+}	Stable in 1 mM EDTA, SDS, DTT and 0.1 % detergents	Li and Zhang, 2005
Bacillus stearothermophilus MC7	pH 7.5-9; 75-80ºC	pH 7-11; 30 min at 70ºC	Tributyrin; *p*NP-palmitate	Enhanced by Ca^{2+}, inhibited by Fe^{2+}, Zn^{2+}, Mg^{2+} and Mn^{2+}	Sorbitol enhanced activity	Kambourova *et al*., 2003
Bacillus sp. RSJ-1	pH 8-9; 50ºC	120 min at pH 8-9; 30 min at 75ºC	Not reported	Enhanced by Ca^{2+}, Mg^{2+}, Ba^{2+}, inhibited by Zn^{2+} and K^{+}	EDTA and Triton X-100 had no effect	Sharma *et al*., 2002
Bacillus stearothermophilus P1 (cloned)	pH 8.5; 55ºC	Half live is 2h at 55ºC	Tricaprylin; *p*NP-caprate	Enhanced by Mg^{2+} and inhibited by Zn^{2+}	Stable in 0.1 CHAPS and Triton X-100. Inhibited by EDTA and PMSF	Sinchaikul *et al*., 2001
Bacillius thermoleovorans ID-1 (cloned)	pH 9; 60-65ºC	Neutral pH (room temperature); 30 min at 70ºC	Tricaprylin	Enhanced by Ca^{2+}, Co^{2+} and Mn^{2+}	EDTA inhibited lipase, ethanol enhanced activity	Lee *et al*., 2001
Bacillus THL027	pH 7; 70ºC	Stable neutral pH fro 24 h at room temperature	Tricaprylin, rice bran and olive oil	Not reported	Not reported	Dharmsthiti and Luchai, 1999

Table 5.1b Characteristics of the lipases belonging to the family 1.5 lipsaes

Organism	Optimum pH / temperature	pH/ temperature stabillity	Substrate preference	Influence of metals on activity	Influence of other chemicals	Reference
Bacillius stearothermophiluss Li (cloned)	pH 7-10; 60-65ºC	Stable for 24h at neutral pH at 4ºC; Stable for 30 min at 55ºC	Beef tallow and palm oil; *p*NP-caprylate	Ca^{2+} had no effect; Zn^{2+} and Cu^{2+} inhibited enzyme	Inhibited by EDTA and Triton X-100 (1 %) with almost no activity present with SDS	Kim *et al*., 1998
Bacillus thermocatenulatus	pH 8-9.5; 50-75ºC (depending on substrate used)	Stable for 14h at pH 9-11 at 30ºC	Tributyrin; *p*NP-caprate	Not reported	Stable in 30 % (v/v) ethanol, isopropanol, ethanol and methanol; Triton X-100 and CHAPS enhanced activity; SDS deactivated protein	Rua *et al*., 1997

5.2.3.2 pNPP assay

The *p*NPP assay was used as described in Section 3.2.3.2.2.

5.2.3.3 pH-stat assay

The pH stat assay as described by Sarda and Desneulle (1958) and the adapted buffer system as described by Carrière *et al*. (1997) was used.

A Metrohm 641 Impolsomat pH stat system was used with a thermostatically controlled titration vessel at 65ºC. The assay buffer consisted of 1 mM Tris-HCl, pH 8.0, 0.1 M NaCl and 5 mM $CaCl_2$. The liquid triacylglycerol, usually tributyrin, was added directly to the titration vessel and the emulsion formed by vigorous stirring using a propeller agitator. Longer chain triacylglycerols were emulsified before addition as described in section 3.2.3.2.1. Substrate (0.5 ml) and buffer (14.5 ml) were mixed in the titration vessel, the reaction was pretitrated to pH 8.0 and the appropriate enzyme dilution was added. The hydrolysis rates of the emulsified triglycerides were measured potentiometrically by titrating the liberated fatty acids with standarised 50 mM NaOH.

A unit (U) of enzyme activity was defined as the amount of enzyme that would release 1 µmole of butyric acid per minute under the defined assay conditions.

5.2.4 Structural characterization of the G. thermoleovorans lipase

Structural analysis was performed with the amino acid sequence obtained in Chapter 4. The alignments were performed using DNAssist v2.0 (Patterton and Grave, 1999) with known lipase sequences obtained from the NCBI website.

Homology modelling was performed with *B. stearothermophilus* lipase L1 (PDB accession number 1KU0) as template using WHATIF (Vriend, 1990) and the model was optimised by simulated annealing using YASARA software (Di Nola *et al*., 1991).

5.2.5 Kinetic characterization of the lipases

Appropriate controls were included for all the experiments done. The amount of repetitions done will be specified for each experiment.

5.2.5.1 Effects of temperature

5.2.5.1.1 Optimum temperature

The optimum temperature was determined for the native, LipA, N-tagged, Detagged and C-tagged lipases over a range of 40 - 70ºC. In each case the assay buffer (1 mM Tris-HCl, pH 8.0; 100 mM NaCl) was equilibrated at the required temperature before addition of enzyme. Olive oil assays (Section 5.2.3.1) were done in duplicate together with a blank at each temperature. Experiments were repeated three times with duplicate values for each repeat.

Experiments examining the influence of calcium and zinc ions on the optimum temperature were performed using the N-tagged and Detagged lipase pre-incubated at room temperature with 1 mM $CaCl_2$, 1 mM $ZnSO_4$ or a combination of the two. This experiment was done in duplicate.

5.2.5.1.2 Temperature stability

The temperature stability was determined for the N-tagged, Detagged and C-tagged lipases at temperatures ranging from 40 - 70ºC. The enzyme was incubated at different temperatures and aliquots were periodically withdrawn and immediately placed on ice. The residual activity was assayed using the *p*NPP assay (Section 5.2.3.2). Assays were done in 50 mM Tris-HCl buffer, pH 8.0.

Experiments examining the influence of calcium and zinc ions on the temperature stability were performed using the N-tagged and Detagged lipase pre-incubated at room temperature with 1 mM $CaCl_2$, 1 mM $ZnSO_4$ or a combination of the two.

5.2.5.1.3 Determination of activation energy for the hydrolysis of olive oil

The activation energy was determined for the Detagged lipase in the presence and absence of 0.1 mM $CaCl_2$, 0.1 mM $ZnSO_4$, a combination of both, 40 mM EDTA and 2 mM N,N,N',N'-tetrakis(2-pyridylmethyl)ethylene-diamine (TPEN). Assays were performed under the same conditions as described in section 5.2.3.2 in 50 mM Tris-HCl buffer, pH 8.0. The experiments were done in duplicate.

5.2.5.2 Effects of pH

5.2.5.2.1 Optimum pH

The activity was determined for all the lipases over a range of pH 2.5 - 10 using the olive oil assay described in Section 5.2.3.1. The pH range was constructed by adjusting the pH of the assay buffer cocktail used, consisting of an equimolar mixture of sodium acetate, Bis-Tris-Propane and glycine (20 mM). The enzyme was added and the reaction was allowed to proceed for 60 min at 65ºC. The experiment was repeated twice with duplicate values for each repeat.

5.2.5.2.2 pH stability

pH stability was tested for the N-tagged, Detaged and C-tagged lipases. The pH of the enzyme solution was adjusted to the required value, between pH 2.5 - 10, using a strong acid or base. The enzyme solution was incubated at 40ºC at the different pH-values and aliquots were periodically withdrawn and immediately placed on ice. The remaining activity was immediately assayed at pH 8.0, ensuring that the pH of the assay was not affected by the enzyme solution, using the *p*NPP assay (Section 5.2.3.2) in 50 mM Tris-HCl buffer, pH 8.0.

5.2.5.3 Effects of metal ions

The effect of metal ions on lipase activity was assessed using Al^{2+}, Ba^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, Hg^{2+}, Mg^{2+}, $Mn^{2+}$and Zn^{2+}. A metal ion concentration range of 0 - 5 mM

was investigated. The enzyme was incubated with different concentrations of metals for 30 min at 40ºC in 50 mM Tris-HCl, pH8.0, and the remaining activity assayed using the *p*NPP assay (Section 5.2.3.2). The assay was performed in 50 mM Tris-HCl, pH 8.0. The experiment was performed in duplicate.

5.2.5.4 Effects of detergents

The effect of detergents on the N-tagged, Detagged and C-tagged lipase activity was tested at concentrations below and above the critical micelle concentration (cmc = the minimum concentration at which detergents begin to form micelles). Four detergents were tested; the anionic detergent sodium dodecylsulphate (SDS) (cmc = 8.2 mM), the cationic detergent cetrimide (hexadecyltrimethyl-ammoniumbromide) (cmc = 0.92 mM), the non-ionic detergent Triton X-100 (cmc = 0.3 mM) and the zwitterionic detergent 3-[(3-Cholamidopropyl)dimethylammonia]-1-propane-sulfonate (CHAPS) (cmc = 6.5 mM). The enzyme was incubated with different concentrations of different detergents for 30 min at 40ºC and the remaining activity was assayed using the *p*NPP assay (Section 5.2.3.2) in 50 mM Tris-HCl buffer, pH 8.0. The experiment was done in duplicate.

5.2.5.5 Effects of organic solvents

The effect of alcohols on lipase activity was tested on the N-tagged, Detagged and C-tagged lipase. The enzymes were incubated with various concentrations of each alcohol varying from 0 - 40 % (v/v) and dimethylsulfoxide (DMSO) at concentrations of 0 - 40 mM. Remaining activity was determined using the *p*NPP assay (Section 5.2.3.2) in 50 mM Tris-HCl buffer, pH 8.0. Experiments were done in duplicate.

5.2.5.6 Effects of other chemical agents

The effects of EDTA and phenanthroline was measured for the N-tagged, Detagged and C-tagged lipases by incubating the enzymes with varying concentrations of 0 - 40 mM of each of the chemicals. Residual activity was

measured using the *p*NPP assay (Section 5.2.3.2) in 50 mM Tris-HCl buffer, pH 8.0. Experiments were performed in duplicate.

The effect of TPEN was measured on the Detagged lipase by incubating the enzyme (50 mM Tris-HCl, pH8.0) with varying concentrations of the TPEN. The samples were incubated at 40ºC and samples taken periodically to measure the remaining activity using the *p*NPP assay done in 50 mM Tris-HCl, pH 8.0.

5.2.5.7 Substrate specificities

The substrate specificity for the native, N-tagged, Detagged and C-tagged and LipA lipases was determined using both synthetic esters and triacylglycerols. Experiments were performed in duplicate

Synthetic esters were tested using the conditions described for the *p*NPP assay (Section 5.2.3.2) using *p*NP-caprylate (C8:0), *p*NP-caprate (C10:0), *p*NP-laurate (C12:0), *p*NP-myristate (C14:0), *p*NP-palmitate (C16:0), *p*NP-stearate (C18:0), *p*NP-palmitoleate (16:1cΔ^{9}), *p*NP-oleate (18:cΔ^{9}), *p*NP-linoleate (18:2$\Delta^{9,12}$), *p*NP-linoleate (18:3c$\Delta^{9,12,15}$), *p*NP-petroselenate (18:3c$\Delta^{6,12,15}$) and *p*NP-eliadate (18:1tΔ^{9}). Assays was performed as described in Section 5.2.3.2. Triacylglycerol specificities were tested using the pH-stat assay (Section 5.2.3.3). The substrates that were evaluated were tripropionin (C3:0), tributyrin (C4:0), tricaprylin (C8:0), triolein (C18:*c*Δ^{9}), trielaidin (C18:*t*Δ^{9}). In these experiments the substrates were emulsified using a Branson Cell Disrupter B-30 to ensure a homogeneous emulsion when stirring was insufficient, especially with the longer chain fatty acids.

5.3 Results

5.3.1 Structural analysis of the G. thermoleovorans lipase

Alignments of the LipA showed a high degree of identity (96 - 99 %) with the lipases from *B. stearothermophilus* and *B. thermoleovorans* (Figure 4.1). An

analysis of the sequences shows the signal peptidase cleavage site of the open reading frame (Figure 5.2 a), yielding the signal peptide and mature lipase. Cleavage is achieved between the alanine amino acids (E-A-A) in the recognition sequence G-R-A-E-A-A-A-S. The catalytic triad residues S113, H358 and D317 were successfully identified and the catalytic serine forms part of the pentapeptide reported (Tyndall *et al*., 2002), consisting of the sequence Ala-His-Ser-Gln-Gly, showing the initial glycine substitution with an alanine residue (Figure 5.2b).

Figure 5.2 The deduced LipA amino acid sequence aligned with reported sequences from *B. stearothermophilus* P1 (gsp1) and L1 (Gsl), and *B. thermoleovorans* T1 (gtt1), LipA (gtla and gta), and IHI-91 as obtained from NCBI. The signal peptide cleavage site, indicated with an arrow (a) and the catalytic serine pentapeptide (b) is shown in the black boxes.

Modelling of the *G. thermoleovorans* GE-7 LipA using the *B. stearothermophilus* L1 lipase (1KU0) as template showed that the two structures were almost identical (Figure 5.3 a), which is expected as the sequence identity is 93 %. The zinc-binding site (Figure 5.3 b) could be identified as consisting of Asp61, His81, His87 and Asp238 as reported by Jeong and co-workers (2002).

The structure showed the elongated loop covering the active site (Figure 5.4). Analysis of the amino acids at the interface of the lid, and the amino acid of the

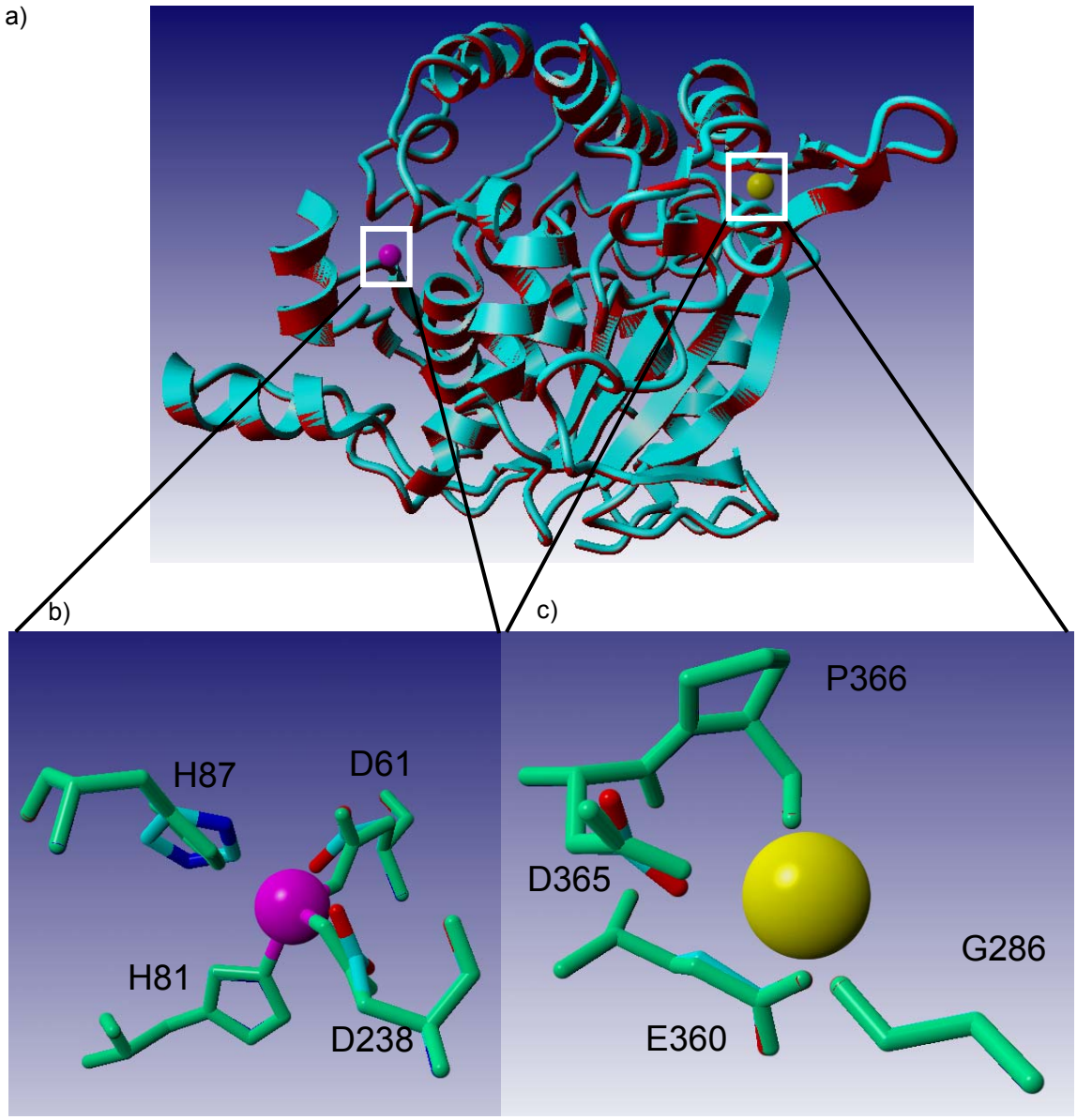


Figure 5.3 Cartoon rendering of the *G. thermoleovorans* GE-7 lipase LipA model (red) superimposed on the *B. stearothermophilus* L1 lipase (cyan) to show a) the complete structure of the thermostable lipase highlighting the novel zinc-binding domain (b) with the associated amino acids and the calcium-biding site (c) with the associated amino acid co-ordinations. The GE-7 lipase is coloured with the normal atom colours and the L1 lipase is coloured cyan.

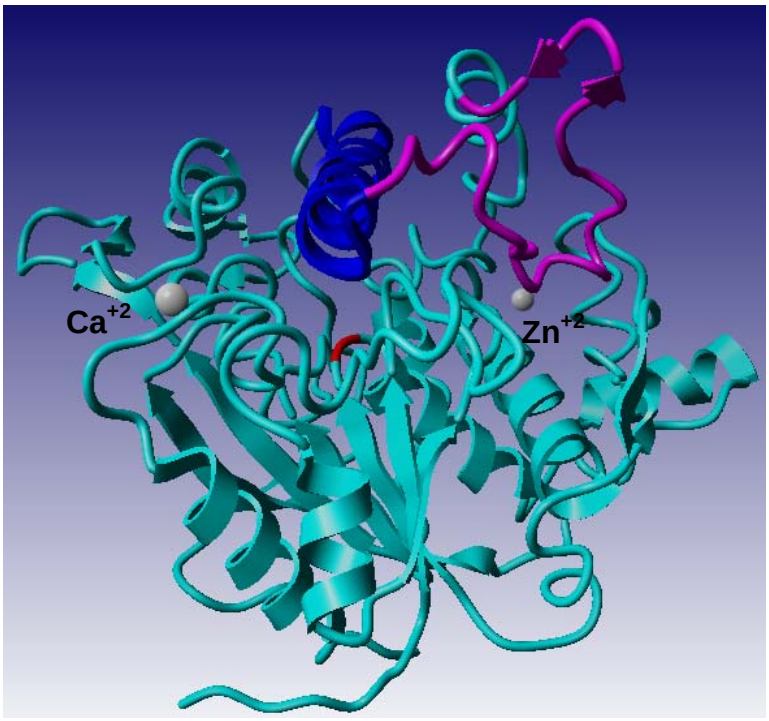


Figure 5.4 Cartoon rendering of *G. thermoleovorans* lipase showing the helix consisting of residues 175-195 (blue) covering the substrate binding cleft with the catalytic serine in red. The extended loop consisting of residues 196 to 221 (magenta) makes contact with the zinc binding domain.

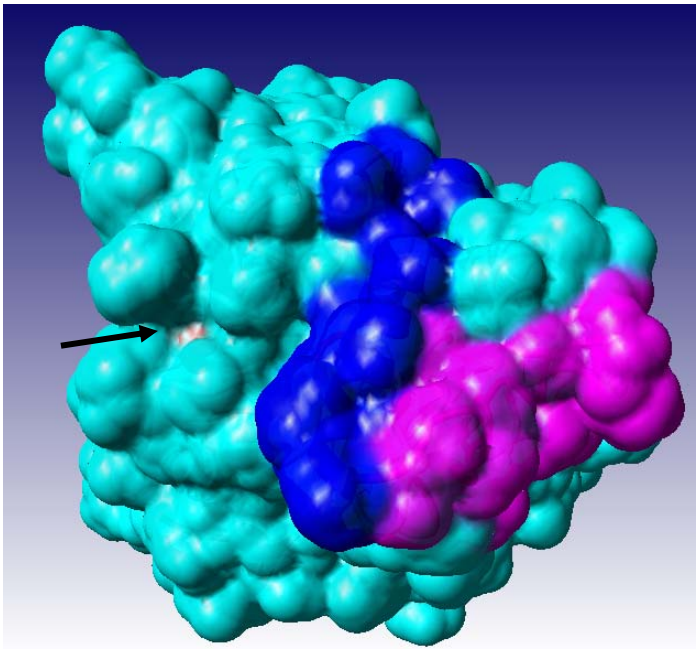


Figure 5.5 Solvent accessible surface of the Lipa lipase showing the Ca^{+2} ion (arrow) exposed to the environment and its relationship with the lid (blue) and the extended loop (magenta).

active site showed a high amount of aromatic and hydrophobic residues, leading to the reported tighter association of the lid with the surrounding α-helices and the active site. The zinc binding site is reported to play a role in the opening of the lid, The proposed calcium-binding site could be identified as including the side chain carboxylates of Glu360, Asp365 and the backbone carboxyl's of Gly286 and Pro366 (Figure 5.3 c). As reported by Jeong *et al*. (2002), the calcium-binding site is exposed to the external environment (Figure 5.5) and might thus not be directly associated with the active site and activity, but rather with the thermostability. The role of the zinc and calcium binding site was further investigated for their role in the opening of the lid, and will be discussed later in this chapter.

5.3.2 Kinetic characterization of the lipases

5.3.2.1 Effects of temperature on the lipases

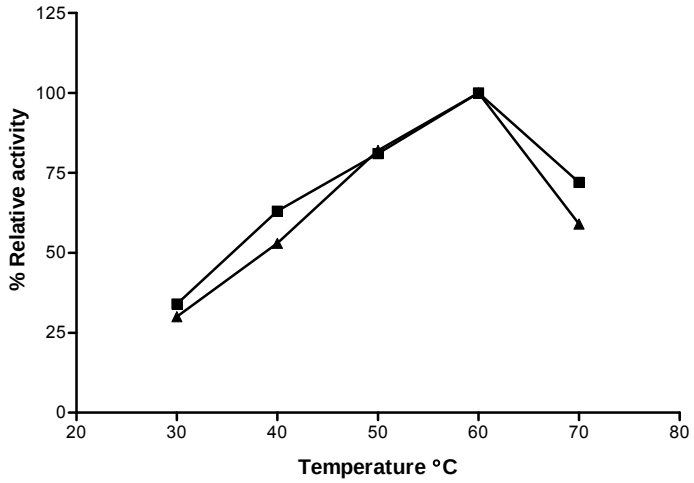
The optimum temperature profiles obtained for the 5 lipases (native, LipA, N-tagged, Detagged and C-tagged) showed that the optimum temperature for the C-tagged lipase was 55ºC while the rest of the enzymes had optimum temperatures of 60ºC (Figure 5.6).

Keeping in mind that the C-tagged lipase is in the presence of 1 mM each of $CaCl_2$ and $ZnSO_4$ the influence of the metal ions was investigated for the Detagged and N-tagged lipases. These experiments were not performed on the partially pure native and LipA lipases. The results obtained showed that the addition of either 1 mM $CaCl_2$, 1 mM $ZnSO_4$ or a combination of the two metal ions had a positive effect on the lipases, raising the optimum temperatures to 65ºC for the N-tagged (Figure 5.7) and Detagged lipase (Figure 5.8). The addition of 1 mM $ZnSO_4$ to the N-tagged lipase not only shifted the optimum temperature but also seemed to enhance the activity above 65ºC, possibly indicative of an improvement in the stability of the enzyme.

The activation energy for the hydrolysis of the triacylglycerol was determined for the Detagged lipase to ascertain whether these cations had any effect on the

temperature dependence of catalysis. Especially the Zn^{2+} was implicated by Jeong *et al*. (2002) in the possible functioning of a temperature switch effect of the lid helix. The hydrolysis of olive oil at different temperatures below the optimum temperature was monitored for the Detagged enzyme in the presence of 0.1 mM $CaCl_2$, 0.1 mM $ZnSO_4$, a combination of the two metal ions and 2 mM TPEN was measured. Involvement of any of the cations in the temperature dependence of the lipase catalysis should lead to alterations in the Arrhenius plot if the Ca or Zinc ions were removed by EDTA or TPEN, which is a strong Zn^{2+} chelator, respectively.

a)



b)

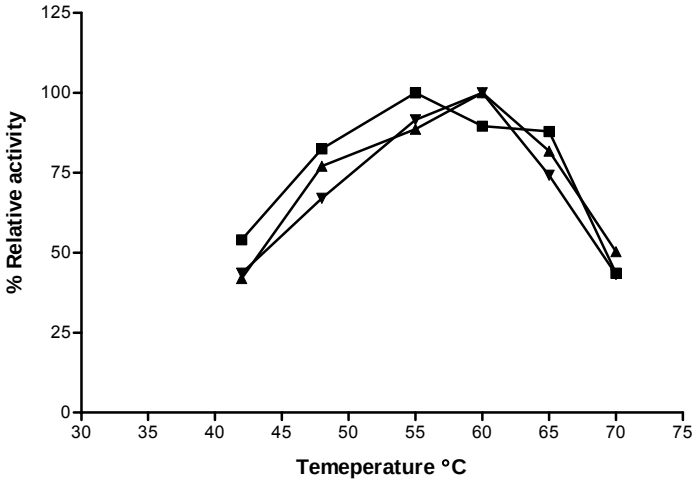


Figure 5.6 Optimum temperature profiles obtained for a) the native (■) and LipA (▲) lipases and b) the C-tagged (■), N-tagged (▲) and Detagged (▼) lipases.

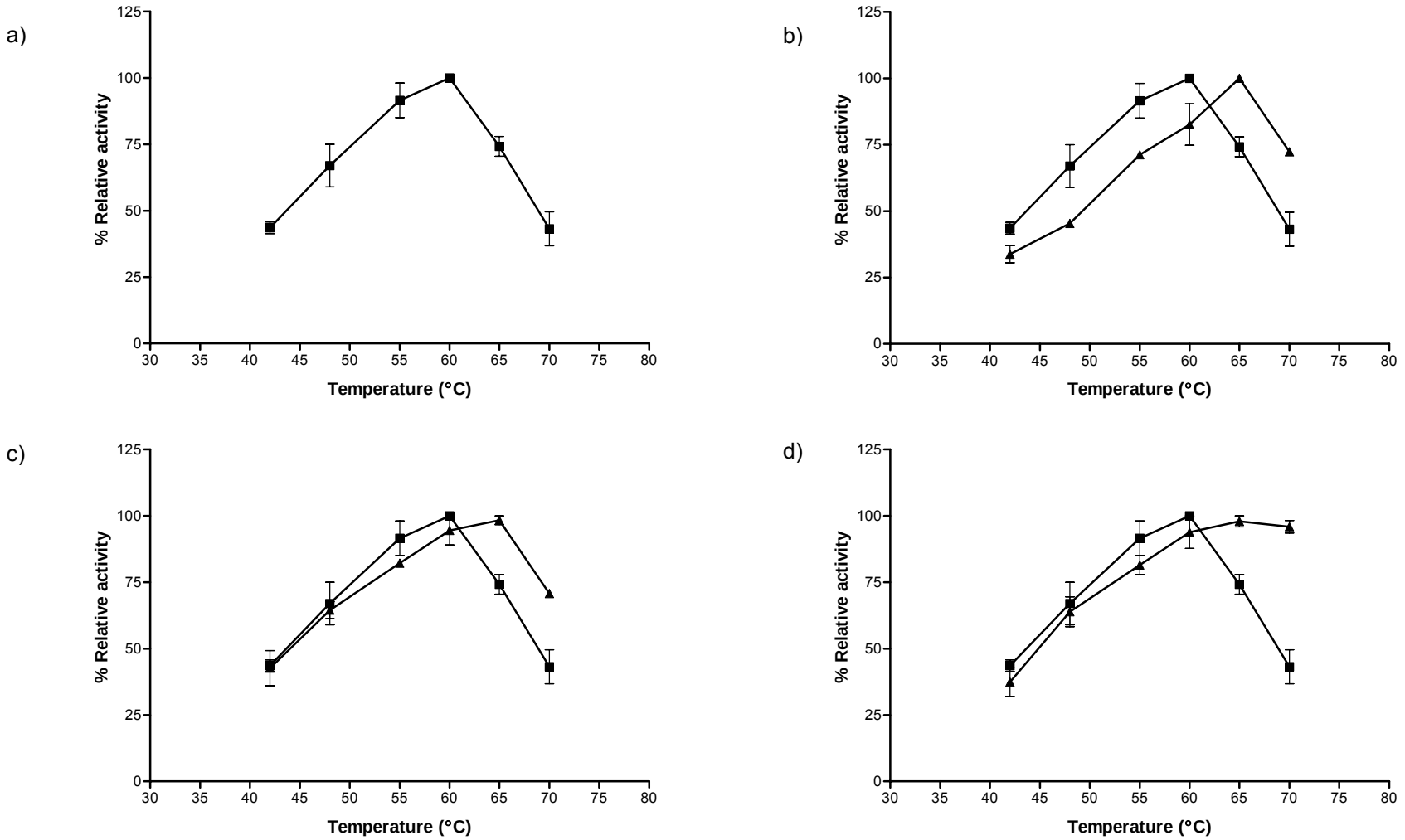


Figure 5.7 Effects of $CaCl_2$ and $ZnSO_4$ on the optimum temperature profile of the N-tagged lipase showing a) the optimum temperature profile obtained for the N-tagged lipase alone; b) the optimum temperature profile obtained for the N-tagged enzyme with the addition of 1 mM $CaCl_2$ (▲), c) 1mM $ZnSO_4$ (▲) and d) 1mM each of the $CaCl_2$ and $ZnSO_4$ (▲) added. In each graph the profile obtained for the N-tagged lipase is shown (■) for comparison.

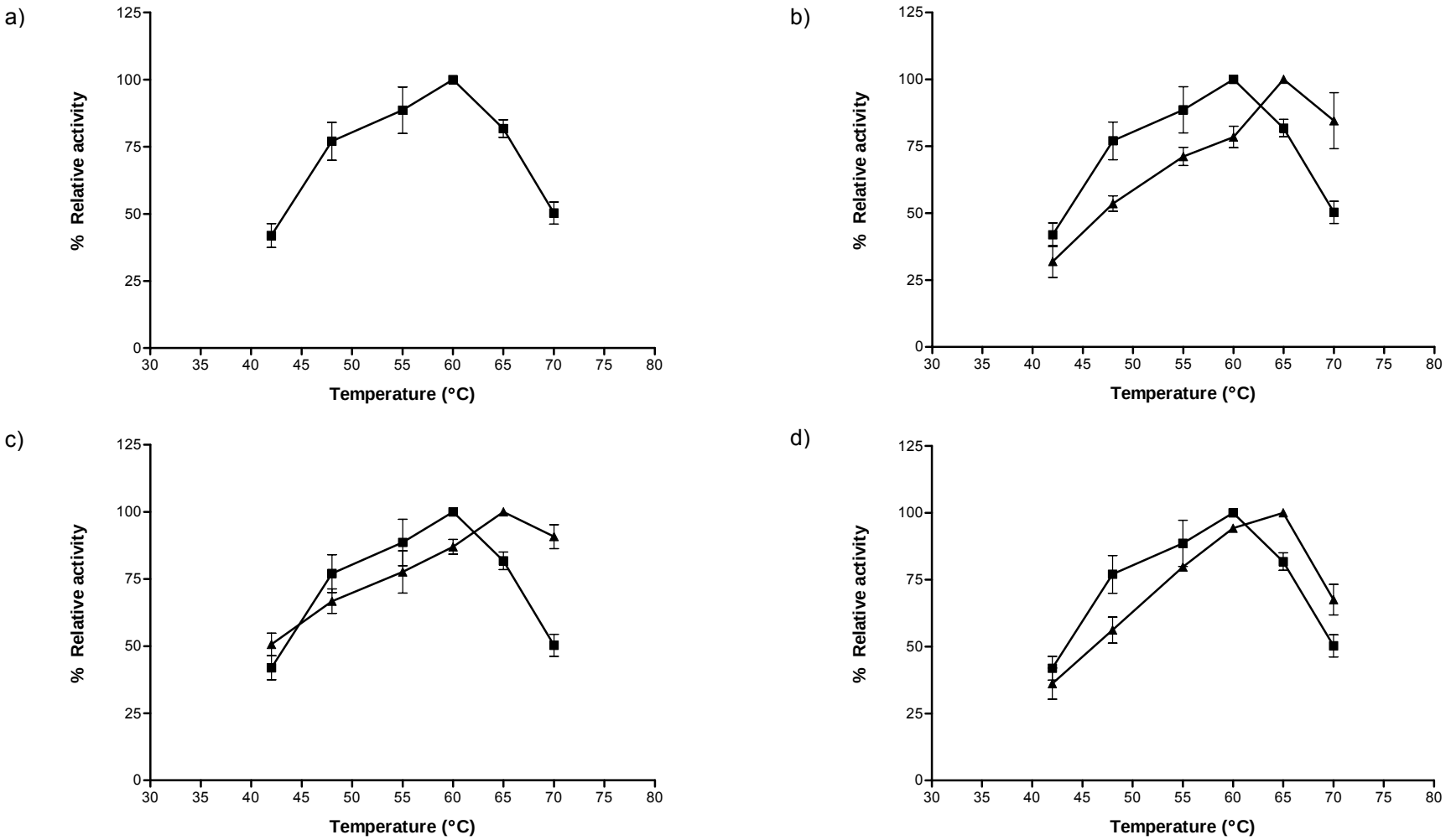


Figure 5.8 Effects of $CaCl_2$ and $ZnSO_4$ on the optimum temperature profile of the Detagged lipase showing a) the optimum temperature profile obtained for the Detagged lipase alone; b) the optimum temperature profile obtained for the Detagged enzyme with the addition of 1 mM $CaCl_2$ (▲), c) 1mM $ZnSO_4$ (▲) and d) 1mM each of the $CaCl_2$ and $ZnSO_4$ (▲) added. In each graph the profile obtained for the Detagged lipase is shown (■) for comparison.

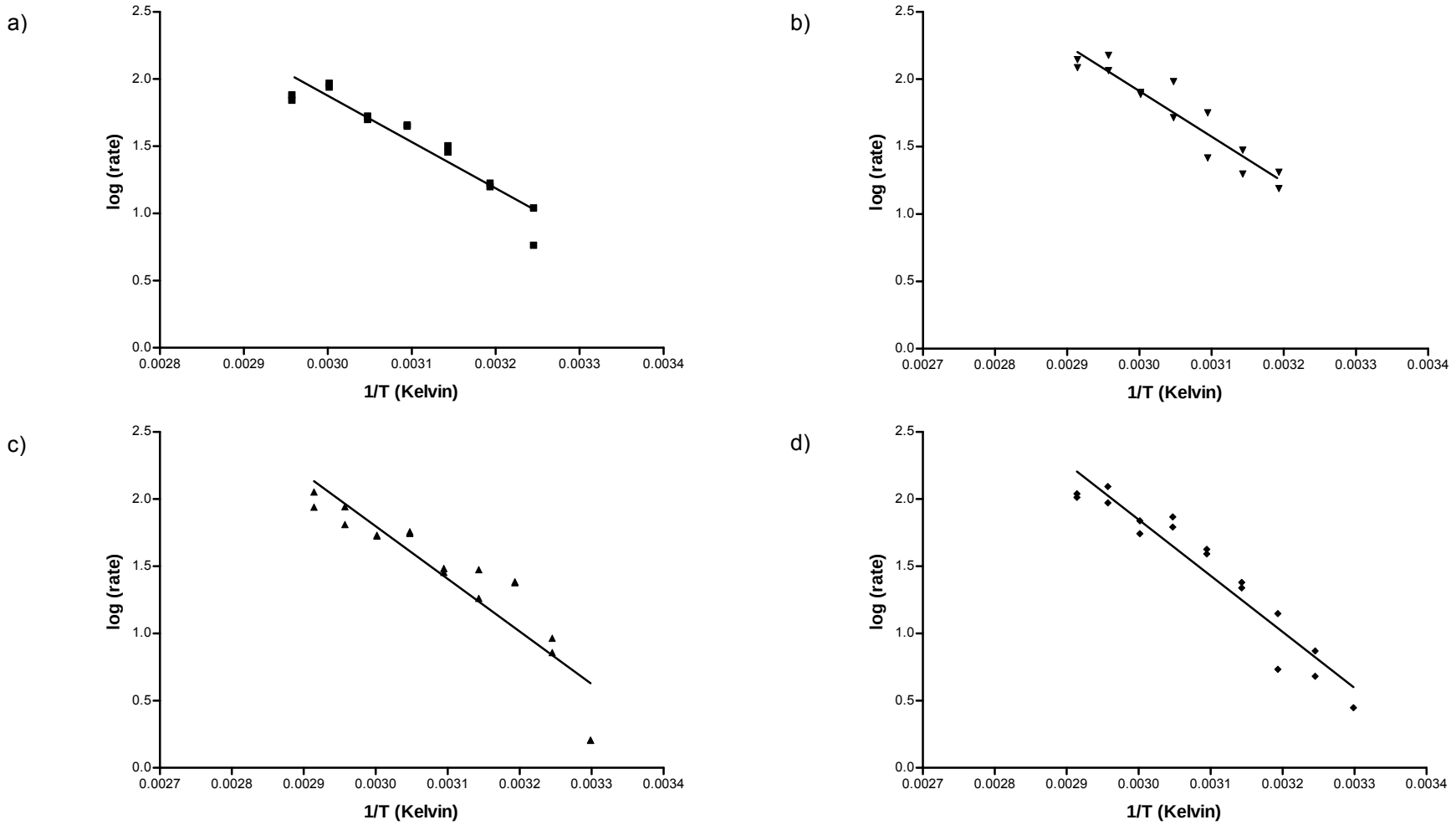
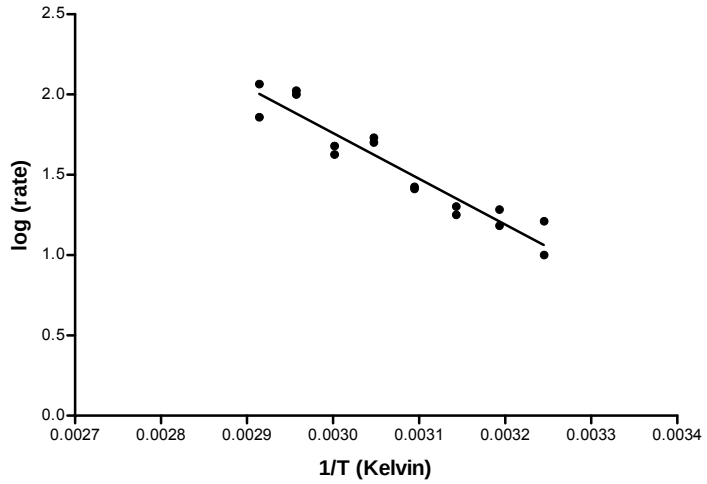


Figure 5.9 Arrhenius plots for the Detagged lipase (a) showing the effect of $CaCl_2$ (b), $ZnSO_4$ (c) and a combination of both divalent ions on the activation energies of the Detagged lipase (d).



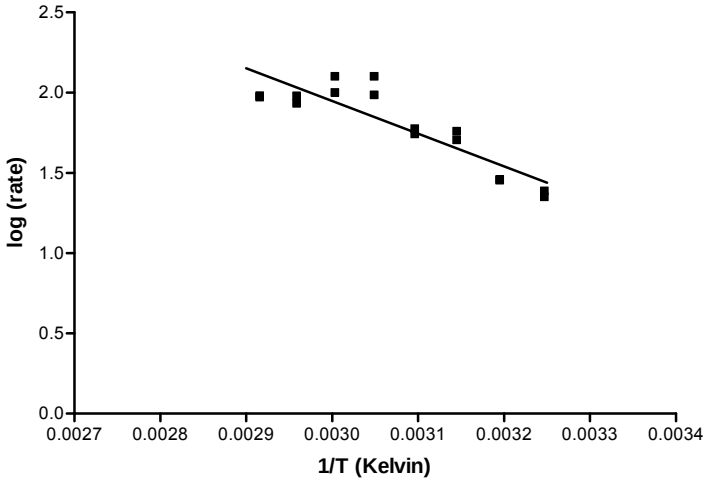


Figure 5.9 (cont) e) Arrhenius plot for the Detagged lipase in the presence of 2 mM TPEN showing the effect of TPEN and f) the effect of 40 mM EDTA on the activation energy of the Detagged lipase.

Table 5.2 Activation energies obtained for the Detagged lipase incubated with different divalent metals and chelators.

	Gradient	y-intercept	Activation energy kJ/mol
Nothing added	8811	13.28	73.3
$CaCl_2$ added	6850	12.03	64.6
$ZnSO_4$ added	7938	13.53	74.9
$CaCl_2$ + $ZnSO_4$ added	8470	14.3	79.9
TPEN added	5774	10.29	54.5
EDTA added	4693	8.063	39

Surprisingly the removal of the metal ions by complexation did not affect the temperature dependence of the catalysis by the lipase (Figure 5.9, Table 5.2). The activation energies and the Y-intercepts (which are representative of the frequency factor), were not significantly affected by the presence of the metal ions. TPEN and EDTA (removal of the metal ions) both lead to a decrease in activation energy, but the significance of this is questionable. Both metals could possibly be involved in stabilising the enzyme rather than influencing its catalysis. Differential scanning calorimetry (DSC) will have be used to study the true effect the ions might have on enzyme stability.

To determine if the metal ions had an effect, the loss of activity at various temperatures was monitored with time. The temperature stability experiments were only performed on the pure lipases, with additional stability experiments performed on the Detagged and N-tagged lipases to again explore the influences the metal ions might elicit on the lipase. Figure 5.10 shows the profile obtained for the C-tagged lipase

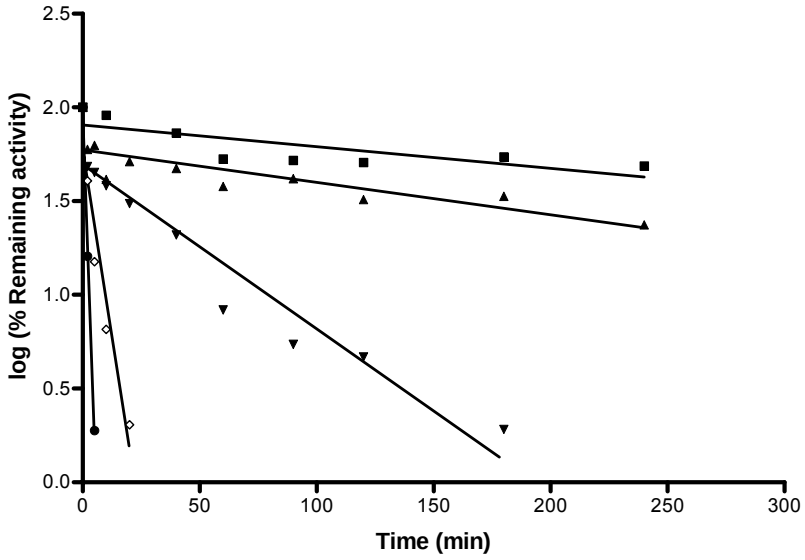


Figure 5.10 Temperature stability graph for the C-tagged lipase incubated at 40ºC (■), 50ºC (▲), 60 ºC (▼), 65ºC (◊) and 70ºC (●).

The half-lives calculated for the C-tagged lipase showed that this lipase was stable for 39 min at 60ºC, 5 min at 60ºC and only 2 min at 70ºC (Table 5.3). The

activation energy for inactivation of the C-tagged lipase was calculated as 210.81 kJ/mol (Table 5.4, Figure 5.11). The N-tagged lipase temperature stability experiments were performed using the conditions described with various combinations of metal ions. It is clear from the data obtained for the temperature stability (Figure 5.12) that the enzymes stability is greatly enhanced by the addition of calcium to the enzyme, making the enzyme indefinitely stable at 40ºC and increasing the half life of the enzyme from 0.5 min to 0.9 h (54 min) at 70ºC (Table 5.3). Results obtained with the addition of zinc ions did not noticeably stabilize the enzyme.

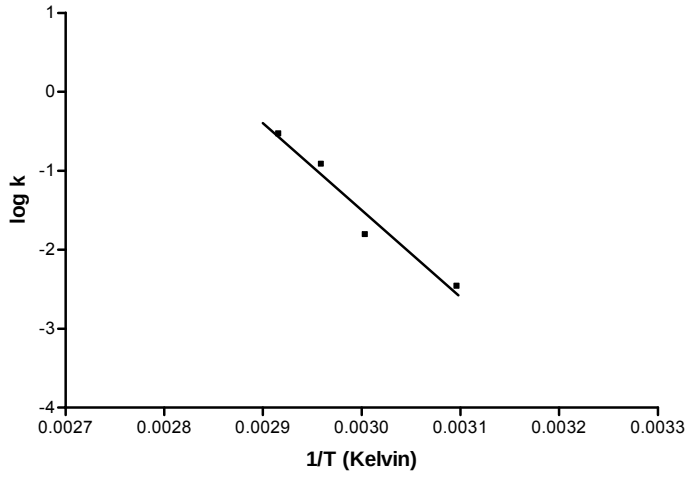


Figure 5.11 Arrhenius plot for the inactivation of the C-tagged lipase.

The activation energy for the inactivation of the different N-tagged lipases (Table 5.4, Figure 5.13) was similar, ranging from 286 kJ/ mol for the N-tagged lipase with calcium, 254 kJ/mol for the N-tagged lipase with no metal ions added and 230/1 kJ/mol for the N-tagged lipase incubated with the zinc and calcium ions. It could thus be seen that the calcium had a slight stabilising effect and the zinc an inhibitory effect when compared to the N-tagged lipase alone.

The results obtained for the temperature stability of the Detagged lipase incubated with calcium and zinc ions (Figure 5.14) showed similar results as the N-tagged lipase. The addition of calcium to the enzyme drastically enhanced the half-life of the enzymes at 60 - 70ºC (Table 5.3). Once again the zinc ions did not seem to

play a role in the stability of the enzyme, in fact from the results obtained it seemed to inhibit the detagged enzyme.

Table 5.3 Half-lives obtained for the *G. thermoleovorans* GE-7 lipases at various temperatures with the addition of 1 mM $CaCl_2$, 1 mM $ZnSO_4$ and 1 mM of each divalent ion. The rate constant is given by k

		No divalent ions		**$CaCl_2$**		**$ZnSO_4$**		**$CaCl_2$ + $ZnSO_4$**	
	ºC	**k X 10^{-3}**	**Half life**	**k X 10^{-3}**	**Half life**	**k X 10^{-3}**	**Half life**	**k X 10^{-3}**	**Half life**
Ntag	40	0.0603	191.4h	0	∞	0.267	43.2h	0.135	85.3
	50	1.82	6.35h	0.032	156.7h	10.5	1.1h	15.9	43.6min
	60	3.22	3.6h	0.178	28.2h	150.4	4.6min	84.9	8.2min
	65	148.8	4.65min	2.22	2.25h	450	1.54min	207.3	3.3min
	70	1289	0.53min	5.367	56min	548.8	1.26min	503.7	1.37min
Detag	40	0.0862	13.4h	0.729	15.8h	6.167	1.9h	2.98	3.87h
	50	1.056	10.9h	0.761	15.2h	8.55	1.35h	7.02	1.65h
	60	2.379	4.9h	0.927	12.45h	153.86	4.5min	25.56	27.1min
	65	54.7	12.7min	4.32	2.67h	367.3	1.88min	239.9	2.9min
	70	598.78	1.2min	37.29	18.6min	649.2	1.1min	1280	0.54min
Ctag	40							2.66	4.3h
	50							3.98	2.9h
	60							17.9	38.6min
	65							139.8	5min
	70							337.2	2.1min

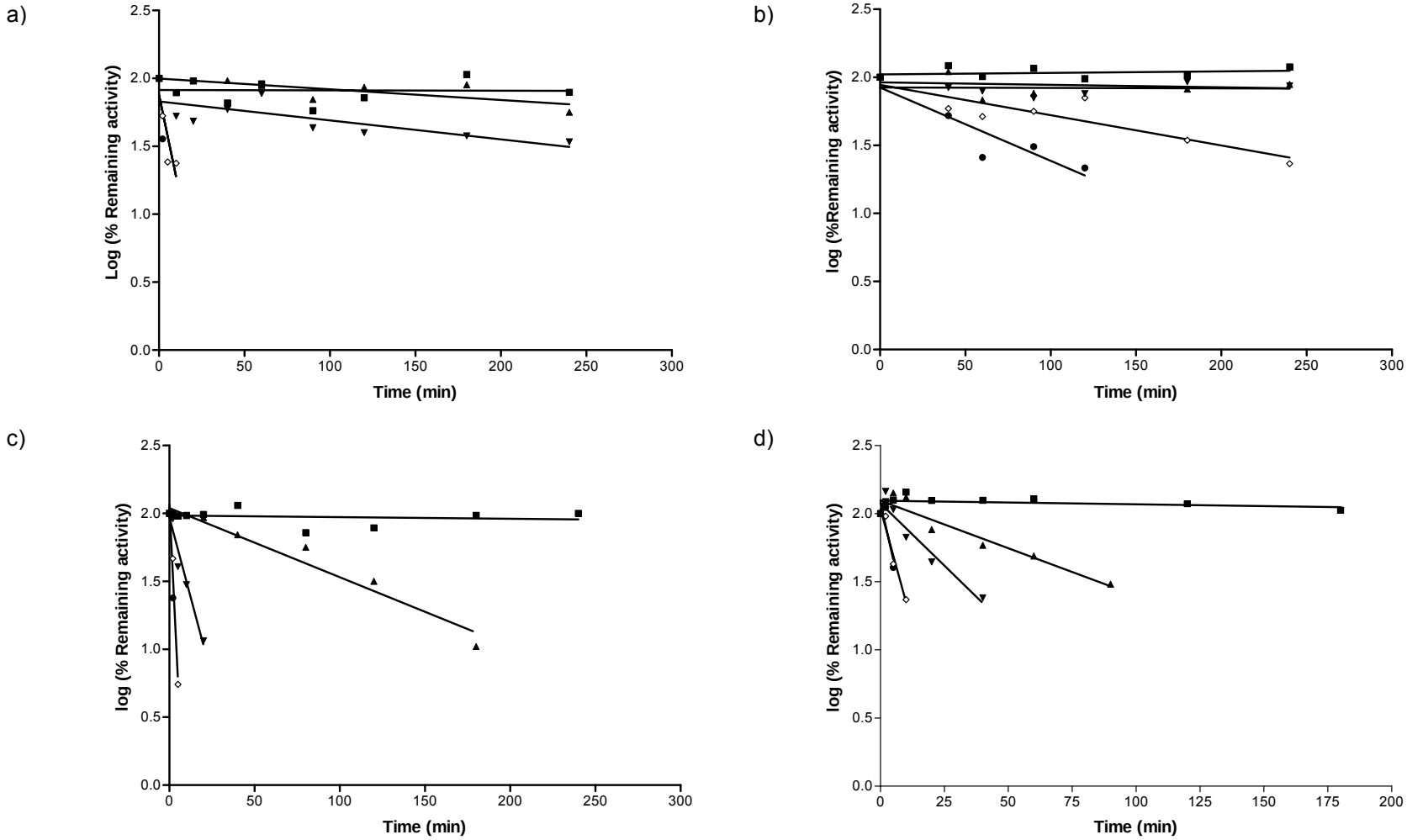


Figure 5.12 Temperature stability graph for the N-tagged lipase incubated at 40ºC (■), 50ºC (▲), 60 ºC (▼), 65ºC (◊) and 70ºC (●) showing a) the N-tagged with no metal added, b) the addition of 1 mM $CaCl_2$, c) the addition of 1 mM $ZnSO_4$ and d) a combination of 1 mM $CaCl_2$ and $ZnSO_4$.

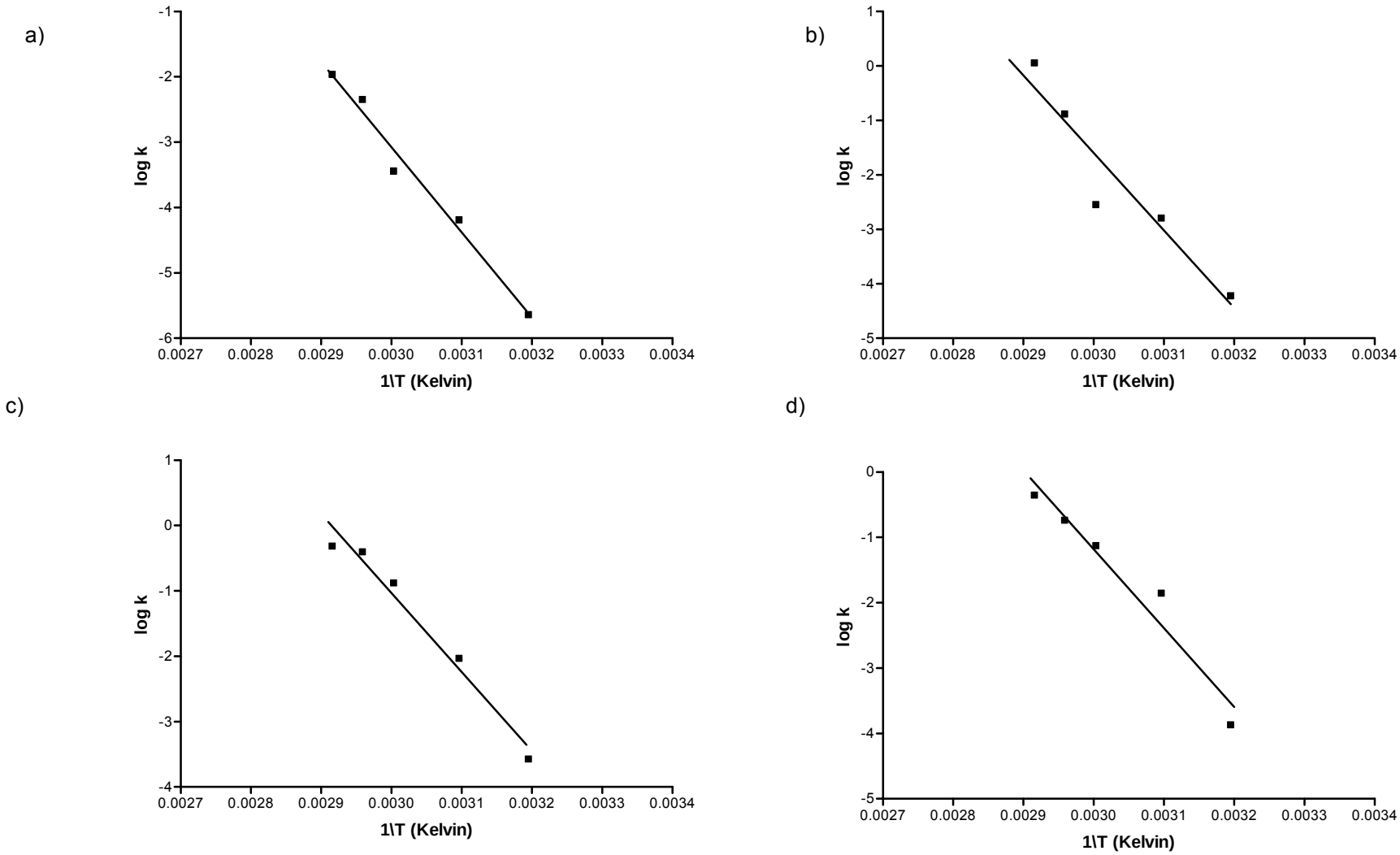


Figure 5.13 Arrhenius plot for the inactivation of N-tagged lipase incubated with no metal ions (a), with 1 mM $CaCl_2$ (b), 1 mM $ZnSO_4$ (c) and a combination of 1 mM of $CaCl_2$ and $ZnSO_4$.

The construction of the Arrhenius plot (Figure 5.13) for the activation energy for the inactivation of the different lipases (Table 5.4) was similar, ranging from 301 kJ/mol for the Detagged lipase with calcium, 288.9 kJ/mol for the Detagged lipase with no metal ions added and 203 kJ/mol for the Detagged lipase incubated with the zinc and 239 kJ/mol for the Detagged lipase incubated with zinc and calcium ions (Table 5.4). Although the activation energy values are very similar, the calcium appeared to have a moderate stabilising effect, generally leading to longer half-lives at higher temperatures. Zinc appeared to have a slight destabilising effect. The role of the metals in the stability and the catalysis of the enzyme are unfortunately not clear and might be better understood in experiments using DSC.

Table 5.4 Activation energy for inactivation obtained from the Arrhenius plot for the *G. thermoleovorans* GE-7 lipase under different assay conditions.

Enzyme	Incubation condition	Gradient	y-intercept	Activation energy kJ/mol
Ntag	Nothing added	30031	36.05	249.7
	$CaCl_2$ added	32771	41.09	272.5
	$ZnSO_4$ added	27820	35.	231.3
	$CaCl_2$ + $ZnSO_4$ added	27774	35.1	230.9
Detag	Nothing added	34752	43.36	288.9
	$CaCl_2$ added	38690	47.84	301
	$ZnSO_4$ added	24527	30.95	203.9
	$CaCl_2$ + $ZnSO_4$ added	28764	36.27	239.14
Ctag	$CaCl_2$ + $ZnSO_4$ added	25236	31.49	210.81

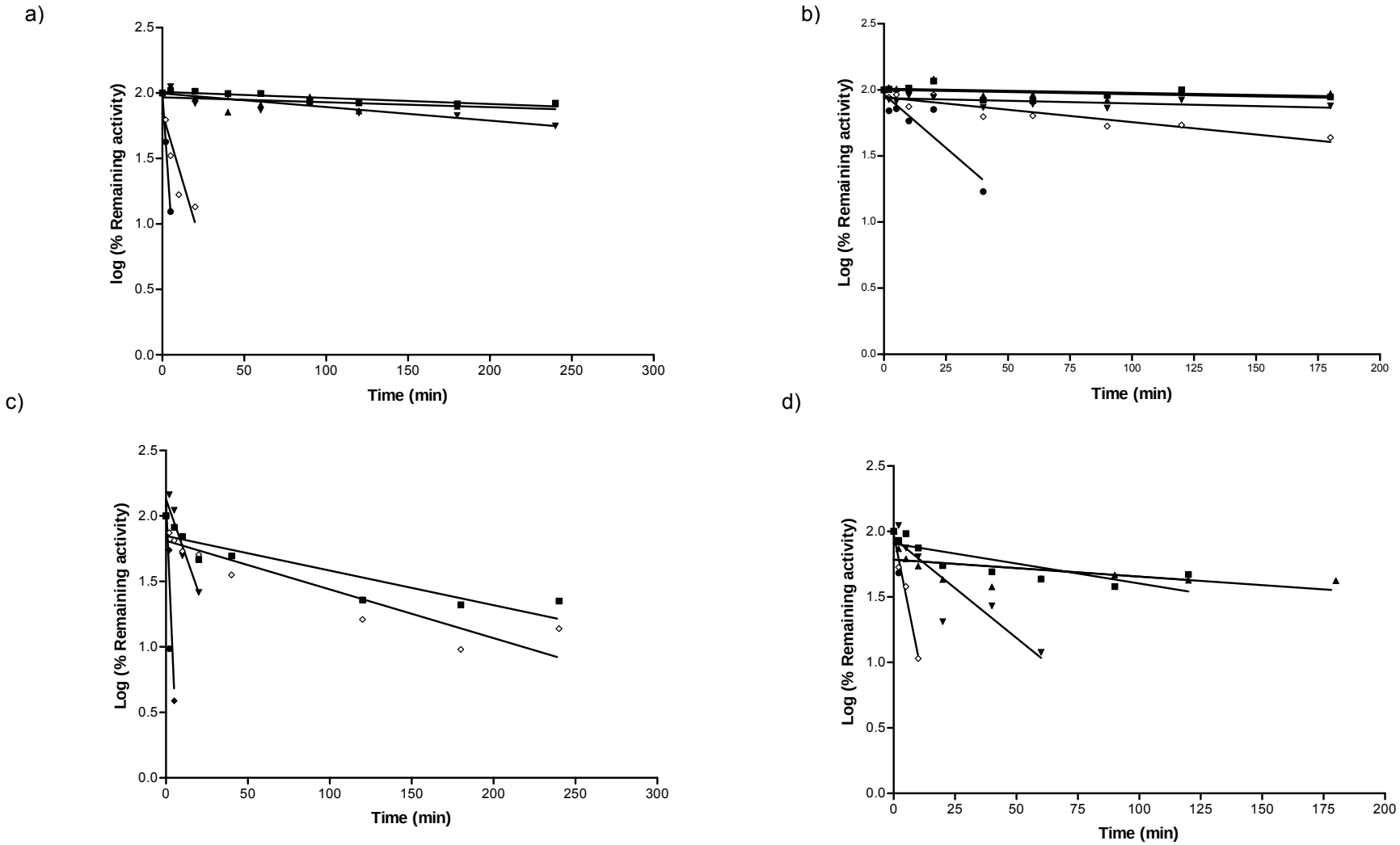


Figure 5.14 Temperature stability graph for the Detagged lipase incubated at 40ºC (■), 50ºC (▲), 60 ºC (▼), 65ºC (◊) and 70ºC (●) showing a) the N-tagged with no metal added, b) the addition of 1 mM $CaCl_2$, c) the addition of 1 mM $ZnSO_4$ and d) a combination of 1 mM $CaCl_2$ and $ZnSO_4$.

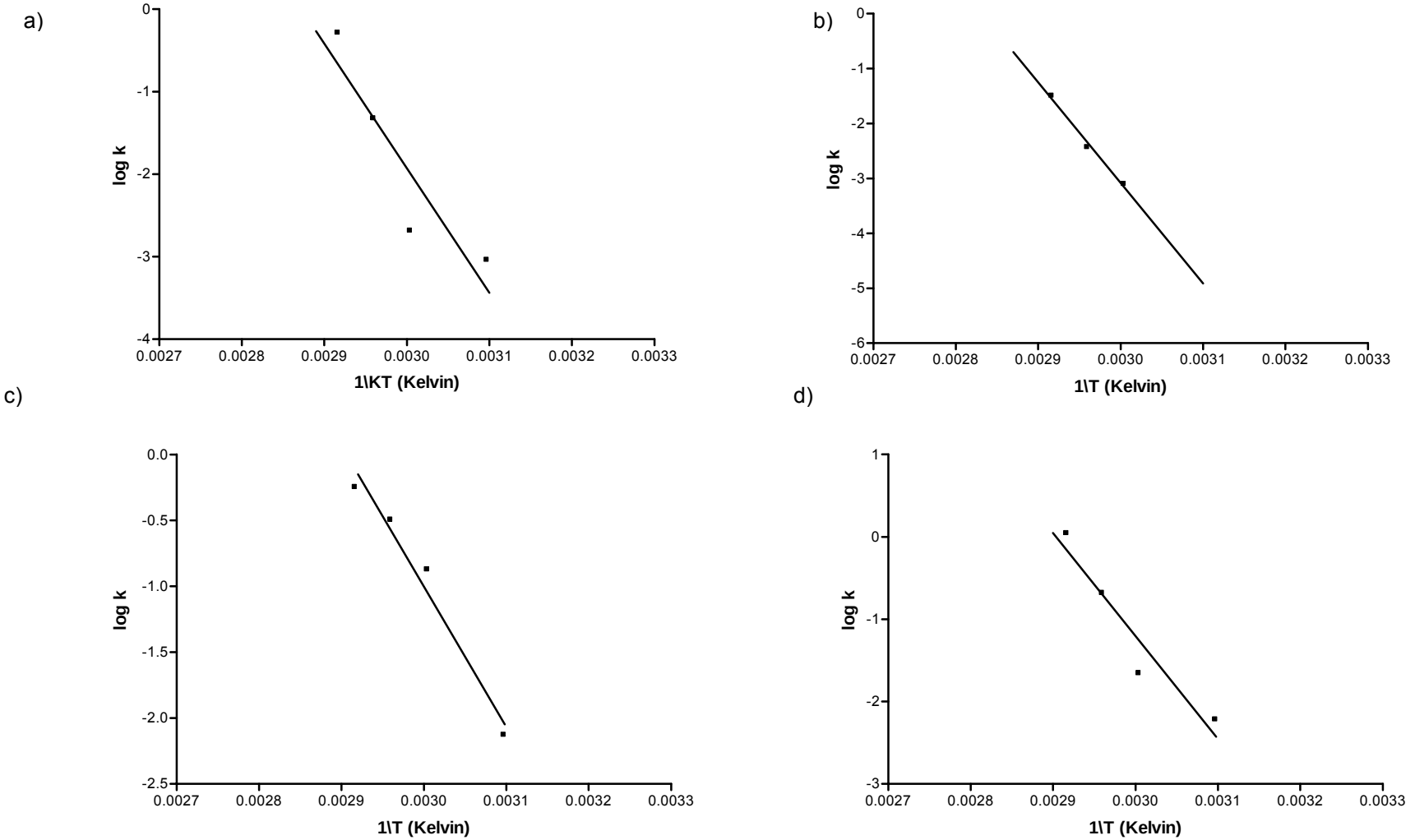


Figure 5.15 Arrhenius plot for the inactivation of the Detagged lipase incubated with no metal ions (a), with 1 mM $CaCl_2$ (b), 1 mM $ZnSO_4$ (c) and a combination of 1 mM of $CaCl_2$ and $ZnSO_4$ (d).

5.3.2.2 Effects of pH on the lipases

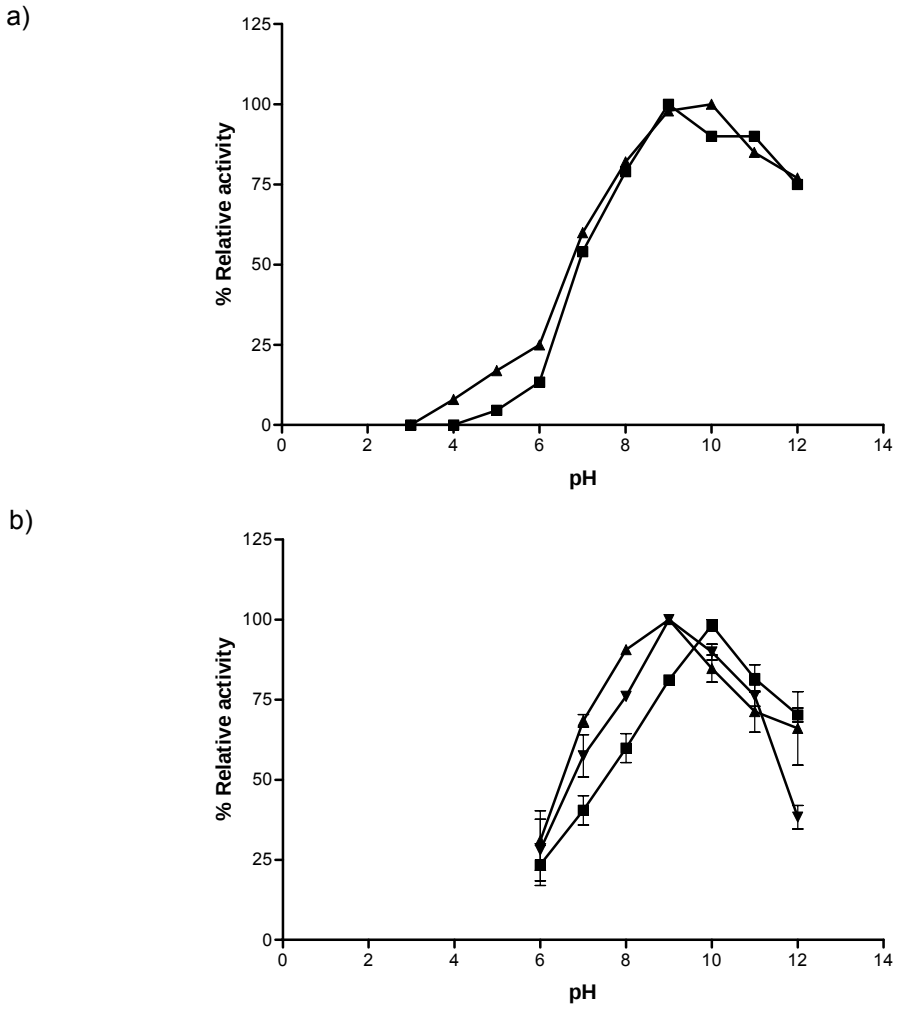


Figure 5.16 The optimum pH profiles for a) the native (■) and LipA (▲) lipases and b) the N-tagged (■), Detagged (▲) and C-tagged (▼) lipases using olive oil as substrate.

The pH optima (Figure 5.16) were determined for all five of the lipases and were found to be in the region of pH 9-10. The experiments for the pH stability were only performed on the C-tagged, N-tagged and Detagged lipases. The C-tagged lipases had a half-life of 12 hours at pH 8.8, but was not stable at higher pH's with a half life of only 17 min at pH 12.22 (Figure 5.17, Table 5.5).

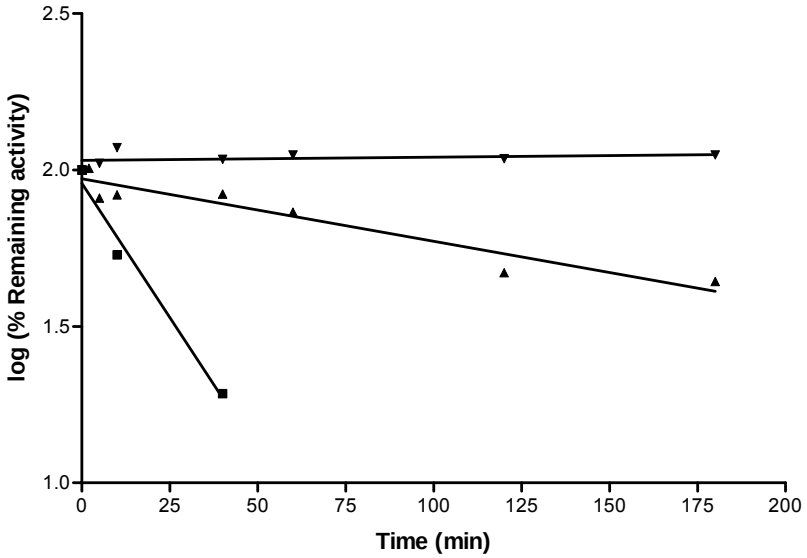


Figure 5.17 pH stability graph for the C-tagged lipase at pH 12.22 (■), pH 8.8 (▼) and pH 6.7 (▲).

The experiments for the Detagged and N-tagged lipases were performed for the enzyme alone and in the presence of 1 mM $CaCl_2$. It was found that the in the case of the N-tagged lipase the calcium seemed to lower the pH stability, whereas the detagged lipase was somewhat stabilised by the addition of the calcium ions (Table 5.5, Figure 3.18).

Table 5.5 Half-lives obtained for the N-tagged, Detagged and C-tagged lipases at varying pH's with and without calcium added

pH	N-tagged – Ca^{2+}	N-tagged + Ca^{2+}	Detagged – Ca^{2+}	Detagged + Ca^{2+}	C-tagged + Ca^{2+} + Zn^{2+}
~2.5	0.24 min	11 min	3.5 min	5.7 min	
~6.7	7.1 h	6.8 h	7.8 h	11.3 h	2.5 h
~8.8	19.5 h	11.9 h	6.3 h	9.66 h	∞
~12.2	25.8 min	23.3 min	6.9 min	41.3 min	17 min

5.3.2.3 Effects of metal ions on lipase activity

The effects of selected metal ions were tested on the N-tagged, C-tagged and Detagged lipases (Figure 5.19, 5.20 and 5.21). Contrary to expected, the $CaCl_2$

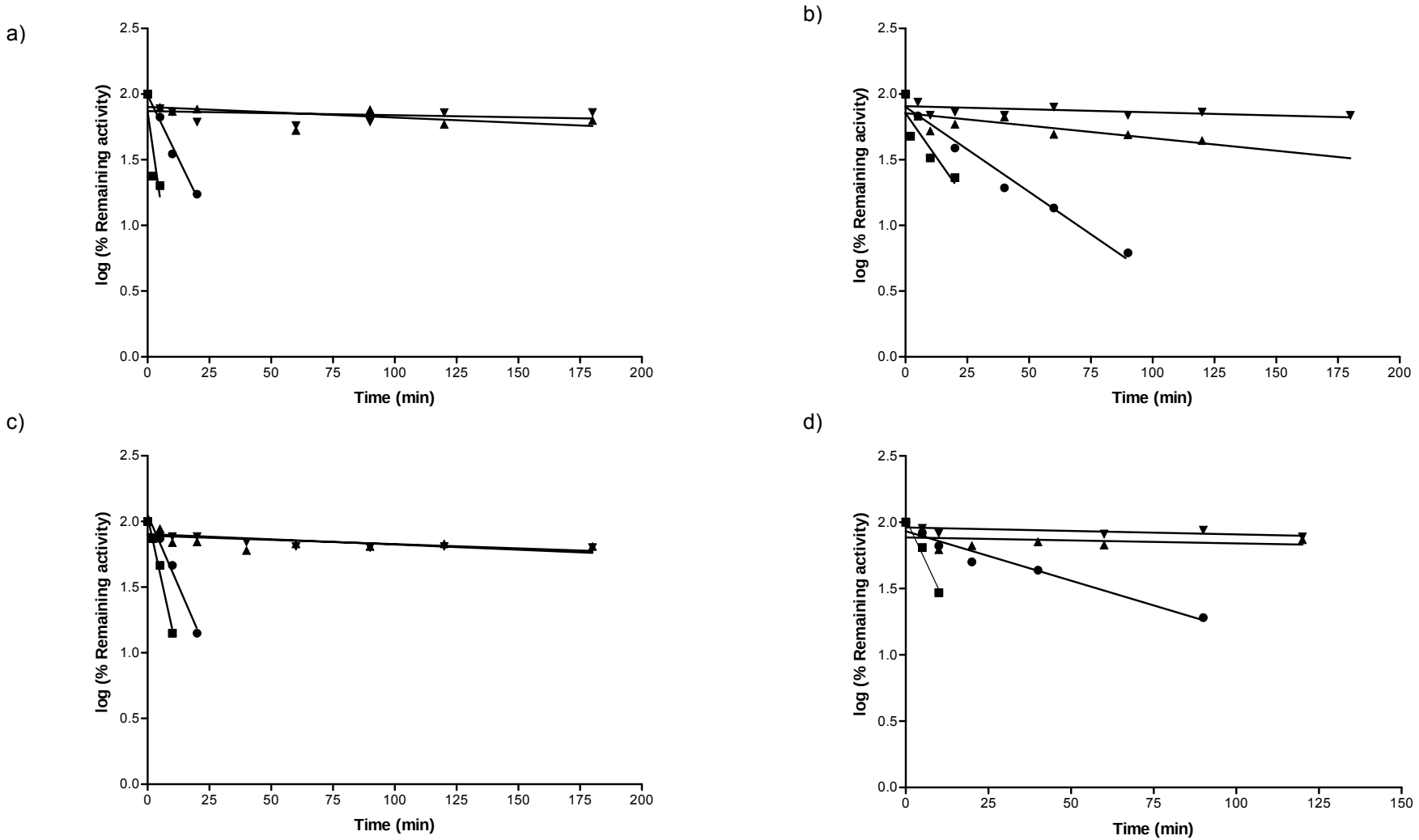


Figure 5.18 pH stability graph for the N-tagged lipase (a), N-tagged lipase with 1 mM $CaCl_2$ (b), Detagged lipase (c) and Detagged lipase with 1 mM $CaCl_2$ at pH 2.4 (■),pH 6.7 (▲). pH 8.8 (▼) and 12.24 (●).

seemed to inhibit the detagged lipase, even at concentrations of 1 mM, only 62% of the activity was left. The effect on the N-tagged and C-tagged lipase was not inhibitory, the activity was similar, up to concentrations of 5 mM. At this concentration N-tagged had 99 % activity remaining and the C-tagged 92 % activity remaining (Figure 5.19 a). Zn^{2+} ions enhanced the activity of C-tagged lipase at concentrations of 0.1 mM, but above this concentration the activity was inhibited. The detagged lipase showed slight inhibition with $ZnSO_4$, but the most notable effect was on the N-tagged lipase, even at 0.1 mM $ZnSO_4$ there was only 38% activity remaining (Figure 5.19 b).

As expected, mercury had a strong inhibitory effect on all three of the lipases (Figure 5.20 a), and at a concentration of 5 mM almost no activity could be detected for the C-tagged and N-tagged lipases. The detagged lipase was more resistant to the effect of mercury and had 77 % activity at 1 mM $HgCl_2$. At concentrations of 0.1 mM, $MgCl_2$ enhanced the activity of the N-tagged and C-tagged lipase, but had a mild inhibitory effect on the Detagged lipase (Figure 5.19 c). The enzymes were however not totally inhibited by the presence of $MgCl_2$ and still had ~80 % activity in the presence of 5 mM.

Manganese also had a slight inhibitory effect on the Detagged lipase (Figure 5.19 d), as there was only approximately 65 % remaining activity at 0.1 mM of $MnCl_2$. The presence of manganese did not drastically influence the C-tagged and N-tagged lipases at concentrations up to 1 mM (~92 - 94 % remaining activity). Copper had a slight activating effect on the C-tagged lipase at low concentrations (0.1 mM), but all of the lipases exhibited lower activity in the presence of 1 mM and 5 mM copper (Figure 5.20 c). Similar results were obtained for nickel, with the C-tagged lipase losing only ~10 % activity even in the presence of 5 mM nickel, but the Detagged and N-tagged lipase lost 20 - 30 % of their activity in 1 mM nickel (Figure 5.20 b).

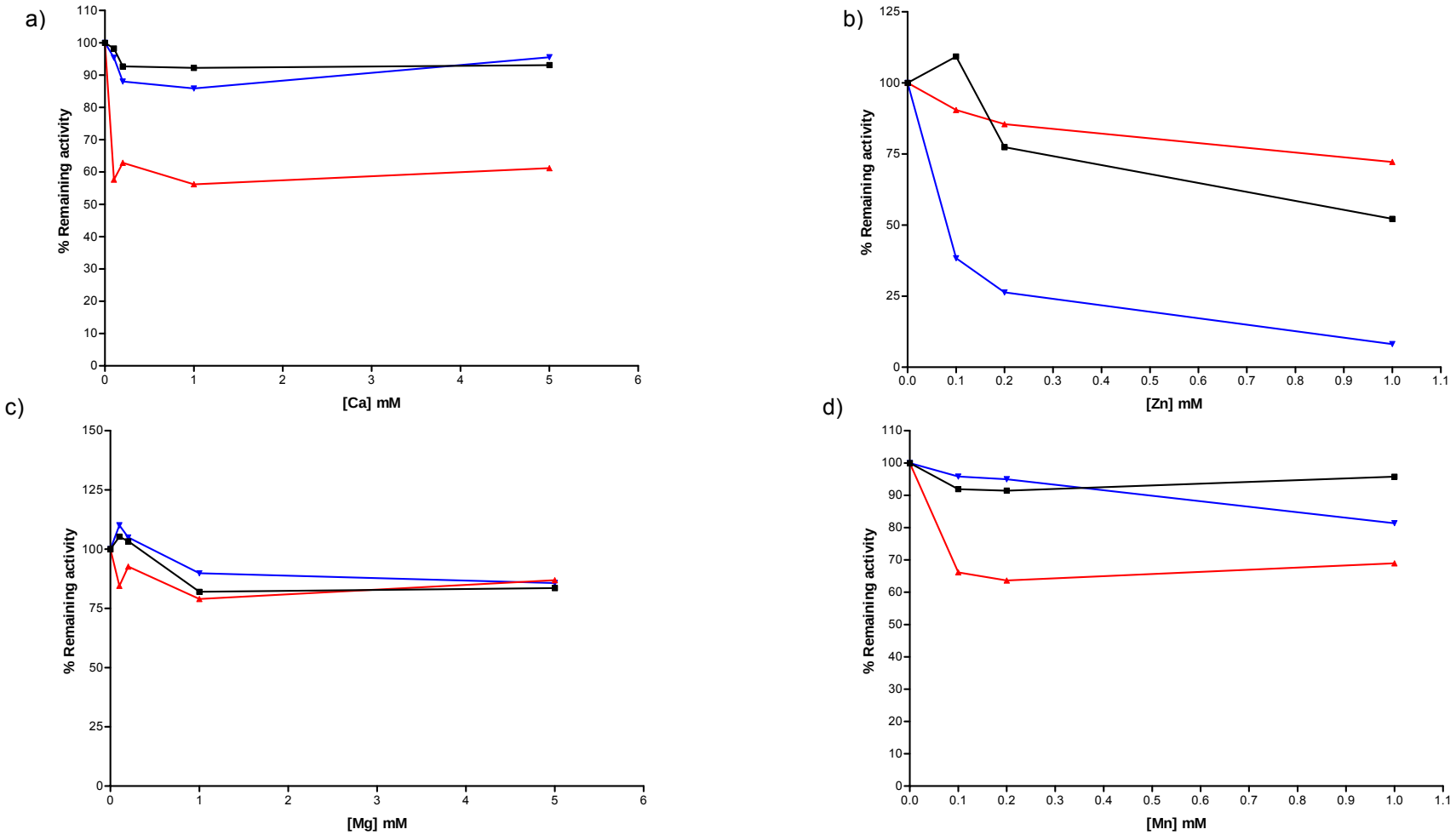


Figure 5.19 Graphs obtained for the Detagged (red), N-tagged (blue) and C-tagged (black) lipases incubated with varying concentrations of a) $CaCl_2$, b) $ZnSO_4$, c) $MgCl_2$ and d) $MnCl_2$.

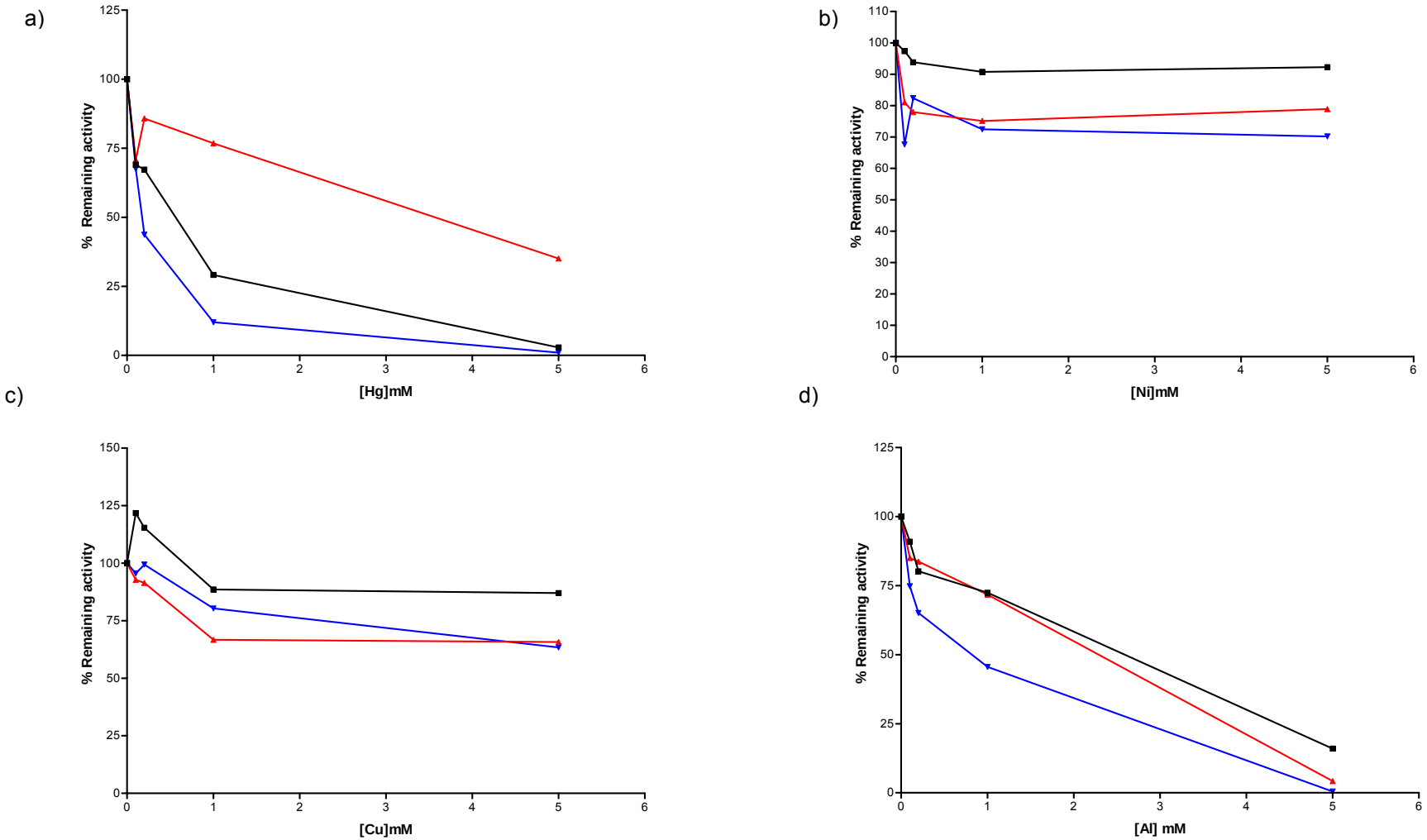


Figure 5.20 Graphs obtained for the Detagged (red), N-tagged (blue) and C-tagged (black) lipases incubated with varying concentrations of a) $HgCl_2$, b) $NiSO_4$, c) $CuCl_2$ and d) $AlCl_2$.

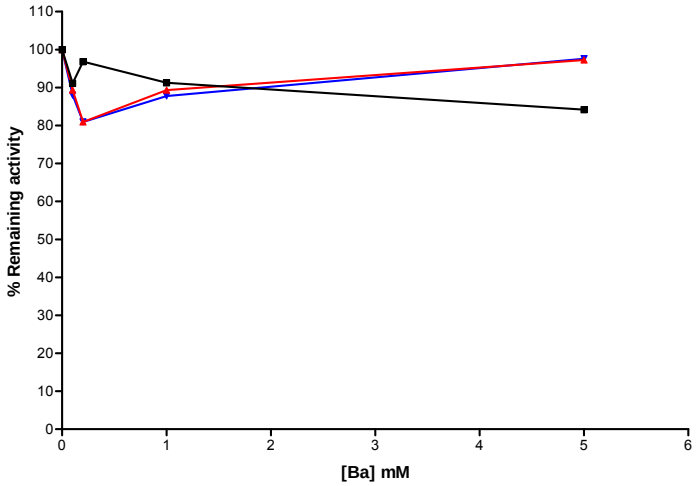


Figure 5.21 Graphs of the Detagged (red), N-tagged (blue) and C-tagged (black) lipases incubated with differing concentrations of $BaCl_2$.

The effect of aluminium was more pronounced with all three of the enzymes. The C-tagged and Detagged lipase had ~75 % activity left in the presence of 1 mM aluminium and the N-tagged with only 48 % activity left under the same conditions, with all the enzymes having less than 20 % activity remaining in the presence of 5 mM aluminium (Figure 5.20 d). The addition of barium chloride did not significantly effect the Detagged and N-tagged lipase activity is not influenced (97 % remaining activity in the presence of 5 mM $BaCl_2$). The effect was the opposite in the case of the C-tagged enzyme with an initial positive effect (0.1 mM) but showing inhibition with higher concentrations (5 mM) (Figure 5.21).

5.3.2.4 Effect of detergents on lipase activity

The effect of detergents was only performed on the pure N-tagged, Detagged and C-tagged lipases (Figure 5.22). The anionic detergent (SDS) enhanced the activity of the Detagged lipase below the cmc of the detergent (8.2 mM), but above the cmc the activity was drastically inhibited. The C-tagged lipase activity was significantly inhibited, even below the cmc. The N-tagged lipase retained 88 % of it's activity in the presence of 10 mM SDS. The Cetrimide

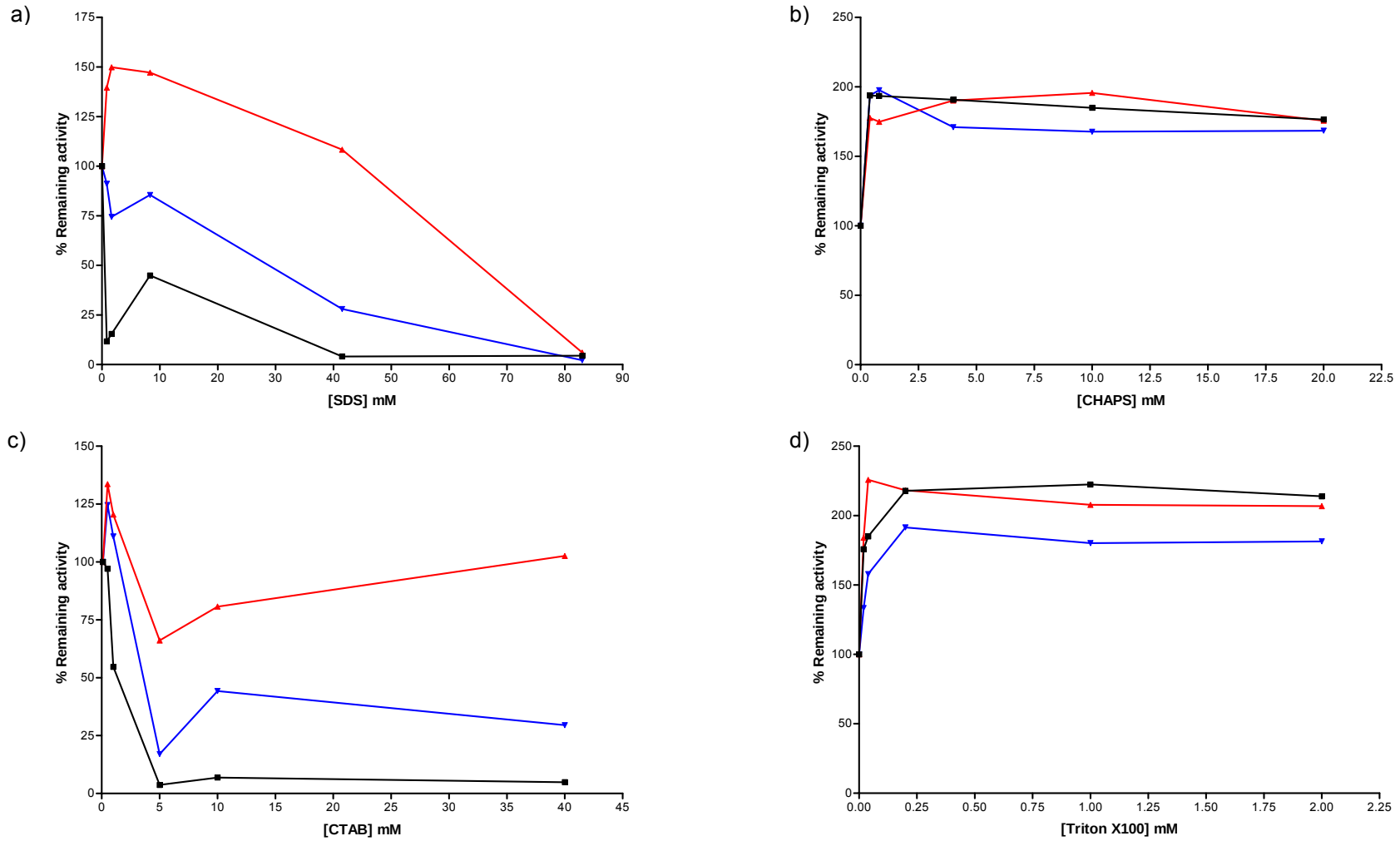


Figure 5.22 Graphs of the Detagged (red), N-tagged (blue) and C-tagged (black) lipases incubated with varying concentrations of a) SDS, b) Cetrimide, c) CHAPS and d) Triton X-100.

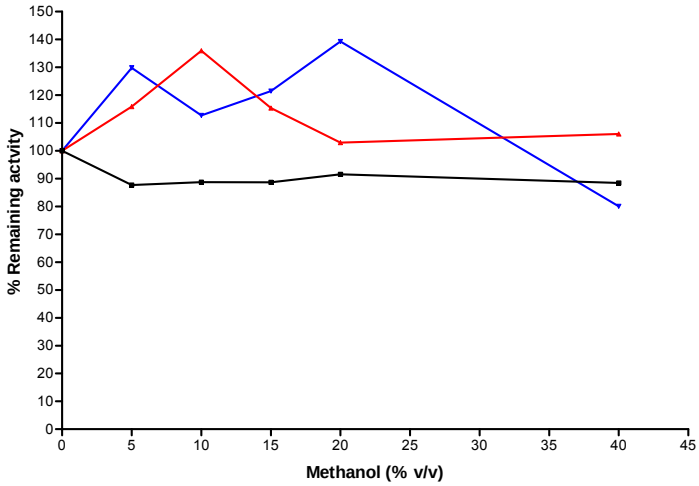
(cationic detergent) enhanced the activity of the N-tagged and Detagged lipase below it's cmc (0.92 mM), thereafter the activity of the N-tagged lipase decreased to below 25 % remaining activity. The effect of the cetrimide was harsher on the C-tagged lipase, leading to immediate inhibition of lipase activity to almost 0 % at concentrations of 5 mM of the detergent.
higher.

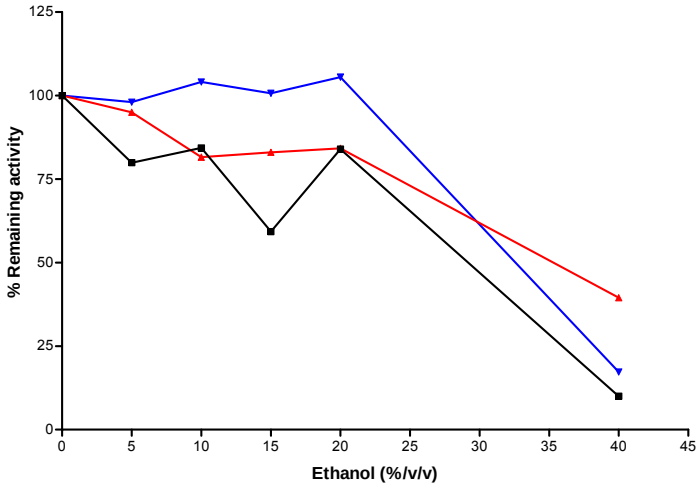
5.3.2.5 Effects of organic solvents on lipase activity

The alcohols methanol, ethanol and isopropanol were used in this experiment, once again only using the Detagged, N-tagged and C-tagged lipases (Figure 5.23). All three lipases were extremely stable in methanol, up to concentrations of 40 % (v/v). The ethanol and isopropanol could only be used up to concentrations of 20 % (v/v), after which the activity decreased for the C-tagged and N-tagged lipase. The Detagged lipase however did not show any decrease in the presence of 40 % isopropanol, but decreased to levels of ~40 % (v/v) in the presence of similar concentrations of ethanol. With the DMSO (Figure 5.24), some inhibition of the enzyme was seen, but not below levels of 75 % remaining activity, even at the highest concentrations of DMSO used. It is thus evident that the deatgged is more stable in up to 40 % methanol and isopropanol.

5.3.2.6 Effects of other chemicals

The experiments were done on the C-tagged, Detagged and N-tagged lipase and the effect of the mild iron chelator, phenantroline, the calcium chelator EDTA (Figure 5.24). EDTA decreased the activity of the lipases to levels below 50 % of the remaining activity in the presence of 10 - 20 mM EDTA. The phenantroline did not have the expected effect on the lipases with all three enzymes retaining above 75 % activity in the presence of 15 - 20 mM phenantroline.





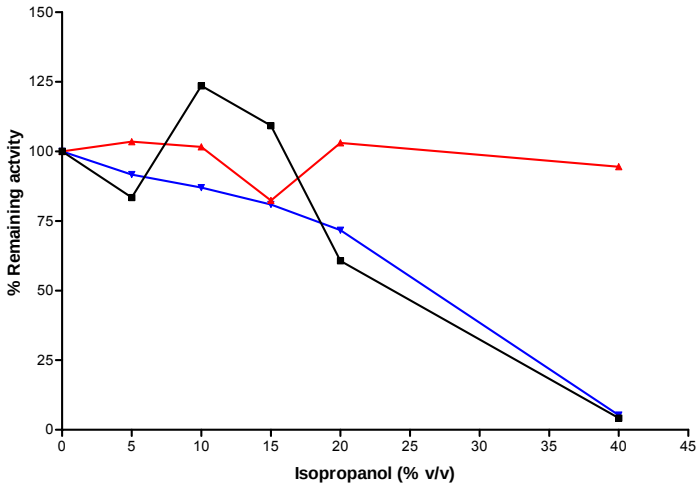


Figure 5.23 Effects of a) methanol, b) ethanol and c) isopropanol on the Detagged (red), N-tagged (blue) and C-tagged lipase (black) at varying concentrations.

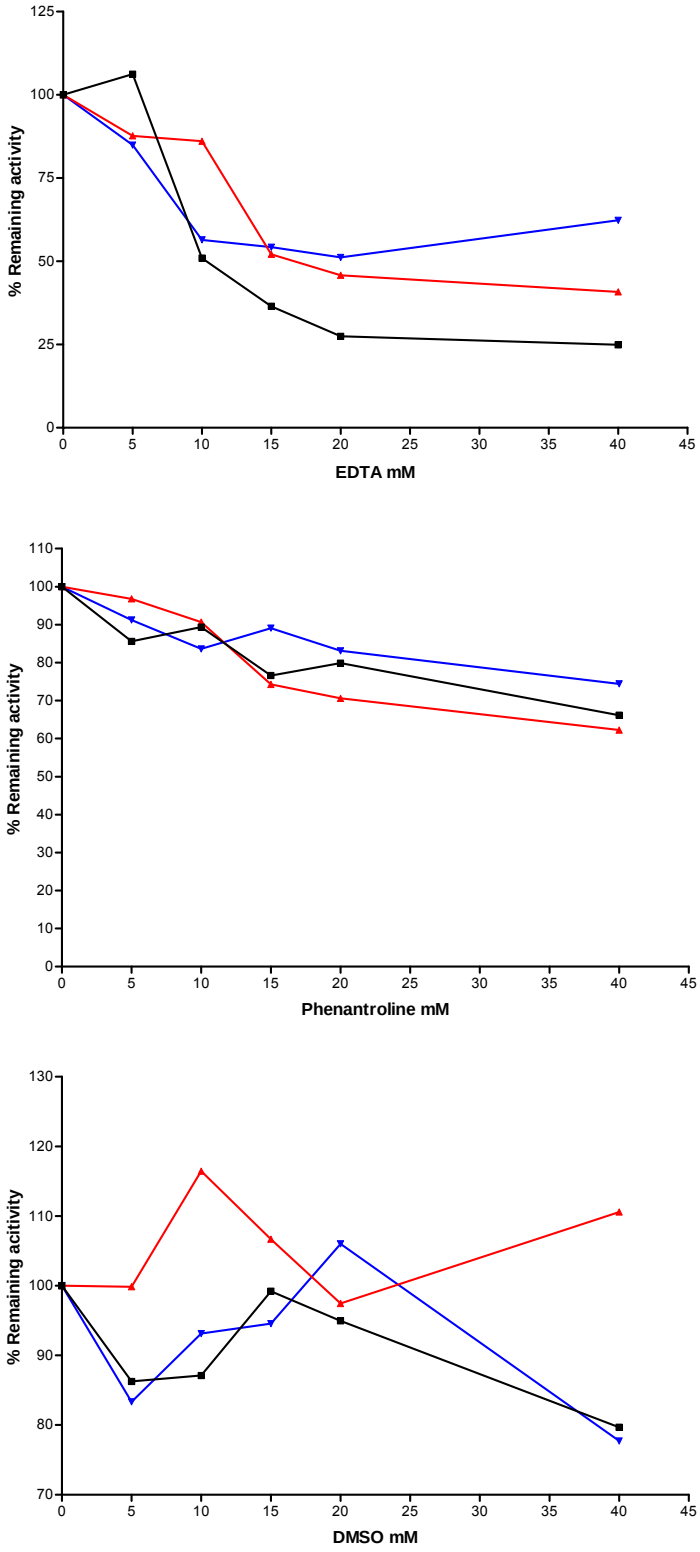


Figure 5.24 Effects of a) EDTA, b) phenantroline and c) DMSO on the Detagged (red), N-tagged (blue) and C-tagged lipase (black) at varying concentrations.

The effect of the strong zinc chelator, TPEN, was tested by incubating the Detagged enzyme with varying concentration of the chelator at 40^{o}C and periodically withdrawing samples to asses the influence on the enzyme (Figure 5.25). From the data obtained it could be seen that even at high concentrations, there was no effect on the lipase activity.

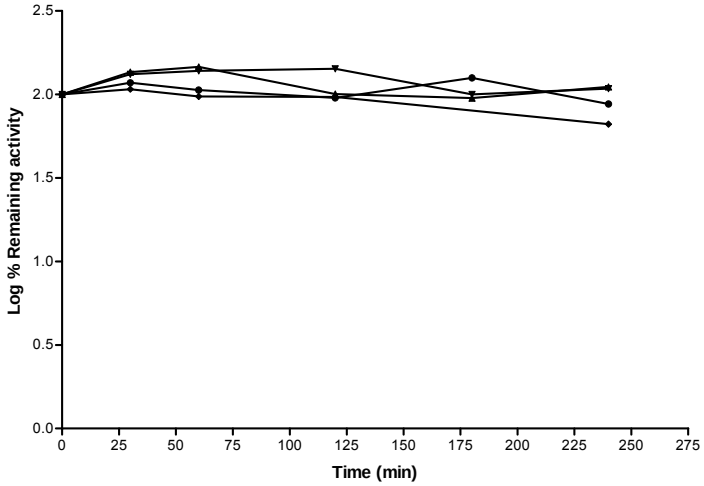


Figure 5.25 Effect of 0.2 mM (▲), 0.5 mM (▼), 1 mM (◊) and 2 mM (●) TPEN on the detagged lipase activity.

5.3.2.7 Substrate specificities of the lipases

All five enzymes were tested for substrate preference using both synthetic esters and triacylglycerols. The tests demonstrated that the lipases have a preference for the saturated shorter fatty acid chain lengths. In all cases the preference was for the C8 - C10 chain lengths, with the exception of the C-tagged; this enzyme prefers the shorter fatty acid (Figure 5.26). The lipases showed preference for the shorter fatty acid chain lengths when triacylglycerols were tested (Figure 5.27). The activity for the native lipase could not be determined due to the low enzyme concentrations.

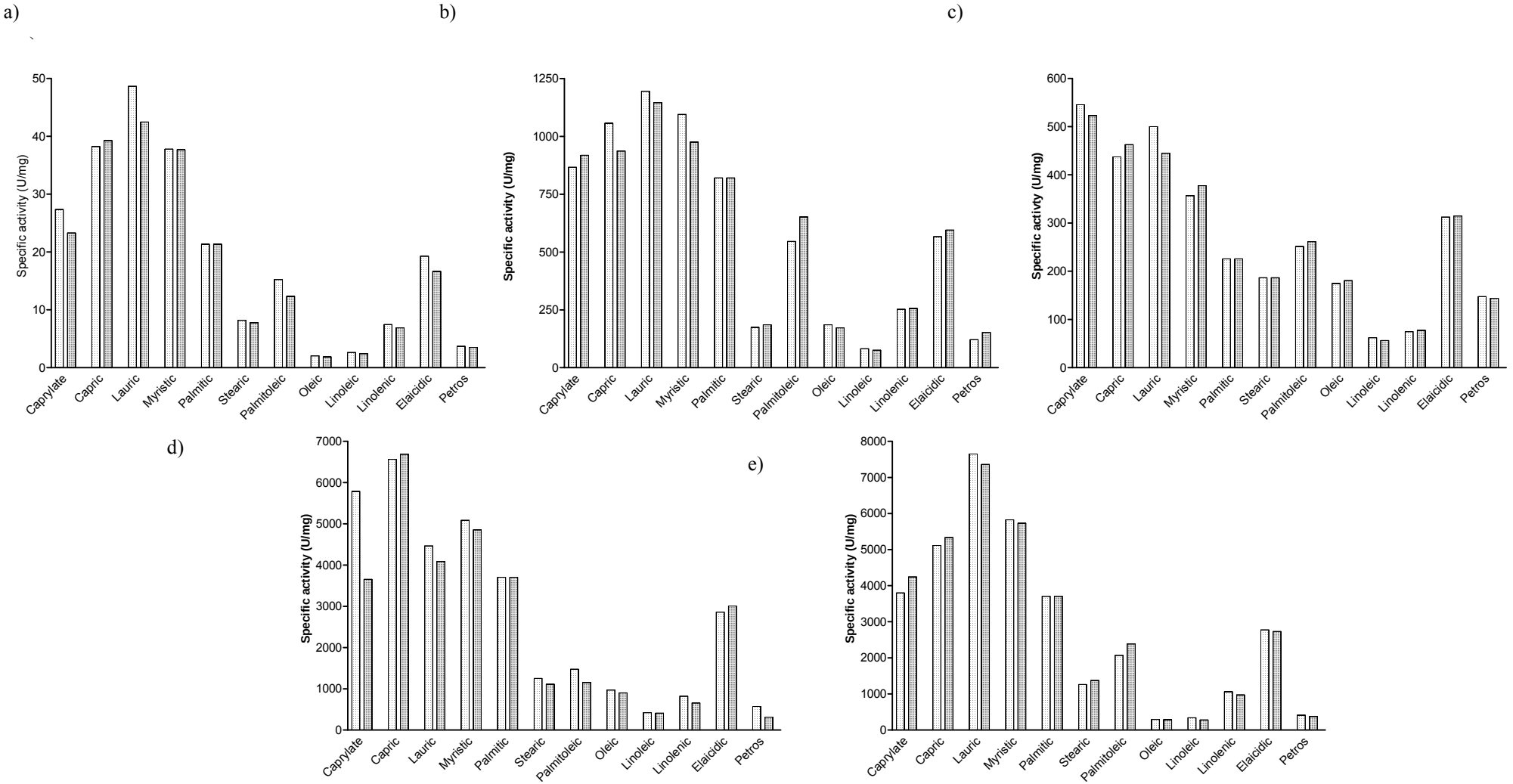


Figure 5.26 Substrate preference profiles obtained for the native (a), LipA (b), C-tagged (c), N-tagged (d) and Detagged (e) lipases using *p*-nitrophenyl esters as substrates. The experiments were done in duplicate and the combined results of both experiments are reported.

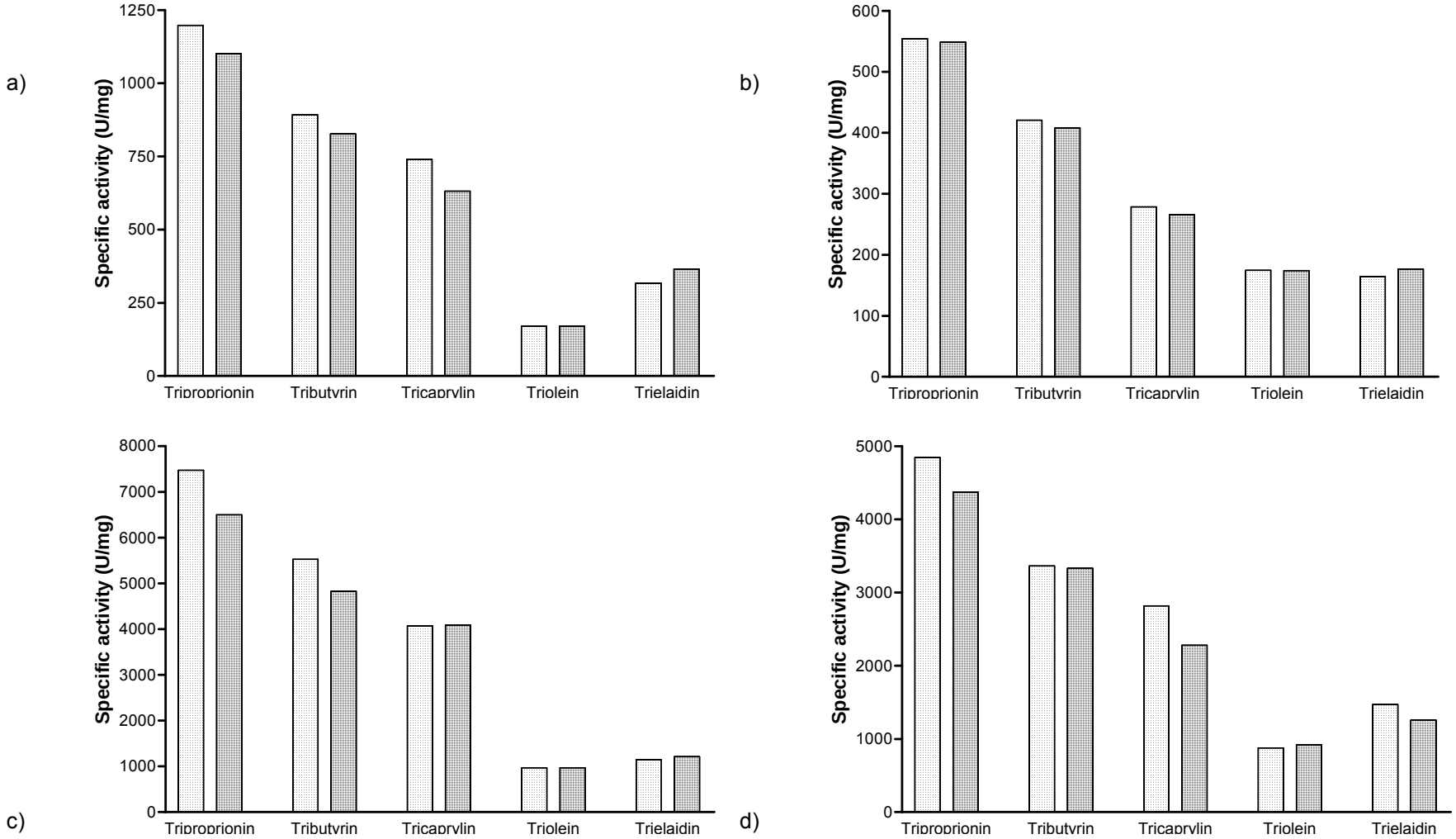


Figure 5.27 Substrate preference profiles obtained for (a), LipA (b), C-tagged (c), N-tagged (d) and Detagged lipases using triacylglycerols as substrates. The experiments were done in duplicate and the combined results of both experiments are reported.

5.4 Discussion

The results obtained from the sequencing and modelling of the LipA lipase shows that the *G. thermoleovorans* GE-7 has a high identity with other *Geobacillus* lipases, at the genetic, amino acid, and even the structural level. The lipases was shown to contain the elongated lid region, the additional zinc-binding domain and the catalytic triad, and pentapeptide described by Jeong *et al*. (2002) and Tyndall *et al*. (2002).

The data obtained showed that the enzymes do indeed display optimal activity at elevated temperatures. Divalent metal ions appeared to increase the optimum temperature of the enzyme, possibly by increasing its stability. The inactivation kinetics confirmed this, as inclusion of metal ions, especially Ca^{2+},in the buffer led to increases in the half-lives of the enzyme at higher temperatures affecting the activation energy for the inactivation process. The optimum temperatures obtained in the absence of, or with incubation of the enzymes with various metals, correlated well with those reported in the literature, i.e. 60 - 65ºC (Sinchaikul *et al*., 2001; Sharma *et al*., 2002).

Kim and co-workers (2000) found a similar increase in the optimum temperature of the *B. stearothermophilus* lipase whilst investigating the effects of calcium on thermostability. They reported a shift of 8 - 10ºC in the optimum temperature for the *B. stearothermophilus* lipase when calcium was added. The data is however dependent on the substrate used, as Rua *et al*. (1997) showed that a shift in optimum temperature could be achieved using triolein versus tributyrin for the *B. thermocatenulatus* lipase. The stability of the lipases at the oil-water interphase is dependant on substrate, and determination of enzyme stability based on enzyme activity measurements and does not reveal intrinsic properties of the lipases.The reported stability for the *Geobacillus* lipases varies significantly between *Geobacillus* species and even strains within the same species. Researchers have reported half lives ranging from 2 min at 70ºC for *B. stearothermophilus* (Kim *et al*., 2000), 30 min at 60ºC for the *B. stearothermophilus* L1 lipase to 2h at 60ºC and 30 min at 70ºC for the

B. thermoleovorans ID-1 lipase (Lee *et al*., 1999). Our results showed that for the enzymes without any added metal ions, the enzyme was not stable for longer than a few minutes at 65 - 70ºC (on average half lives of less than 2 min). Kim and co-workers (2000) reported that above 58ºC the *Geobacillus* lipases undergo significant unfolding, but reported that the enzymes unfolding temperature could be increased to 66ºC in the presence of calcium. Our data supports these findings.

Data obtained from the Arrhenius plots for the activation energy for inactivation of the lipases showed that the addition of metal ions to the Detagged and N-tagged lipase did not significantly effect the energy requirement, although an increase in activation energy for inactivation was seen in the presence of calcium ions. Comparison with the C-tagged lipase showed that the C-tagged lipase did have lower activation energy than the other lipases. It should be kept in mind that the C-tagged lipase contained zinc and calcium ions, and the activation energy for inactivation (210 kJ/mol) correlates well with the data obtained for the N-tagged (230 kJ/mol) and Detagged (203 kJ/mol) lipases in the presence of zinc and calcium ions.

The lipases from *G. thermoleovorans* GE7 are active over a wide range of pH values, with the optimum pH similar to reports found in the literature for other *Geobacillus* sp., with the pH optima's ranging from 8 - 9 (Leow *et al*., 2004; Sinchaikul *et al*., 2001). It has also been reported that the *Geobacillus* lipases are stable at pH 8.0 for extended periods of time (Lee *et al*., 2001), similar to results obtained.

Bacterial lipases usually do not require the presence of a cofactor for catalysis, but divalent ions, such as calcium, usually stimulate the enzyme activity (Gupta *et al*., 2004), but the influence of metal ions on the activity of the lipases from *Geobacillus* species seems to differ between the different strains and species. The Detagged lipase activity was inhibited by calcium ions, contrary to what was found in the literature, where most authors show either an increased activity in the presence of calcium ions (Lee *et al*., 2001) or no effect on lipase activity (Kim *et al*, 1998). One other study however (Finkelstein *et al*. ,1970) reported on the inhibitory effect of

calcium on the *P. aeruginosa* 10145 lipase. Sharma and co-workers (2002), studying the lipase from *Bacillus* sp. RSJ-1, reported that the lipase activity was stabilised in low calcium concentrations (>1 mM), but that the half-life drastically decreased with increasing concentrations (up to 10 mM) of the divalent ion. It might thus be the case that the ratio of enzyme to calcium was not optimal for the Detagged lipase. The N-tagged and C-tagged lipase was not inhibited by up to 5 mM $CaCl_2$. The presence of zinc ions enhanced the enzymes' activity at low concentrations. This was supported by various authors (Sinchaikul *et al*.,2001; Lee *et al*., 2001; Kim *et al*., 1998; Dharmsthiti and Luchai, 1999). Sinchaikul and co-workers (2002) found that the *B. stearothermophilus* P1 lipase had only 35 % activity remaining after incubation of the lipase with 1 mM $ZnCl_2$. It should be noted that this was not the case for the C-tagged lipase; the presence of both calcium and zinc stabilised the enzyme. The same trend was observed with the divalent ions of Mg^{2+} and Mn^{2+} inhibiting the Detagged enzyme whilst having almost no influence on the C-tagged and N-tagged lipase.

The lipase activity was strongly inhibited by the cationic and anionic detergents, although below the cmc of the anionic detergent there was a slight activation of the enzymes activity. The inhibition by the anionic detergent, SDS, should be reversible, since activity could be recovered after SDS-PAGE with the renaturation of the enzyme. Similar results were reported for these detergents, with Lee *et al*. (2001) showing that no activity could be detected for the *B. thermoleovorans* ID-1 lipase after incubation of the lipase with 1 % (w/v) SDS. Most reports concerning the use of zwitterionic and non-ionic detergents showed that the lipase was either activated or not inhibited by these detergents (Sinchaikul *et al*., 2001). Our results are similar, with both CHAPS and Triton X-100 enhancing the activity of the lipases. It has been reported that the presence of non-ionic detergents might have a substrate mimicking effect on lipases (Hermoso *et al*., 1996) shifting the ratio of inactive / closed conformation to active / open conformation of the enzyme. In some cases these detergents have led to a slight decrease in activity as reported by Kim and co-workers (1998) working on the *B. stearothermophilus* lipase. It only had 83 % remaining activity after incubation with 1 % (w/v) Triton X-100.

Results obtained for the effect of alcohols on lipase activity was supported by work reported by Lee *et al*. (2001) on the *B. thermoleovorans* ID-1 lipase showing that low concentrations (1 % v/v) of ethanol and isopropanol enhanced the activity by 24 - 35 %. No reports could however be found on the influence of higher concentrations of alcohol on *Geobacillus* lipase activity. The Zn^{2+} chelators used in the experiments showed that even in the presence of a strong Zn^{2+} chelator such as TPEN, there was no marked reduction in residual activity, suggesting that the Zn^{2+} does not play a role in catalysis.

The results obtained for the substrate specificities showed that all the lipases preferred the saturated shorter fatty acid chain lengths, both for the synthetic esters and triacylglycerols. What was interesting to note was that for the triacylglycerols, the enzymes preferred the shorter lengths (C3 - C4) versus the synthetic esters which preferred C6 - C10 chain lengths were preferred. Kim and co-workers (1998) reported the same for the *B. stearothermophilus* lipase L1, but in general the thermophillic *Geobacillus* lipases show a preference for substrates with chain lengths ranging from C6 - C12 (Sinchaikul *et al*., 2001; Lee *et al*., 1999; Rua *et al*., 1997). What was interesting to note for all five the lipases tested was the increased activity, and thus preference for *trans* configuration, when comparing the ρNP-elaidate versus the ρNP-oleate specific activities. Similar to what was reported in literature for the *B. thermocatenulatus* (Rua *et al*., 1997), and *B. stearothermophilus* (Sinchaikul *et al*., 2001) lipases, we found an underestimation in the activity and specific activity when comparing the C16 to C10 fatty acid chain length substrates used. The *p*NP-palmitate however was used for these experiments because it is more stable at higher temperatures than the other substrates.

The data thus shows that although the results were comparable for the five enzymes tested, the N-tagged lipase is the lipase of choice as it is more stable and generally has a higher substrate specificity than the other lipases, something also observed with the monolayer experiments, discussed in the next chapter.

CHAPTER 6

Surface Kinetics of *Geobacillus thermoleovorans* GE7 lipase

6.1 Introduction

Under normal conditions lipases catalyze hydrolysis of the ester bonds at the interface between a pure lipid substrate and the water phase. This process is usually monitored in bulk phase using techniques such as the olive oil- (Duncombe, 1963) or pH-stat assay (Beisson *et al*., 2000). A new field of research was opened in 1935 when Hughes, for the first time, used the monolayer technique for the study of lipolytic enzymes at the air/water interface (Hughes, 1935). He observed that the rate of the phospholipase A-catalyzed hydrolysis of a lecithin film, measured in terms of the decrease in surface potential, decreased considerably when the number of lecithin molecules per square centimeter increased. Since this early study, several laboratories have used the monolayer technique to monitor lipolytic activities using phospholipids and galactolipids as substrates (Sias *et al*., 2004)

The technique has gained wide popularity, with the main advantages reported (Verger and de Haas, 1976; Verger, 1980; Verger and Pattus, 1982; Pièroni *et al.,* 1990; Ransac *et al.,* 1991) including the high sensitivity of the monolayer technique, the need for small amounts of lipid to obtain reliable kinetic measurements and the ease with which reactions can be followed whilst monitoring several physicochemical parameters characteristic of the monolayer film, such as surface pressure (the interfacial free energy), potential and density. (Beisson *et al*., 2000). Using lipid monolayers one can also monitor the quality of the interfacee, which depends on the nature of the lipids forming the monolayer, the orientation and conformation of the molecules, the molecular and charge

densities, the water structure and the viscosity. The lipid packing of a monomolecular film of substrate can be kept constant during the course of hydrolysis using the surface barostat, and it is therefore possible to obtain accurate pre-steady state kinetic measurements with minimal perturbation caused by increasing amounts of reaction products. Lastly, inhibition of lipase activity by water-insoluble substrate analogues can be precisely estimated using a zero-order trough and mixed monomolecular films in the absence of any synthetic, non-physiological detergent.

Despite the high sensitivity of the monolayer technique, this technique requires as much lipase as the pH-stat method, ranging from 0.1 - 1μg lipase per assay (Beisson *et al*., 2000). Another pre-requisite for using this technique is that the substrate must be insoluble in the aqueous sub-phase and must form stable monomolecular films so that enzymatic hydrolysis can be followed by measuring the fall of surface pressure (Lagocki *et al.,* 1973). Examples of such substrates are the dicaprin isomers reported by Rogalska and co-workers (1995), tricaprin reported by Nannelli and co-workers (2002) and the dilaurin isomers used by Cernia *et al*. (2004). The natural substrates of lipases are however esters of long-chain lipids, generating partly water soluble products remaining transiently at the interface (Ivanova *et al*., 2002), which can induce molecular reorganization leading to enzyme inhibition causing a decrease in hydrolysis rates. This problem was overcome by Laurent *et al*. (1994) using β-cyclodextrin, a non surface-active agent, to trap the long-chain lipolytic products generated from the hydrolysis of long chain neutral acylglycerols in the monomolecular films. Ivanova *et al*. (2002, 2004) reported on the successful use of β-cyclodextrin in monolayer experiments using both monoolein and diolein to follow hydrolysis by the *Humicola lanuginose* lipase.

Kinetic models for interfacial lipolysis cannot adhere to the classical Michaelis-Menten kinetics model, as this model relies on the assumptions that the enzymatic reaction must take place in an isotropic medium (i.e., both the enzyme and the substrate must be in the same phase). This model therefore cannot be used to study lipolytic enzymes acting mainly at the interface between a water phase and an insoluble lipid phase. The mechanism of the chemical reactions carried out at the interfaces in a heterogeneous medium depends strongly on the interfacial

organization, steric coordination, and physical interaction between the reacting molecules. The chemical interactions are coupled with processes of adsorption, desorption, convection, and molecular diffusion of the reaction molecules and products of the reaction, etc. One or any combination of these processes may be rate determining.

Although several models were proposed to adapt the Michaelis-Menten kinetic scheme to interfacial lipolysis, the simplest model was described by Verger *et al.,* (1973), where an instantaneous solubilisation of the products of the reaction is assumed (Figure 6.1). This model is based on the hypothesis that an enzyme-substrate complex might be formed at the interface and consists of two successive steps, starting with the reversible penetration of a water-soluble enzyme (E) into the lipid substrate at the interface. The enzyme is fixed at the interface by an adsorption-desorption molecular mechanism. One formal consequence of this stage is the dimensional change in the enzyme concentration. The penetration stage (adsorption) probably involves enzyme activation (e.g. via the opening of the amphiphilic lid covering the active site). This penetration step, leading to a more favorable energy state of the enzyme (E*), is followed by a two-dimensional Michaelis-Menten kinetic scheme. The enzyme in the interface E* binds a substrate molecule S to form the E*S complex followed by its decomposition. The product of reaction P is soluble in the water phase; it desorbs instantaneously, and induces no change with time in the physicochemical properties of the interface. An important aspect of this model is that to be consistent with the fact that the enzyme-catalyzed reaction occurs at the interface, the concentration of E*, E*S and S must be expressed as surface concentration units.

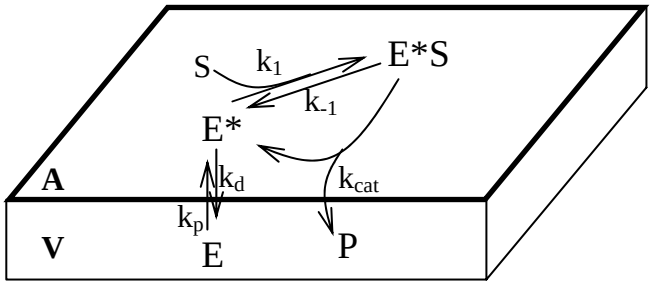


Figure 6.1: Proposed model for lipase kinetics at the interfaces (Adapted from Verger and de Haas, 1976).

The reaction rate dP/dT (product concentration change with time) is given as:

$$v = \frac{dP}{dT} = k_{cat} E_o \frac{S}{K_m^* \left[1 + \frac{k_d}{k_p (A/V)} \right] + S} \qquad [6.1]$$

Where (in figure 6.1 and equation 6.1):

A = total interfacial area in (surface area units)

E_0 = total enzyme concentration (molecules/volume)

k_1 = rate constant for the complex formation E*S (molecules/surface)$^{-1}$ (time)$^{-1}$

k_{-1} = rate constant for the dissociation of E*S into E* + S (time)$^{-1}$

k_{cat} = catalytic rate constant (time)$^{-1}$

k_d = desorption rate constant (time)$^{-1}$

K_m* = interfacial 'Michaelis-Menten' constant (molecules/surface)

k_p = penetration rate constant (volume/surface)

P = product concentration (molecules/surface)

S = two-dimensional surface concentration of substrate (molecules/surface)

V = total volume (volume).

Biological lipids, which self-organize and orientate at interfaces, are chiral molecules, and their chirality is expected to play an important role in the molecular interaction between proteins and bio-membranes. The most unusual aspect of acylglycerol hydrolysis catalysed by pure lipases is its particular stereochemistry (Morley *et al.,* 1974; Rogalska *et al.,* 1990; Cernia *et al.,* 1994). Lipases can encounter both chiral forms of their substrates, as well as molecules that are prochiral. Physicochemical factors such as temperature (Holmberg and Hunt, 1991), solvent hydrophobicity (Wu *et al.,* 1990; Parida and Dordick, 1991) or the hydrostatic pressure (Kamat *et al.,* 1993), which can affect the stereo selectivity of the reaction, may influence the mechanism whereby an enzyme differentiates between two antipodes of a chiral substrate. Achieving a measurable impact of hydrostatic pressure on a protein in a bulk solution would require pressures in the order of 3 kbar; monitoring the enzyme activity under these conditions is difficult. Utilizing the monolayer technique overcomes this predicament as the surface pressure is easy to manipulate and its effects on the enzyme activity can be readily controlled. Rogalska *et al.,* (1993) investigated the assumption that the stereoselectivity, which is one of the basic factors involved in enzymatic catalysis, may be surface pressure dependent. When working with bulk solutions, the external pressure is not a practical variable because liquids are highly incompressible, whereas the monolayer surface pressure is easy to manipulate.

Authors, such as Ransac *et al*. (1990), Rogalska *et al*. (1995) and Douchet *et al*. (2003), have utilized these properties of monolayers to investigate the stereo- and regioselective properties of lipases from various sources against pseudoglycerides such as 1,2-didecanoyl-2-deoxyamino-*sn*-glycerol and 1,3-didecanoyl-2-deoxyamino-*sn*-glycerol (Ransac *et al*., 1990), enantiomeric pairs of didecanoyl-deoxyamino-O-methyl glycerol (Douchet *et al*., 2003) and dicaprin isomers (Rogalska *et al*., 1995). Several selectivity patterns were distinguished and Rogalska and co-workers (1995) termed the phrases Vicinity Index (V.I.) and Stereoselectivity Index (S.I.) to determine the regio- and stereoselectivity of the lipases tested.

Testing of the lipases by Rogalska *et al*. (1995) showed that using the surface pressure profiles obtained for the enzyme velocity for each of the dicaprin isomers, gave kinetic patterns that could be grouped into four distinct classes.

The monolayer technique is thus a useful tool for the investigation of interfacial catalysis and enzyme kinetic parameters, such as stereo- and regioselectivity and substrate preference, some of the traits investigated in this chapter.

6.2 Materials and methods

The experimental work was done at the C.N.R.S, Laboratory of Enzymology at Interfaces and Physiology of Lipolysis, Marseille, France.

6.2.1 Instruments used

Experiments were performed using the KSV 2200 Surface Barostat and a "zero-order" Teflon trough (Figure 6.2; KSV, Helsinki, Finland).

6.2.1.1 The surface barostat

The KSV 2200 Surface Barostat has been designed to measure the surface pressure of a monolayer at gas-liquid interface, defined as the decrease in the interfacial tension produced by the monolayer, i.e. $\pi = \gamma_o - \gamma_m$ where γ_o and γ_m are the interfacial tension in the absence and presence of the monolayer. Control of the surface pressure is done by the user (Figure 6.2). The surface pressure is measured with a KSV 2200 Surface Balance connected to a platinum Wilhelmy plate (Figure 6.3). The information on the surface pressure is received by the Dynamic Film Control (DFC) system, which regulates a step-by-step-motor driven

mobile Teflon barrier, thereby controlling the surface area occupied by the monolayer and thus subsequently controlling the surface pressure.

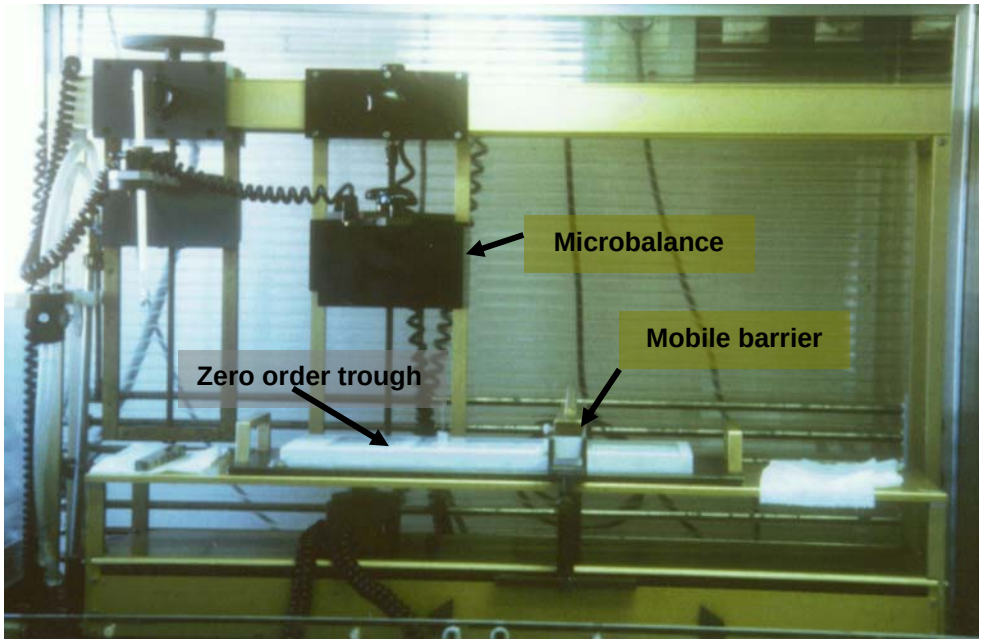


Figure 6.2: KSV 2200 Barostat equipped with “zero-order” Teflon trough for kinetic studies.

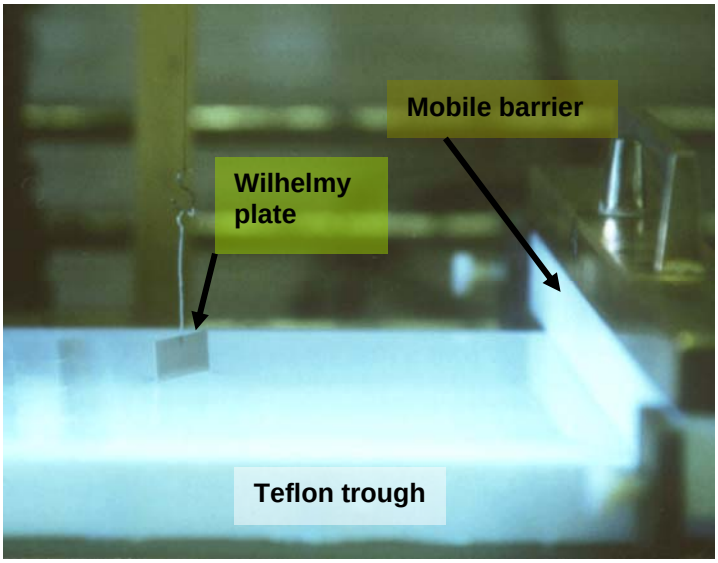


Figure 6.3: The Wilhelmy plate suspended by wire leading to an electromicrobalance to control the movement of the mobile barrier, which in turn controls the surface pressure.

6.2.1.2 The zero-order trough

Several types of troughs have been used to study enzyme kinetics. The simplest of these is made of Teflon that is rectangular in shape (Figure 6.4 a) but gives non-linear kinetics (Zografi *et al*., 1971). To obtain rate constants, a semi-logarithmic transformation of data is required. This drawback was overcome by a new through design (Figure 6.4 b) consisting of a substrate reservoir and a reaction compartment containing the enzyme solution (Verger and de Haas, 1973). A narrow surface canal made of etched glass connects the two compartments. The kinetic recordings obtained with this trough are linear, unlike the non-linear plots obtained with the usual one-compartment trough. The surface pressure can automatically be kept constant by the surface barostat method (Verger and de Haas, 1973).

The width of the reservoir compartment used was 147,9 mm, the length 249,2 mm and the surface area 36 815 mm^2. The surface area of the reaction compartment was 10 205 mm^2. The depth of the trough was 8 mm. The reaction compartment was equipped with 2 magnetic stir bars operating at 250 rpm.

6.2.2 Surface kinetics experiments

The buffer (aqueous sub-phase) used was composed of 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA and 10 mM $CaCl_2$. The buffers were prepared with double distilled water and were filtered through a 0.45 µm Millipore filter. Any residual surface-active impurities were removed before each assay by sweeping the surface of the buffer with a clean Teflon® bar and suction of the surface with a Pasteur pipette connected to a tap aspirator. The reactions were performed at ambient temperature (25ºC). Experiments were performed using the N-tagged and Detagged lipases. The enzyme solution was injected through the film with a Hamilton syringe over the magnetic stirrers.

a)

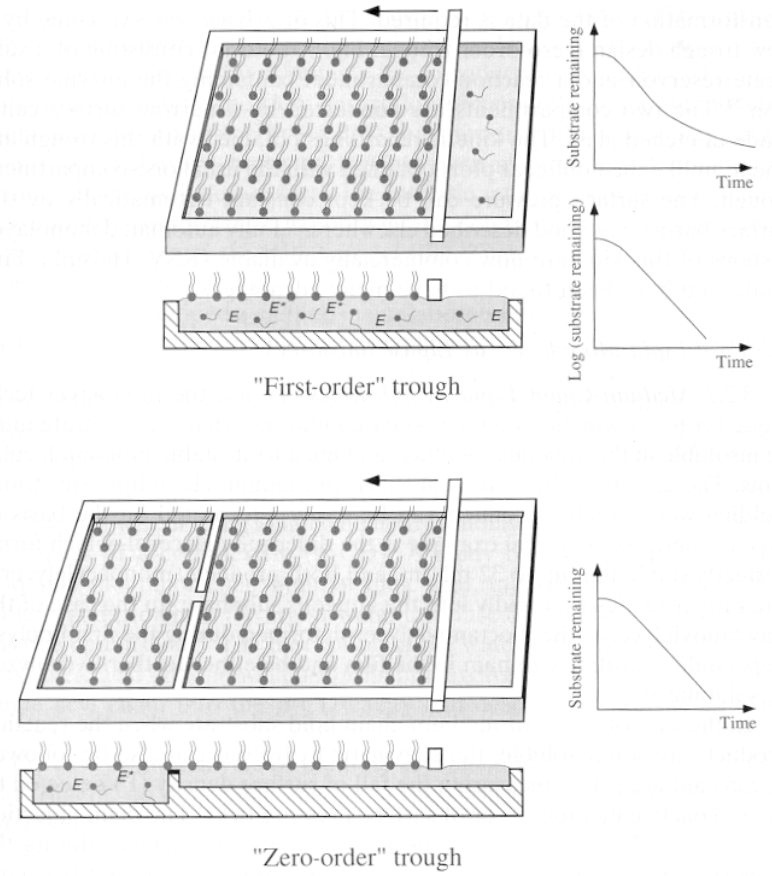


Figure 6.4: Comparison of lipase kinetics obtained with a first-order trough (a) and a zero-order trough (b) (Taken from Ransac *et al.,* 1997)

Before each use, the Teflon trough (Figure 6.4) used to form the monolayer film was cleaned with water, then gently brushed with distilled ethanol, washed again with tap water and finally rinsed with double distilled water.

6.2.2.1 Positional preference and stereospecificity

The lipase stereoselectivity and regioselectivity experiments were performed as described by Rogalska and co-workers (1995) using optically pure dicaprin enantiomers 1,2-*sn*-dicaprin (Sigma), 2,3-*sn*-dicaprin (SRL, London) and 1,3-*sn*-dicaprin (Synthesised by G.H. de Haas and H.M. Verheij, Utrecht-NL). The former two isomers are optically active antipodes (enantiomers), forming stable films up to

40 mN/m, while the latter one is a prochiral compound, which collapses at a surface pressure of 32 mN/m (Figure 6.5).

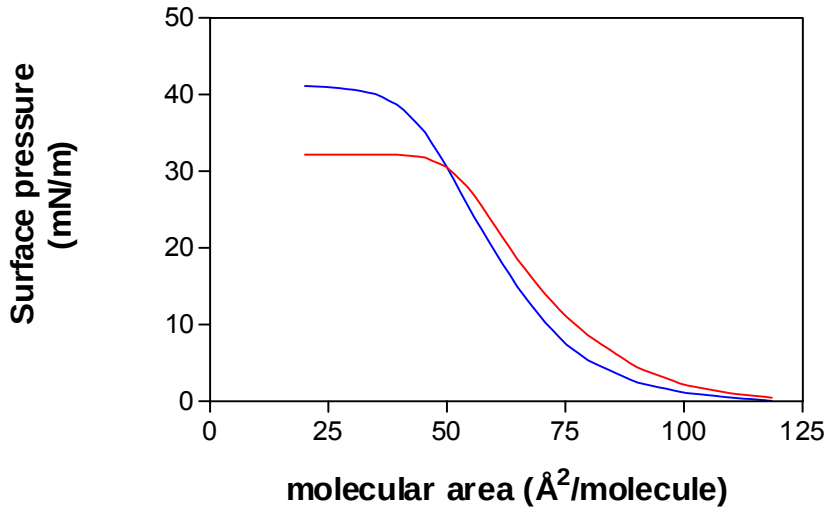


Figure 6.5: Surface pressure versus molecular area in monomolecular films (*sn*-1,2-dicaprin or *sn*-2,3-dicaprin (——) and *sn*-1,3-dicaprin (——) (Taken from van Heerden *et al*., 2002).

Specific activity was expressed as mole.cm^{-2}.min^{-1}.M^{-1} and was determined as follows:

$$Activity = \frac{V_{cm/\min} \times l \times mole.cm^{-2}}{A_t \times \pi} \qquad [6.2a]$$

where

$$\pi = \frac{C \times V_l}{Mw \times V_t} \qquad [6.2b]$$

and

$V_{cm/min}$ = barrier speed

l	=	length of “zero-order” trough
mole.cm^{-2}	=	dicaprin constant at specific surface pressure
A_t	=	surface area of “zero-order” trough
C	=	concentration enzyme added
V_{ll}	=	volume of enzyme added
Mw	=	molecular weight of enzyme
V_t	=	volume of “zero-order” trough

Surface pressures, as a function of the mean molecular area isotherms were measured for all compounds. Stock solutions of individual lipids were prepared in distilled $CHCl_3$. Fifty μl of a lipid solution with an accurately known concentration was spread on the aqueous buffer. The monolayer was allowed to stabilise for 5 min, enzyme added and surface pressure kept constant using the mobile Teflon barrier, compensating for the substrate molecules removed from the film by enzyme hydrolysis (monocaprin and capric acid are soluble in water). Every 3 seconds data were collected and analysed with proprietary software from KSV Instruments (Helsinki).

6.2.2.2 Interactions of lipases with phospholipids

The hydrolytic capabilities of *G. thermoleovorans* GE-7 lipases against phospholipids were tested as described in Section 6.2.2.1 using 1,2-didodecanoyl-*sn*-glycerol-phospatidylglycerol (1,2 $DiC_{12}PG$), 1,2-didodecanoyl-*sn*-glycerol-phosphotidylethanoamine (1,2 $DiC_{12}PE$) and 1,2-didodecanoyl-*sn*-glycerol-phospatidylcholine (1,2 $DiC_{12}PC$) (Avanti polar lipids).

6.2.2.3 Interactions with galactolipids

The hydrolytic capabilities of *G. thermoleovorans* GE7 lipases against galactolipids were tested as described in Section 6.2.2.1 using 1,2-di-*O*-dodecanoyl-3-*O*-β-D-

galactopyranosyl-*sn*-glycerol (MGDG) synthesised as described by Sias and co-workers (2004).

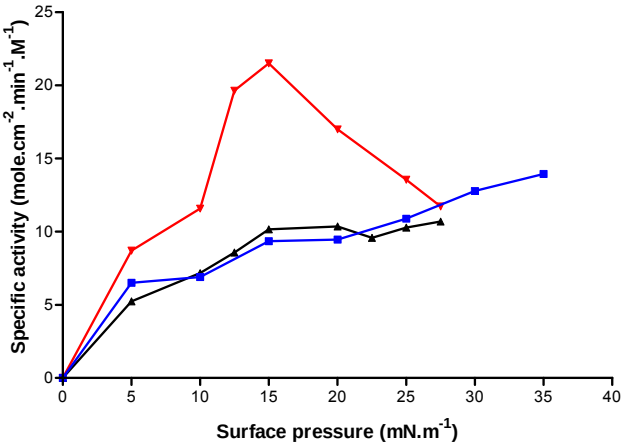
6.3 Results

6.3.1 Positional preference and stereospecificity

It is important to consider that assays were performed at 25ºC, which is 40ºC below the optimum temperature of the enzymes. At this temperature (Chapter 5) the enzyme has approximately 20-25 % of its activity. The results of these experiments showed that the N-tagged lipase (Figure 6.6 a) preferentially hydrolysed the *sn*-1,3-dicaprin at lower surface pressures, with a decrease in hydrolytic activity against this substrate at higher surface pressures. Similar results were obtained for the detagged lipase (Figure 6.6 b) with the exception that hydrolysis of the *sn*-1,3-dicaprin was not as enhanced at surface pressures of 5-20mN/m, showing the clear substrate preference for the *sn*-1,3 dicaprin in the case of the N-tagged lipase.

Using the surface pressure profile obtained for a study of 23 lipases, Rogalska *et al*. (1995) divided the lipases into four distinct groups. The N-tagged and Detagged lipases were grouped into group C. The lipases tested show a low surface pressure threshold, as maximum activity could be detected at 5 mN/m. Rogalska *et al*. (1995) used the terms vicinity index (V.I.) to describe the regioselectivity (Equation 6.3) and stereoselectivity index (S.I.) to illustrated the stereoselectivity (Equation 6.4) of lipases against the dicaprin isomers. In this study, we calculated the V.I. and S.I. at all the surface pressures tested and found that the N-tagged lipase (Figure 6.7 a) has a clear preference for the *sn*-1,3-dicaprin, especially at 15 mN/m, although the selectivity decreases with the higher surface pressures. The Detagged lipase (Figure 6.7 b) showed a lower selectivity toward the *sn*-1,3-dicaprin, with the same decrease in selectivity, accompanied with the decreasing surface pressure. It also showed an absence of stereochemistry effects with *sn*-1,2- and *sn*-1,3-dicaprin.

a)



b)

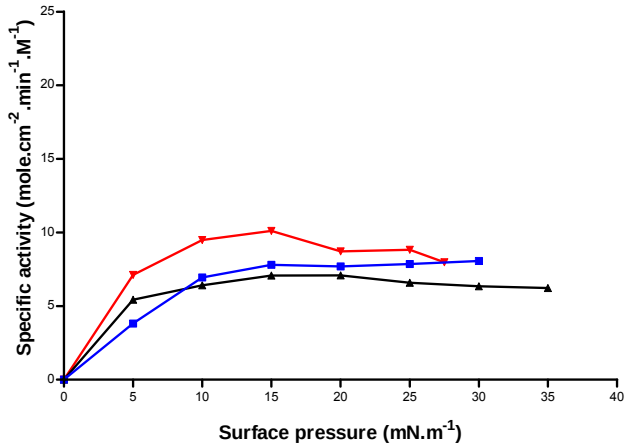


Figure 6.6 Surface pressure profiles of *Geobacillus thermoleovorans* N-tagged (a) and Detagged (b) lipase using dicaprin substrates (*sn*-1,2-dicaprin (blue), *sn*-2,3-dicaprin (black) and *sn*-1,3-dicaprin (red).

$$S.I.= {(A_{2,3} - A_{1,2})}\Big/{(A_{2,3} + A_{1,2})}$$

[6.3]

$$V.I.= {[A_{1,3} - 0.5(A_{2,3} + A_{1,2})]}\Big/{[A_{1,3} + 0.5(A_{2,3} + A_{1,2})]}$$

[6.4]

$A_{x,y}$ are the measured rates at identical surface pressures for the diacylglycerol substrates where X and Y indicate the position of the fatty acid.

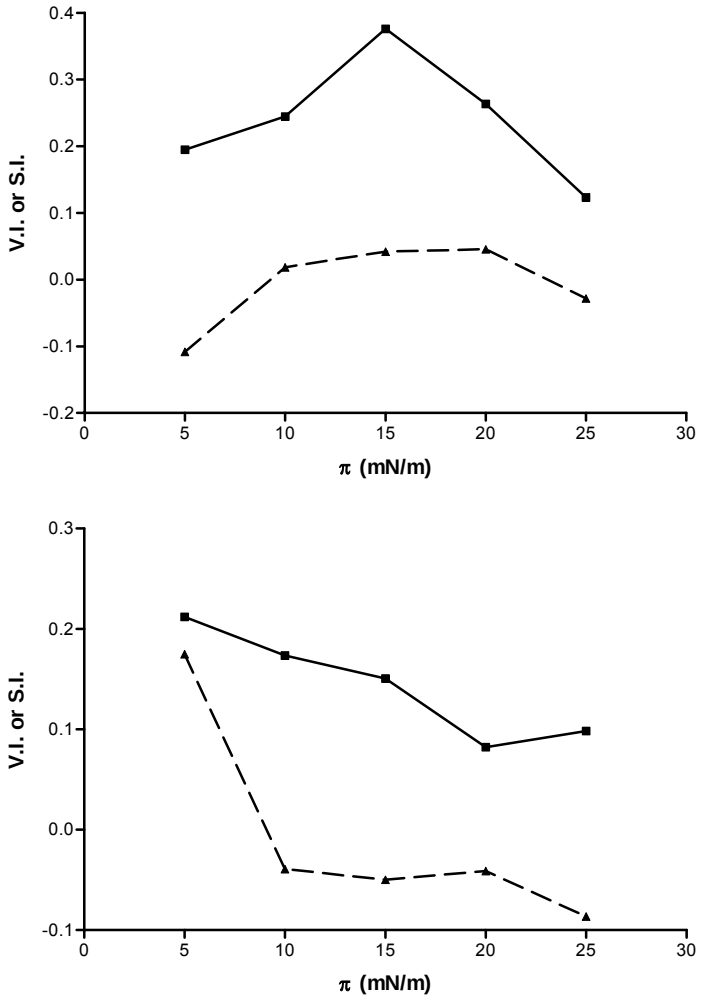


Figure 6.7 The V.I. (■) or S.I. (▲) versus surface pressure as calculated from the specific activities measured at different surface pressures respectively for a) the N-tagged lipase and b) the detagged lipase.

The N-tagged and Detagged lipases both showed low stereoselectivity at all the surface pressures investigated, with the exception of the Detagged lipase at 5 mN/m, showing some stereoselectivity towards the *sn*-1,3 positions. The only significant effect seems to be due to the presence of the His_6-tag when the *sn*-1,3-dicaprin was used.

6.3.2 Interactions of lipases with phospholipids

The experiments were performed the same as for the dicaprin isomers at varying surface pressures, but no activity could be detected against either the 1,2

$DiC_{12}PG$, 1,2 $DiC_{12}PE$ or 1,2 $DiC_{12}PC$ substrates. The *G. thermoleovorans* GE-7 lipases thus do not have any hydrolytic activity against phospholipids.

6.3.3 Interactions with galactolipids

No activity could be detected against MGDG as substrate with either the N-tagged or Detagged lipase, showing that the *G. thermoleovorans* GE-7 lipases do not have galactolipase activity.

6.4 Discussion

According to the surface pressure profiles described by Rogalska *et al*. (1995), the N-tagged lipase was classified into group C lipases showing maximum activity with a “bell-shaped” surface pressure profile. A similar profile was obtained for the porcine pancreatic lipase. The surface pressure-activity profiles as well as the optimum activity values varied with the particular lipase-substrate combination used. This was best seen when the hydrolysis of *sn*-1,3-dicaprin by the N-tagged and Detagged lipases were compared. Both the enzymes hydrolysed the *sn*-1,2- and *sn*-2,3-dicaprin at similar rates showing a preference for the *sn*-1,3-dicaprin.

An important observation made was the low surface pressure threshold (SPT) of the enzymes, being most active below 10 mN/m in the case of the Detagged and 15 mN/m for the N-tagged lipase. It was reported by Graham and Phillips (1979) that low surface pressure is equivalent to high surface free energy and that proteins can unfold under these conditions. Typically enzymes resistant to low surface pressures are good candidates for use in the detergent industry as they can withstand the presence of tensio-active agents. Lipases active at low surface pressures are possibly more resistant to denaturing conditions (Rogalska *et al*., 1995), something that was seen for the *Geobacillus* lipases (Chapter 5).

The regioselectivity of the lipases showed that the lipase preferred the *sn*-1,3-dicaprin, but with the preference declining at higher surface pressures. It is thus

clear that in the case of 1,3-diglycerides, the “tuning fork” conformation (Grochulski *et al.,* 1994) does not have an influence on lipase regioselectivity.

Although this is the first report of *Geobacillus* lipase activity being tested using the monolayer technique, other reports have described the positional specificities of these lipases. Lee *et al*. (2001) found the *B. thermoleovorans* ID-1 lipase to be positionally non-specific using triolein as substrate, while Dharmstiti and Luchai (1999) and Rua *et al*. (1997) found the *Bacillus* sp THL027 and *B. thermocatenulatus* preferred the 1,3-ester bonds of triolein. These experiments were analysed using thin layer chromatography after hydrolysis was done at 60ºC. Rogalska *et al*. (1995) reported that the stereopreference for either the *sn*-1 *or sn*-3 position on glycerides is generally maintained in both di- and triglycerides. Taking this, and the fact that experiments were performed at 25ºC, into consideration, we obtained similar results with the *G. thermoleovorans* GE-7 lipases prefering for the 1,3-bonds of the diglycerides tested.

Microbial lipases showing phospholipase activity are rare; although some bacterial enzymes have been isolated showing this activity, such as the *Staphylococcus hyicus* lipase (Simons *et al*., 1998). No reports have been made of naturally occurring thermophillic lipases with phospholipase activity. Kauffmann and Schmidt-Danert (2001) however reported on a *B. thermocatenulatus* strain that was modified via random mutagenesis to yield an enzyme showing phopholipase activity against 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. Galacticolipase activity is not commonly found among microbial lipases. The fungus *Rhizopus delemar* (*arrhizus*) is the only reported microbial lipase with galactolipase activity. No such activity could be detected for either of the *G. thermoleovorans* GE-7 lipases tested.

A significant results of this study is the enhanced activity of the N-tagged lipase towards *sn*-1,3-dicaprin. The detagged enzyme is identical to the native one, except that the N-terminal amino acids are Gly-Ser-His followed by the mature lipase amino acids. A possible explanation for the enhanced activity of the N-tagged lipase against the *sn*-1,3-dicaprin might be the addition of the N-terminal histidines and thrombin cleavage site aiding in the orientation of the substrates. It

has been shown in the literature that the N-terminal region of lipases is important for their proper function (Sayari *et al*., 2005). How this would happen is however not obvious as the N-terminal part, which contains the His_6 tag is situated away from the binding cleft of the enzyme.

Considering the results presented in the previous chapter (Chapter 5), it can be clearly seen that the hypothesis of only a temperature switch controlling the opening of the lid for *Geobacillus* lipases is probably not correct, as the interaction with the interface at different temperatures should be considered. Cajal and co-workers (2003), working on the *Thermomyces lanuginosa* lipase, showed with dicaprin monolayers at temperatures ranging from 15ºC – 42ºC there was a linear correlation between increasing temperature and activity. Similar to the proposals of Jeong *et al*. (2002), Cajal *et al*. (2003) suggested that with increasing temperatures, the rotational freedom of the lipase residues would increase due to thermal excitation, favouring the opening of the lid. It is thus clear that the interplay between "interfacial activation" and increased temperature is needed for the optimum functioning of thermophillic lipases.

The exact catalytic mechanism for the *Geobacillus* lipase at the interface still needs to be investigated, something that might be done using tricaprin- (Nanneli *et al*., 2002), monoolein-, diolein- (Ivanova et al., 2002 and 2004) or dilaurin monolayers (Cernia et al., 2004) to more accurately investigate the substrate preference of these lipases. Although these experiments will have to be done at sub-optimum temperatures, it will give an indication of what might be happening at the interface. Quantification of the lipase in the lipid layer will also help to estimate the specific activity, and thus the amount responsible for catalysis. Similar experiments can be performed using bulk phase experiments with the subsequent estimation of enzyme associated with the lipid phase. This might provide interesting information concerning the mode of catalysis at elevated temperatures, since the exact conformation of the lipid/water interface at these temperatures are not completely understood. Resolution of the *Geobacillus* lipase 3D structure with the lid in its open conformation is also a major target for a future understanding of these enzymes stability and activity at high temperatures.

CHAPTER 7

General Discussion

Various authors have reported on the inducible production of lipases by *B. stearothermophilus* (Sinchaikul *et al*., 2001), *Bacillus thermocatenulatus* (Rua *et al*., 1997) and *Bacillus thermoleovorans* (Lee *et al*., 1999) and were able to not only isolate the gene coding for this lipase, but also heterologously overexpress them in various hosts. *Geobacillus thermoleovorans* GE-7 was shown to produce lipase when tributyrin, olive oil and stearic acid were used as inducers. The lipase open reading frame, coding for the signal peptide and the mature lipase, could be successfully amplified from genomic DNA, the PCR product cloned into the pGEM®T-easy cloning vector, transformed into *Escherichia coli* and the colonies screened for lipase activity using tributyrin plates with almost all the colonies testing positive for lipase activity. This method could thus be applied to environmental samples, screening for family 1.5 lipases. Modification of the lipase genes was successful using a combination of PCR and the pET 28a expression vector incorporating a histidine tag at either the N- or C-terminus of the lipase. Sequencing results obtained for the different constructs had high similarities with other known *Geobacillus* lipases, at both nucleotide and amino acid (deduced sequence) level.

Confirming reports by Sonnleitner and Fiechter (1983) concerning the low production levels of enzymes by thermophiles, it was found that under optimised induction procedures, using stearic acid as inducer, the highest levels of lipase produced was 1531 U/l (Table 7.1). Overexpressing the lipase gene in the mesophillic host, *E. coli*, could produce higher levels of this lipase. Expression of the constructs pGEMGtherm-LipA (LipA), pETGtherm-LipA(C) (C-tagged) and pETGtherm-LipA(N) (N-tagged) (Chapter 4) yielded expression levels of 5770 U/l for LipA, 5020 U/l for the C-tagged and 37340 U/l in the case of the N-tagged lipase. In the case of the N-tagged lipase 23,4 times more lipase could be

produced in a shorter time when compared with optimised *G. thermoleovorans* GE-7 lipase production protocols.

Production of the C-tagged lipase proved to need more optimization, as almost all this lipase was located extracellularly when the recommended protocol was used. Changing the growth and induction conditions (i.e. temperature and IPTG concentration) resulted in accumulation of the lipase in the intracellular environment. Expression of the tagged recombinant proteins is usually preferred in the cytoplasm since the production yields are higher and preparation of the sample for chromatographic steps is less cumbersome (Sorenson and Mortenson, 2005). The LipA could be produced in *E. coli* without the addition of any inducers, although most of the lipase was located in the extracellular media. Combining these results with initial N-terminal sequencing results obtained for LipA indicated the ability of *E. coli* to correctly process and transport the lipases out of the cells if the signal peptide is present. Almost no extracellular N-tagged lipase could be detected, something that was expected as the signal peptide was not part of the construct.

Table 7.1 Comparison of some of the characteristics obtained for the different lipases studied.

	Native Lipase	**N-tagged**	**Detagged**	**C-tagged**	**LipA**
Induction levels	Best level was 1531 U/l	37340 U/l		5024 U/l	5776 U/l
Molecular weight (SDS)	~40 kDa	~ 40.1 kDa	~39 kDa	~ 42 kDa	~ 41 kDa
pI	~ 7	~ 7.1	~ 6.8	~ 7.3	Not determined
Optimum temperature	60ºC	60ºC 65ºC with Ca^{2+} added	60ºC 65ºC with Ca^{2+} added	55ºC with Ca^{2+} and Zn^{2+} added	60ºC
Optimum pH	9-10	9-10	9-10	9-10	9-10

Purification of the native lipase proved to be difficult and although various authors have reported the successful purification of *Geobacillus* lipases, this author was unsuccessful in purifying the lipase to electrophoretic homogeneity using a variety,

and different combinations, of purification steps. The best results were obtained with the addition of Triton X-100 to the mobile phase of the Super-Q chromatographic resins, seemingly separating the lipase from ~50 % of the contaminating proteins. Problems however were experienced with the removal of the detergent. A major factor that has to be taken into consideration for the purification of the native lipase is the low amount of enzyme produced, hindering the use of high number of consecutive purification steps. Similar problems were experienced with the purification of the LipA recombinant lipase, as the protein could only be partially purified. Even though higher amounts of lipase were available for the purification process, 75 % of the activity was lost after only 4 purification steps.

This problem was overcome with the use of the histidine tagged lipases. Using only one chromatographic step the N-tagged and C-tagged lipase could be purified to electrophoretic homogeneity with high yields of the enzyme, using the Ni-NTA resin. Initial loss of activity for the C-tagged lipase could be overcome with the addition of both Ca^{2+} and Zn^{2+} to the enzyme before storage. Removal of the N-terminal histidine tag was accomplished with the help of the incorporated thrombin cleavage site, followed again by only one chromatographic step to successfully separate the Detagged lipase from the cleaved histidine tag with on average a yield of 90-95 % of the lipase. In all the cases stated above, lipolytic activity could be correlated to a specific protein band on SDS-PAGE gels using zymograms with either olive oil or tributyrin.

The sizes of the various lipases produced correlated to what was reported in literature (Table 3.1), showing expected sizes of 39-42 kDa (Table 7.1). Small differences in sizes obtained can be attributed to the modifications made to the recombinant lipases. The optimum temperatures obtained ranged from 55ºC for the C-tagged lipase to 60ºC for the rest of the lipases tested. Interesting results were obtained for the N-tagged and Detagged lipases optimum temperature when the enzymes were pre-incubated with Ca^{2+}, Zn^{2+} or a combination of the two, leading to a optimum temperature of 65ºC. The optimum pH seen for all the lipases were extremely basic lying at pH 9-10.

The stability of the C-tagged, N-tagged and Detagged lipases were investigated in terms of temperature and pH, and it was found that the presence of calcium contributed drastically to the temperature stability of the N-tagged and Detagged lipases. The N-tagged lipase had a half-life of 23 h at 65ºC and almost a hour at 70ºC with the Detagged enzyme exhibiting a half-live of 2.67 h at 65ºC and only 19 min at 70ºC. The C-tagged lipase was not as stable at elevated temperature and showed a half-life of only 2 min at 70ºC. All three enzymes were not stable at lower pH values, but were stable close to their optimum pH, with reported half-lives of 19.5 h for the N-tagged and 9.7 h for the Detagged lipase.

The effect of metal ions on the lipases tested (C-tagged, N-tagged and Detagged) showed that although Ca^{2+}, Mg^{2+}, and Mn^{2+}, up to concentrations of 5 mM, had an enhancing effect on the activity of the C-tagged and N-tagged lipases, they slightly inhibited the Detagged lipases activity at concentrations of 0.1 mM. The lipases were all inhibited by 5 mM Hg^{2+} and Al^{2+}, but the activity was almost unchanged in the presence of 5 mM Ba^{2+}. All three of the lipases were strongly stimulated by the non-ionic detergent Triton X-100 and the zwitterionic detergent CHAPS, but were inhibited by the anionic (SDS) and cationic (cetrimide) detergents. Of the alcohols tested it was found that the lipases preferred the presence of methanol (up to 40 % v/v) but could tolerate up to 20 % (v/v) ethanol and isopropanol.

As was expected, the presence of EDTA (15 mM) led to a loss of activity in all three the enzymes, but contrary to what was expected, the presence of Zn^{2+} chelators such as TPEN had no effect on the activity. Considering this and the results obtained for the optimum temperature experiments done with the Detagged and N-tagged lipases and the activation energy determinations for the Detagged lipase, it can be concluded that the Ca^{2+} ions play a role in the enzyme stability and are not involved in catalyses. This was confirmed by modelling experiments of the lipase structure, which revealed the Ca^{2+} binding site to be exposed to the environment, and not closely associated with the active site. The novel Zn^{2+} binding site present in this family of enzymes seems to play a role in keeping the

enzymes conformation stable rather than in catalysis itself. What was interesting was that although there is this novel Zn^{2+} binding site, the addition of Zn^{2+} led to only a slight loss in activity.

All the lipases (native, LipA, N-tagged, C-tagged and Detagged) showed preference for the shorter saturated fatty acids (C10-14) when synthetic esters were used, but preferred the shorter saturated fatty acids when triacylglycerols were used. This aspect of the study however needs further investigation, as only 3 of these substrates were available for testing. Another interesting observation was the fact that all the enzymes tested preferred the *trans* position when the $C18\Delta^1$ unsaturated fatty acids were tested. Results obtained from the monolayer experiments showed that the N-tagged and Detagged lipases preferred the *sn*-1,3 position of triacylglycerols, and that the additional N-terminal histidines and thrombin clevage site enhanced the activity of this lipase at 15 mN/m.

Reports made by Tandyll *et al*. (2002) and Jeong *et al*. (2002) on the structure of *Geobacillus* lipase pointed towards a temperature switch for the opening of the lid covering the active site. Reports by Cajal and co-workes (2003), working on the *Thermomyces lanuginosa* lipase, support the hypoyhesis that the lid opening is rather controlled by an interplay between temperature and “interfacial activation”. Thus at higher temperature the lid will be opened with more ease due to thermal excitation. Palamo and co-workers (2004 a and b) showed that by binding *Geobacillus* and *Thermus* liapses Octadecyl-Sepabeads-TAL using “interfacial activation” not only lead to enhanced stability, but the activity of the lipases could be enhanced by a 50-fold factor at mesophillic temperatures. It is thus clear that both factors are needed for optimal lipase activity.

The influence of the N-terminal His_6 additions can not yet be explained, but it is clear from the data obtained and presented here that the N-tagged lipase is not only the most stable of the 5 lipases, but is also produced in higher quantities than the other enzymes tested. The author was thus sucessful in complying with the aims set out at the start of this project, with the exception that the native lipase could only be partially purified.

CHAPTER 8

Summary

Geobacillus thermoleovorans GE-7 was isolated from the West-Driefontein goldmine in South Africa. It is a Gram positive rod showing optimal growth at 65ºC. Lipase production by the organism can be induced using tributyrin, Tween 80 and stearic acid. We were able to partially purify this thermophilic lipase (native lipase) using hydrophobic (Phenyl-Toyopearl), anionic (Super-Q and DEAE-Toyopearl) and size exclusion (Sephacryl S-100/200-HR) chromatographic resins. Using tributyrin zymograms, the size of the protein was estimated to be 40 kDa with a pI of 7. The lipase open reading frame (1251 bp), coding for the signal peptide and mature lipase, could be succesfully amplified from genomic DNA using polymerase chain reaction and the PCR product was cloned into the pGEM®T-easy cloning vector (pGEMGtherm-LipA; LipA). The gene was modified with PCR and cloned into the pET 28a expression vector to yield a hexa histidine tag at the C- (pETGtherm-LipA(C)) and N-terminus (pETGtherm-LipA(N)) of the lipase gene. All three clones could be succesfully expressed in *E. coli* JM109(DE3), with expression levels of the N-tagged lipase 23.4 times higher when compared to optimised lipase production by *G. thermoleovorans* GE-7. The N-tagged and C-tagged lipase could be sucessfully purified using affinity chromatography (Ni-NTA resin) whilst the LipA lipase was only partially purified using the same purification protocols used for the native lipase. The N-terminal His_6 tag was removed using thrombin targeting the incorporated thrombin cleavage site, resulting in the Detagged lipase which could be separated from the cleaved His_6 tag using the Ni-NTA resin. The N- and Detagged lipase was stable during storage but the C-tagged lipase had to be stored in with 1 mM $CaCl_2$ and 1 mM $ZnSO_4$ to retain activity. The different lipase sizes and pI's were comparable, ranging from 42 kDa (pI 7.3) for the C-tagged lipase, 41.1 kDa (pI 7.1) for the N-tagged lipase, 39 kDa (pI 6.8) for the Detagged lipase and 41 kDa for LipA. The optimum temperatures for the lipases varied from 55ºC (C-tagged) to 60ºC (N-tagged, Detagged, native,

LipA) but could be increased to 65ºC with the addition of 1 mM $CaCl_2$ (Detagged and N-tagged lipase). All the lipases showed an optimum pH of 9-10 and had a preference for the shorter chain saturated fatty acids. In the presence of 1 mM $CaCl_2$ the N-tagged lipase had a half-life of 1 h at 70ºC and 23 h at 65ºC while the Detagged lipase had a half-life of 19 min at 70ºC and 2.7 h at 65ºC. The C-tagged lipase had a half-life of only 2 min at this temperature. Initial interest was in the production of a lipase by an extremophillic bacterium, but addition of the N-terminal histidine tag also lead to a higher enzyme stability at higher temperatures.The activity of the C-, N- and Detagged lipase was enhanced by the presence of Triton X-100 and CHAPS, but was inhibited by SDS and cetrimide, and were stable in up to 20 % (v/v) of various alcohols. Ca^{2+}, Mg^{2+}, and Mn^{2+} (up to 5 mM) enhanced the activity of the C- and N-tagged lipase but slightly inhibited the Detagged lipase. All three enzymes showed slight inhibition in the presence of Zn^{2+} but was inhibited by Al^{2+} and Hg^{2+}. EDTA had an inhibitory effect on lipase activity, but TPEN showed no effect. The N-tagged and Detagged lipases showed preference for *sn*-1,3-dicaprin isomers with monolayer experiments but did not exhibit any phospho- or galactolipase activity.

CHAPTER 9

Opsomming

Geobacillus thermoleovorans GE-7 is uit die Wes-Driefontein-goudmyn in Suid-Afrika geïsoleer. Dit in ‘n Gram-negatiewe staaf met ‘n optimale groeitemperatuur van 65^{O}C. Lipase-produksie deur die organisme kon met tributirien, Tween 80 en steariensuur geïnduseer word. Ons kon daarin slaag om die termofiliese (natuurlike) lipase gedeeltelik te suiwer deur van hidrofobiese- (Phenyl-Toyopearl), anioniese- (Super-Q en DEAE-Toyopearl) en uitsluitings- (Sephacryl S-100/200-HR) chromatografiemedia gebruik te maak. Deur van tributirien simogramme gebruik te maak, kon die grootte van die proteïen op ongeveer 40 kDa geskat word met ‘n pI van 7. Die oopleesraam (1251 bp) wat vir die seinpeptied sowel as die geprossesseerde lipase koder, kon suksesvol vaaf die genomiese DNA deur middel van die polimerase kettingreaksie geamplifiseer word. Die PKR-produk is in die pGEM®T-easy kloneringsvektor ingevoeg (pGEMGtherm-LipA; LipA). Die geen is ook gemodifiseer deur ‘n PKR reaksietegniek te gebruik en in die pET 28a uitdrukkingsvektor ingevoeg, om die heksa-histidien merkstuk aan die C- (pETGtherm-LipA(C)) sowel as die N-terminale (pETGtherm-LipA(N)) punt van die ensiem op te lewer. Al drie klone kon suksesvol uitgedruk word in *E. coli* JM109(DE3). Uitdrukkingsvlakke van die N-gekopelde lipase was 23.4 keer hoër as die van die geoptimeerde vlakke behaal met die *Geobacillus* GE-7 self. Die N-gemerkte en C-gemerkte lipase kon gesuiwer word deur van affiniteitschromatografie met ‘n Ni-NTA chromatografiemedium gebruik te maak. Die LipA kon net gedeeltelik gesuiwer word deur van dieselfde suiweringsprosedure as vir die natuurlike lipase gebruik te maak. Die N-terminale His_6 merkstuk kon verwyder word deur trombien vertering, gerig op die geïnkorporeerde kliewingsetel, gevolg deur Ni-NTA affiniteitschromatigrafie.

Die N-gemerkte lipase en die een waarvan die koppelstuk verwyder is, was stabiel tydens berging maar die C-gemerkte ensiem moes in die teenwoordigheid van 1

mM $CaCl_2$ en 1 mM $ZnSO_4$ geberg word om aktiwiteit te behou. Die groottes en pI-waardes van die verskillende lipases was vergelykbaar en het tussen 42 kDa (pI 7.3) vir die C-gemerkte, 41.1 kDa (pI 7.1) vir die N-gemerkte, 39 kDa (pI 6.8) vir die ensiem waarvan die N-terminale koppelstuk verwyder is en 41 kDa vir LipA gewissel. Die optimum temperature vir die lipases het tussen 55ºC (C-gemerkte) en 60ºC (N-gemerkte, N-terminale merkstuk verwyder, natuurlik, LipA), gewissel maar kon tot 65ºC verhoog word met die byvoeging van 1 mM $CaCl_2$ (N-terminale merkstuk verwyder en N-gemerkte lipase). Al die lipases het ‘n optimum pH van 9-10 vertoon en het kortketting, versadigde vetsuuresters verkies.

In die teenwoordigheid van $CaCl_2$, het die N-gemerkte ensiem ‘n halfleeftyd van 1 h by 70ºC and 23 h by 65ºC getoon terwyl die ensiem waarvan die N-terminale merkstuk verwyder is ‘n halfleeftyd van 19 min by 70ºC en 2.7 h by 65ºC getoon het. Die aktiwiteit van die gemerkte ensim sowel as die waarvan die merkstuk verwyder is, is verhoog deur Triton X-100 en CHAPS maar dit is deur SDS en setramied geïnhibeer. Die ensiem was stabiel in die teenwoordigheid van 20% (v/v) van verskillende alkohole. Ca^{2+}, Mg^{2+}, en Mn^{2+} (tot 5 mM) het die aktiwiteit van die N- en C-gemerkte ensieme verhoog maar het die ensiem waarvan die merkstuk verwyder is, matig geïnhibeer. Al drie ensieme is matig deur Zn^{+2} en sterk deur Al^{2+} en Hg^{2+}. geïnhibeer. EDTA het die ensiem geïnhibeer maar TPEN het geen effek getoon nie. Beide die N-gemerkte en die ensiem waarvan die merkstuk verwyder is, het voorkeur getoon vir die *sn*-1,3-posisie met dikaprien isomere as substraat, in lipied-monolaag eksperimente. Die ensieme het nie fosfolipase- of galaktolipase-aktiwiteit getoon nie.

CHAPTER 10

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