Lipolytic activity in *Geobacillus thermoleovorans* GE7: Molecular and proteomic characterization

by

Matsobane Godfrey Tlou

Submitted in fulfilment of the requirements for the degree of

Philosophiae Doctor

In the

Department of Microbial, Biochemical and Food Biotechnology

Faculty of Science

University of the Free State

Bloemfontein

Republic of South Africa

November 2010

Supervisor: Piater LA (Dr)

Co-supervisor: van Heerden E (Prof.)

ACKNOWLEDGEMENTS

Dr. L.A, Piater, You supplied me with a rope of immeasurable length which made it possible for me to “tarzan out of the thickest of forests”, I’m greatful, thank you. Your guidance during and post this study was(is) a blessing to me.

Prof. E van Heerden, you have opened my eyes to a different way of thinking and for that I am very greatful. Thank you for your invaluable contribution to this study amidst your busy schedule.

Thank you to Prof. J Berenguer and all the members at the “Centro de Biología Molecular Severo Ochoa” in Madrid (Spain), for the opportunity to work in their laboratories. It was a life changing experience indeed.

Thank you Armand for all your help with the protein purification studies and analysis of the MS data. It would have been a mission for me to get to this point if it wasn’t for the time we spent trying to understand what was going on with the data.

To Landi, Sandile, Kamini and Nathlee, we remain soldiers of the same struggle through and through. Lets collaborate one of these days.

To all the friends I have gathered in the department over the years. Thank you for your ears, smiles, chats and moral support.

To my parents and my siblings, I’m grateful for the encouragements and emotional support. Its true, trying and tough situations/times make us stronger.

Special thanks to my high school teachers, Mrs Sibeko, Mr Lukhozi and Mrs Bashele, through your encouragements, I realized that the hurdles that appeared to sorround me were nothing but a figment of my imagination.

My sincere gratitude to the BioPAD/UFS Metagenomics Platform, National Research Foundation and the Oppenheimer Memorial Trust for the financial support.

I would not be where I am today if it wasn’t for your grace and unconditionallove. All this is through you my heavenly Father.

TABLE OF CONTENTS

List of Figures

Figure 1.4: Putative roles of microbial extracellular lipases. Growth: lipolysis might provide carbon sources that the microorganism could use for growth; adhesion: the release of free fatty acids due to lipolytic activity could support cell to cell and/or cell to host tissue adhesion; synergism: a lipase might work hand in hand with another enzyme or it might optimize conditions for other enzymes; unspecific hydrolysis: lipases might posses additional phospholipolytic activity; immune system: lipases and their catalytic end products may have an effect on different immune cells and might initiate inflammatory processes; defense: microorganisms that secrete lipolytic enzymes might have a selection advantage by lysing competing microflora (Stehr *et al*., 2003).

Figure 1.6. **A**: Bacterial preprotein secretion. (**a**) Secretion occurs in three distinct statges, targeting (I), translocation (II) and release (III). The preprotein crosses the membrane through the protein translocase (white box). N' symbolizes the new amino terminus of the secretory protein after cleavage of the leader peptide has taken place. (**b**) The targeting chaperones, preprotein ttranslocase and the leader peptidase. The celluar machinery intimately involved in the three stages of protein secretion in *Escherichia coli* is shown. Several house-keeping chaperones (not shown) can also contribute efficient membrane-targeting. SecA and SecYEG compose the core of the preprotein translocase, wherease SecD and SecF are regulatory subunits (Economou, 1998). **B**: Schematic representation of the Sec-type signal peptide characterized by a tripartite structure: a positively charged N-terminal domain (N), a hydrophobic H-domain (H) and a C-domain (C) containing a cleavage site. Moreover, about 60% of the predicted Sec-type signals contain a helix-breaking praline (P) of glycine (G) residues in the H-domain (Ling *et al*., 2007).

Figure 2.1: **A**, Graph showing the relationship between growth, glucose consumption, % dissolved oxygen (%DO), extracellular lipase activity and the subsequent release and utilization of fatty acids. Cultivation was performed in lipase production media, 2.5g/l olive oil and pH 7 (Knoesen, 2004). **B**, SDS-PAGE gel of the GE-7 culture supernanant applied to the Pheny-Toyopearl column in lane 1 (a) and the

Figure 3.6: **A**, PCR detection of the GE-7 small lipase using primers GTL2N-1F and GTL2N-2R. Lane 1 is the negative control, lanes 2, 3 duplicates of the PCR with GE-7 genomic DNA as a template and M is the molecular weight marker. **B**, PCR amplification of the regions up and downstream the ~ 80 bp GE-7 small lipase gene fragment. Lanes 1, 5, PCR with genomic DNA from *G. kaustophilus* HTA 426 (positive control) as a template. Lanes 2,3 and 6,7 PCR with GE-7 genomic DNA as template (experiment). **C**, PCR amplification of the complete small lipase gene using primers designed base on the genes flanking the gene of interest. Lane 1 represents the positive control (*G. kaustophilus* genomic DNA), lane 2 (experiment), lane 3 (negative control) and lane M (molecular weight marker).

Figure 3.7: **A**, A comparison of the nucleotide sequences encoding the GE-7 small lipase and its homolog from *G. kaustophilus* HTA426, highlighted in grey, is the most conserved feature of these genes. **B**, A comparison of the *G. thermocatenulatus* small lipase N-terminal amino acid sequence to the sequences of its homologs from GE-7 and *G. kaustophilus* HTA426. The amino acid residues in highlighted in grey represent the highly conserved N-terminus that appears to be characteristic to this group of proteins. The underlined sequence block and the amino acid residues indicated by the arrow respectively represent the putative signal peptide and peptidase cleavage site.

Figure 3.8: PCR amplification of the GDSL lipase from the genomic DNA of GE-7. Lane 1, PCR product obtained with the GE7 genomic DNA, lane 2 (molecular weight marker) and lane 3 (negative control).

Figure 3.9: A comparison of the GE-7 GDSL lipase to its homologs from selected *Geobacillus* species. The region shaded in yellow represents the conserved motif and residues highlighted in red represent the catalytic triad. The under lined sequences and the amino acids in bold indicated by the arrow respectively represent the putative signal peptide and the peptidase cleavage site.

Figure 3.11: Batch purification of the GE-7 lipase. Lane M represents the molecular weight marker. Lanes 1 and 2 represent the unbound fraction , while lanes 3-5 represent the eluted fractions.

Figure 3.12: **A**, The elution profile obtained for the GE-7 culture supernatant run on butyl-toyopearl resin (decreasing salt gradient). **B**, A magnification of the elution peak observed in elution profile A. **C**, The elution profile obtained using ethanol as the eluent.

Figure 3.13: SDS-PAGE analysis of the pooled fractions corresponding to the above mentioned protein peaks.

Figure 3.14: **A**, Mass spectrum of the tryptic digest of the ~45 kDa GE-7 protein (precusor ions). **B**, Mass spectrum of one of the product ions that were as a result of the fragmentation of one of the precusor ions (996.520 M/Z) circled in red.

Figure 3.15: **A**, Similarity searches with the amino acid sequence candidates that ranked number 1 from all the spectra analysed using Lutefisk. **B**, Alignment of some of the sequence candidates with the *G. thermoleovorans* LipA amino acid sequence.

Figure 3.17: **A**, Total RNA extracted from GE-7 cells harvested at the 20 and 40 hour interval under induced (I) and uninduced (U) conditions. **B**, PCR amplification of the GDSL lipase gene from cDNA synthesized from the RNA extracted as in A. Lane M, represents the molecular weight marker, lane 1, represents the positive control (GE-7 genomic DNA), lanes 2 (20 h) and 3 (40 h), PCR performed on cDNA from RNA extracted under uninduced conditions and lanes 4 (20 h) and 5 (40 h), PCR with cDNA from RNA harvested under induced conditions. **C (a)**, PCR amplification of LipA gene from cDNA synthesized from RNA extracted under uninduced conditions (Lanes 2 and 3, 20 and 40 hour interval, respectively). **C (b)**, PCR amplification of the LipA gene under induced conditions (Lanes 1 and 2, 20 and 40 hour interval, respectively). Lanes 1 and 3 [C(a) and

C(b), respectively], PCR with total RNA that was not reverse transcribed as a template (negative control).

Figure 4.1: **A**, Lipase production profile (■) and the amount of free fatty acid (♦) when GE-7 was cultured in LPM (olive oil). **B**, Lipase production profile (■) and the amount of free fatty acids when GE-7 was cultured (♦) in LPM with olive oil as the sole carbon source. **C**,GE-7 Lipase production profile (■) in LPM supplemented with stearic stearic acid [(40 uM, stearic acid consumption (♦)].

Figure 4.2: **A**, Agarose gel electrophoresis of the PCR amplified *lipA* promoter region from *G. kaustophilus* HTA426 (C, control) and GE-7 (E, experiment). Lane M and –V represent the molecular weight marker and the negative control, respectively. **B**, Alignment of the *lip*A promoter regions from *G. thermoleovorans, G. thermocatenulatus* and *G. kaustophilus*. The sequence in red represents the putative CRE-box consensus sequence. The -10 and -35, respectively represents the putative conserved promoter regions. **C**, Lipase production (■) and free fatty acids (♦) profile observed when GE-7 was cultured in LPM (olive oil) supplemented with additional glucose at the 10 hour interval (indicated by the arrow).

Figure 4.3: Graph showing the relationship between growth (ж), glucose consumption (•), % dissolved oxygen (%DO, ▲), extra cellular lipase activity (■) and the subsequent release and utilization of fatty acids (♦). Cultivation was performed in lipase production media (2.5 g/l olive oil) and pH 6.5.

Figure 4.4: **A**, Total RNA extracted from the GE-7 cells cultured as in section 4.3.4 harvested at the 30, 40, 50 and 60 hour interval. **B**, Agarose gel electrophoresis of the PCR products amplified from the cDNA that was synthesized from total RNA harvested as mentioned above. Lane M represents the molecular weight marker and lane –V, represents the PCR with DNase treated RNA as a template (negative control).

List of Tables

Chapter 1
Literature Review

1.1 General introduction

The genus *Bacillus* is a large and diverse collection of aerobic to facultative anaerobic, rod-shaped and Gram-positive or Gram variable, endospore forming bacteria (Claus and Berkely, 1986). This genus has undergone considerable reclassification as advances in molecular biology have revealed a high phylogenetic heterogeneity (Ash *et al*., 1991). Certain thermophilic, aerobic, spore-forming bacteria with growth optima in the range of 45 to 75ºC were previously classified into the genera *Alicyclobacillus*, *Brevibacillus*, *Aneurinibacillus, Sulfobacillus* and *Thermobacillus* (Wisotzkey *et al*., 1992; Touzel *et al*., 2000). Molecular analysis, however, showed that the majority of such thermophilic bacteria described in literature belong to the genus *Bacilli* genetic groups 1 and 5 (Ash *et al*., 1991; Rainey *et al*., 1994). Subsequently, group 5 isolates were found to be a phenotypically and phylogenetically coherent group of thermophilic bacteria with a high 16S rRNA sequence similarity (98.5 – 99.2%) (Nazina *et al*., 2001). As a consequence, group 5 was reclassified as comprising members of *Geobacillus gen*. *nov*., meaning earth or soil *Bacillus*, with the well known *Geobacillus* (*Bacillus*) *stearothermophilus* being assigned the type strain (Nazina *et al*., 2001).

Thermophilic bacilli, including *Geobacillus*, are widely distributed and have been successfully isolated from all continents where geothermal areas occur (Sharp *et al*., 1992). Geobacilli are also isolated from shallow marine hot springs and from deep-sea hydrothermal vents, with Maugeri *et al*. (2001) recently describing the isolation of three novel halotolerant and thermophilic *Geobacillus* strains from three separate shallow marine vents off the Eolian Islands, Italy. High temperature oil fields have also yielded strains of *Geobacillus* with Nazina *et al*. (2000; 2001) reporting two novel species of *G. subterraneus* and *G. uzenensis*, isolated from the Uzen oilfield in Kazakhstan. In addition, *Geobacillus* species have also been recovered from artificial hot environments such as hot water pipelines, heat exchangers, water treatment plants, burning coal refuse piles and bioremediation biopiles (Maugeri *et al*., 2001). Moreover, some studies have shown that thermophilic aerobic bacilli could be readily isolated in large numbers from a range of soils from geographically dispersed temperate regions in Europe (McMullan *et al*., 2004).

Industrial interest in *Geobacillus* species has arisen from current applications in biotechnological processes (Table 1.1). *Geobacillus* species also have the potential in generate products for industrial uses such as exopolysaccharides (Moriello *et al*., 2003). In addition, two strains of *G. thermoleovorans* have been described as producing large bacteriocins that exhibited a lytic activity on other strains of *G. thermoleovorans* and also a range of bacteria of medical importance including *Salmonella typhimurium* (Novotny and Perry, 1992). A variety of potential environmental biotechnology applications involving *Geobacillus* species have been described, which is unsurprising given the ubiquitous capability of these species to metabolize hydrocarbons (Bustard *et al*., 2002). Moreover, McMullan and co-workers (2004) identified two novel applications for *Geobacillus* species, firstly in metabolizing herbicides and therefore being potential sources of genes for use in agricultural biotechnology, and secondly having the ability to disrupt quorum sensing in certain Gram-negative bacteria.

Table 1.1: A selection of U.S. patents for *Geobacillus* products or processes
(Zeigler, 2001)

Product or Process	Patent No.
α-arabinofuranosidase	US05434071
acetate kinase	US05610045
alpha-amylase	US05824532, US05849549
arabino furanoside	US05491087
biological indicator for sterilization	US05073488, US05223401, US05252484, US05418167
BsrFI restriction endonuclease	US06066487
catalase	US06022721
cellobiose fermentation	US06102690
DNA polymerase	US05747298, US05830714, US05834253, US05874282, US06013451, US06066483, US06100078, US06238905
ethanol production	US05182199
glucose-6-phosphate dehydrogenase	US04331762
liquefying starch	US05756714
maleate dehydrogenase	US04331762
neutral proteases	US06103512
perillyl compounds	US05487988
polynucleotide phosphorylase	US04331762
prenyl diphosphate synthase	US06225096
pyruvate kinase	US04331762
riboflavin glucoside	US06190888
superoxide dismutase	US05772996
xylanase	US05434071
xylosidase	US05489526

1.1.1 *Geobacillus thermoleovorans* GE-7

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. The organism was isolated from fissure water collected approximately 3.1km below ground surface in East

Driefontein Goldmine situated in the Boonton Shales. The *in situ* rock temperature was measured at 45ºC and the fissure water's pH measured 8. It is an aerobic, rod-shaped (Fig. 1.1), gram-positive, spore-forming bacteria which showed high lipase activity and a broad substrate specificity against triacylglycerides ranging from C4 to C18. This isolate was not only able to grow (specific growth rate of $2.5h^{-1}$) on olive oil as the sole carbon source, but also on a variety of other lipid substrates and even emulsifiers (De Flaun *et al.,* 2007).

The ability of GE-7 to grow on the various lipid substrates indicated that the bacterium produces lipases. Moreover, GE-7 was found to display lipase activity higher than that reported for other *Geobacillus* species (Lee *et al*., 1999; Schmidt-Dannert *et al*., 1994). However, the number and the identity of the lipolytic enzymes responsible for the organism's ability to grow on the various lipid substrates is unknown.

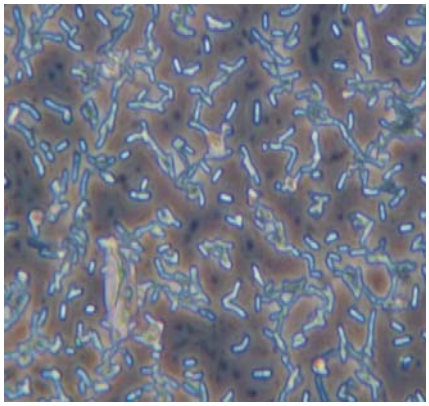


Figure 1.1: Light microscopy photograph of *G. thermoleovorans* during late exponential phase in batch culture [taken from Knoessen, (2003)]

1.2 Lipases

Lipases (E.C. 3.1.1.3) constitute a group of enzymes that catalyze the hydrolysis (and synthesis) of long chain acyl-glycerols at the lipid-water interface. The significant enhancement in the hydrolytic activity of lipases at the lipid-water interface {interfacial activation (Desnuelle, 1961)} distinguishes them from esterases (3.1.1.1) which only act on short chain water-soluble acyl-glycerols. In addition, lipases catalyze the hydrolysis and transesterification of other esters as well as the synthesis of esters and exhibit enantioselective properties. The ability of lipases to perform very specific chemical transformations (biotransformation) has made them very

useful in the food, detergent, cosmetic and pharmaceutical industries (Park *et* al., 2005; Gupta *et* al., 2007; Grbavcic *et* al., 2007; Franken *et al*., 2009).

Lipases have emerged as one of the leading biocatalysts with proven potential for contributing to the multibillion dollar under exploited lipid technology bio-industry and have been used in *in situ* lipid metabolism and *ex situ* multifaceted industrial applications (Joseph *et al*., 2008). The number of available lipases has increased since the 1980s'. This is mainly as a result of the huge achievements made in the cloning and expression of enzymes from microorganisms, as well as the increasing demand for these biocatalysts with novel and specific properties such as substrate specificity, stability, pH and temperature (Bornscheuer *et al*., 2002; Menoncin *et al*., 2009).

1.3 Classification of lipolytic enzymes

Lipolytic enzymes are widely distributed in nature, being found in plants, animals and micro-organisms (Villeneuve *et al.,* 2000). Classification of these enzymes is facilitated by a comparison of the substrate specificities, alignment of their amino acid sequences, comparison of their structural properties or on the bias of their biochemical and physiological properties (Bornscheuer, 2002). A classification scheme for esterases was proposed by Whitaker (1972), based on the specificity of the enzymes for the acid moiety of the substrate, such as the carboxylic ester hydrolases which catalyse the cleavage of the carboxylic acid esters. In addition, to the carboxyl esterases, aryl esterases, acetyl esterases, cholin esterases, cholesterol esterases and lipases also belong to this group of hydrolytic enzymes. Classification of these enzymes by substrate specificity required that the enzymes to be compared be assayed with the same or related substrates under the same reaction conditions (Jaeger *et al*., 1994).

Classification of lipolytic enzymes based on physiological properties is difficult because the physiological functions of many esterases are not clear. This is attributed to the fact that many of them display wide substrate specificity (Jaeger *et al*., 1994), as a result it becomes difficult to assign them a specific physiological function. It has, however, been speculated that several classes of esterases exist: those that have evolved to enable access to carbon sources (Dalrymple *et al*., 1996), those that are involved in catabolic pathways (Ferreira *et al.,* 1993),

some that display biocide detoxification activity (Pohlenz *et al*., 1992) and those that play a pathogenic role (McQueen and Schottel, 1987) etc.

1.3.1 Structure-based classification

Structurally, lipolytic enzymes are classified under the α/β hydrolase fold family originally described by Ollis *et al*., (1992), based on their structural properties (Cygler *et al*., 1993). The α/β hydrolase fold family is a growing superfamily of proteins with a wide range of properties. The α/β-hydrolase fold (Fig. 1.2 A) is characterised by a β-sheet of five to eight strands connected by α-helices to form α/β/α sandwich (Satoh *et al.,* 2002). The members of this family diverged from a common ancestor into a number of hydrolytic enzymes with a wide range of substrate specificity such as acetylcholine esterase (Sussman *et al*., 1991), serine carboxypeptidase (Liao and Remington, 1990) and haloalkane dehalogenase (Franken *et al*., 1991), together with other proteins with no known catalytic function (Hotelier *et al*., 2004). The enzymes catalytic triad residues (serine, histidine and aspartate or glutamate) are found on the loops, of which the one is commonly referred to as the nucleophilic elbow, which contains the active site serine residue, and it is the most conserved feature of the fold (Fig. 1.2 A) with the general conserved consensus sequence (Gly-X-Ser-X-Gly) (Arpigny and Jaeger, 1999). In addition, most lipases consist of a helical lid-like structure covering the active site (Fig. 1.2 B). In these lipases, activation, which is often necessary for the enzymes, involves the movement of the lid. This process is part of the activation of activity, referred to as interfacial activation (Verger, 1997), which takes place above the critical micellar concentration of the substrate. In *Humicola lanuginosa* lipase, the opening of the lid can be described as a hinged bending motion of the helical lid (Brzozowski *et al*., 2000). For other lipases the activation is more complex, e.g., *Candida rugosa* lipase has more than one lid or flap (Grochulski *et al*., 1993). In addition, activation is only present for certain substrate, e.g., *Fusarium solanipisi* cutinase, has a small structural change associated with long chained acyl substrates (Grochulski *et al*., 1993). However, not all lipases belong to the same structural superfamilies, as, e.g, the PLA_2, PLA-D, and some lysophospholipases, having very distant structures compared to the triacylglycerol hydrolases (Svendsen, 2000).

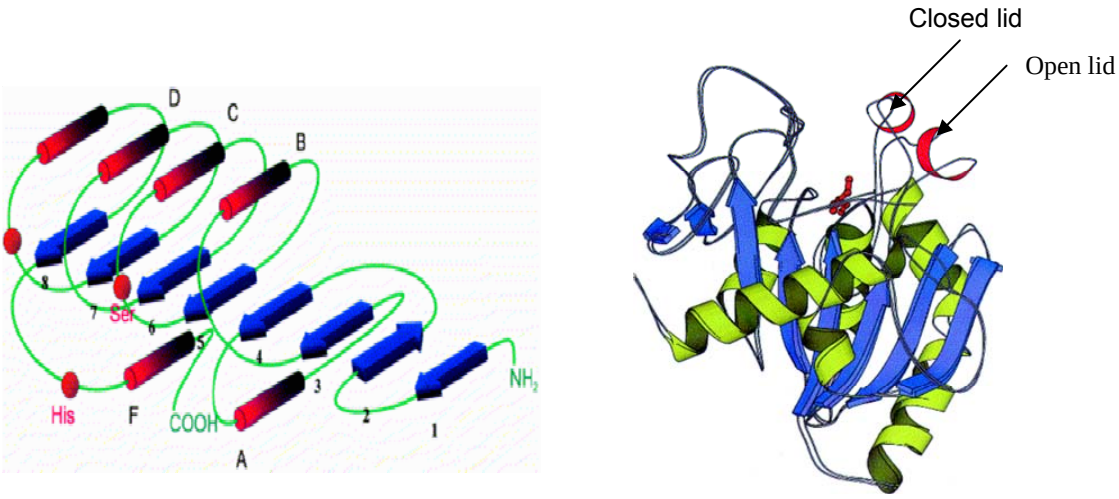


Figure 1.2: **A**, Schematic representation of the α/β-hydrolase fold. β-sheets (1-8) are shown as arrows, α-helices (A-F) as columns. The relative positions of the amino acids of the catalytic triad are indicated as circles (Bornscheuer, 2002). **B,** Lipase overall structure presented using the *Humicola lanuginosa* lipase crystal structure (Lawson *et al*., 1994), and presented in Molscript representation (Kraulis, 1991). The β-sheet is shown in blue, surrounded by some helices (yellow), and the active serine site residue in red sticks and the lid shown in red. Both the open and the close conformation are superimposed (taken from Brzozowski *et al*., 2000).

1.3.2 Sequence based classification

Classification of lipolytic enzymes by sequence comparison is facilitated by the increasing amount of sequence information on the public nucleotide databases. Comparison of amino acid sequence gives an indication of the evolutionary relationships between enzymes from different origins (Arpigny and Jaeger, 1999) and reveals conserved sequence motifs which become characteristic features on which the classifications are based (Fiedler and Simons, 1995; Henikoff *et al*., 1997, Jaeger *et al*., 1999). In some cases, comparing enzyme amino acid sequences complements other forms of classification (i.e. classification by physiological role) by revealing conserved sequence motifs that suggest the ability of an enzyme to carry out a particular physiological function. As an example, the comparison of type B carboxyl esterase from *Peanibacillus* sp. BP-23 (Prim *et al*., 2000) to the phenidipham hydrolase from *Arthrobacter oxydans* P52 (Pohlenz *et al*., 1992), revealed the presence of a β-lactamase signature S-X-X-K (Oefner *et al*., 1990), that suggested that the type B carboxyl esterase could also display biocide detoxification activity. However, high sequence homology cannot be related to enzyme properties such as substrate specificity, stereoselectivity, pH, temperature optima, and in some

cases completely different reactions are catalysed (Pelletier and Altenbuchner, 1995). As an example, a bromoperoxidase from *Streptomyces aureofaciens* (Hetch *et al*., 1994) shares 55% sequence identity to a carboxyl esterase from *Pseudomonas fluorescens* (Pelletier and Altenbuchner, 1995) but they share very low substrate specificity.

1.3.2.1 *Geobacillus* lipolytic enzymes

In 1999, Arpigny and Jaeger devised a classification system based on the amino acid sequence similarities and biochemical properties, grouping bacterial lipolytic enzymes into 8 families. The bacterial true lipases (Family I) were subdivided into six subfamilies, which were further expanded in 2002 by Jaeger and Eggert to seven subfamilies. Subfamilies I.1, I.2 and I.3 contain the true lipases from Gram-negative bacteria with the Gram-positive bacteria lipases divided into subfamilies I.4, I.5, and I.6. Subfamily I.4 comprises *Bacillus* lipases known to have in common that an alanine residue replaces the first glycine in the conserved penta-peptide: Ala-Xaa-Ser-Xaa-Gly.

Schmidt-Dannert (1994) and Kim *et al*., (1998) respectively reported on the production by *G. thermocatenulas* and *G. stearothermophilus* of a lipase with a conserved pentapeptide similar to that reported for subfamily I.4 enzymes. Albeit the similarities in the conserved motif, this group of enzymes which was placed under subfamily I.5 was found to be larger (~ 45 kDa) compared to their mesophilic counterparts (~ 20 kDa, subfamily I.4) and share very low sequence similarity (~ 15 %). However, intra-subfamily I.5 sequence comparison revealed that enzymes comprising this subfamily displayed very high sequence similarity (~ 90%, Fig 1.3 A). The high sequence identity facilitated the cloning of the gene encoding this lipase from related *Geobacillus* species. As a result, this subfamily has expanded over the years with the addition of lipase encoding genes cloned from *G. zalihae* (Rahman *et al*., 2007), *G. thermoleovorans* ID-1 (Lee *et al*., 2001), *G. thermoleovorans* GE-7 (Barnard, 2005), and *G. kaustophilus* HTA426 (Takami *et al*., 2004).

Ewis and co-workers (2004) reported on the molecular cloning and expression of a thermostable esterase from *G. stearothermophilus* (Est50), which has the typical lipase motif G-X-Ser-X-G. The *G. kaustophilus* HTA 426 genome sequencing project (Takami *et al*., 2004), revealed that a homolog to Est50 was present in the genome of this organism. This esterase is classified under the Family VII of bacterial lipolytic enzymes (~ 55 kDa in size) which share

significant homology to eukaryotic actetylcholine esterases and intestine or liver carboxyl-esterases (e.g. pig liver esterase) (Jaeger *et al*., 1999). This family is comprised of biotechnologically significant carboxyl-esterases such as ofloxacin hydrolyzing esterase from *Bacillus niacini*, *p*-nitrobenzyl esterase from *Arthobacer oxydans* active against phenylcarbamide herbicides. Alignment of the amino acids sequences encoding selected members of this family revealed four conserved sequence blocks characteristic to this family (Nthangeni *et al*., 2005). In 2005, Nthangeni and co-workers reported on the use of the conserved blocks as templates for designing primers for the PCR detection of members of this family from *Bacillus* genomes.

Furthermore, genome sequencing projects by Takami *et al*., (2004) and Feng *et al*., (2007) on *G. kaustophilus* HTA 426 and *G. thermodenifricans* NG80-2 respectively, revealed by sequence annotation that these two species harbored, on their genomes, a lipase (~ 25 kDa) with a distinct GDSL sequence motif [characteristic to lipases comprising Family II (Arpigny and Jaeger, 1999)] different from the GxSerxG found in many lipases. Unlike most common lipases, GDSL enzymes do not have the so-called nucleophile elbow (Akoh *et al*., 2004). Studies have shown that GDSL hydrolases have a flexible active site that appears to change conformation in the presence and binding of different substrates, much like the induced fit mechanism proposed by Koshland (1958). Some of the GDSL enzymes have thioesterase, protease, arylesterase and lysophospholipase activity, yet they appear to be the same protein with similar molecular weight (~ 22 – 60 kDa for most esterases), although some have multiple glycosylation sites with higher apparent molecular weight (Akoh *et al*., 2004). These enzymes have four consensus sequence blocks (I-V) and four invariant catalytic residues Ser, Gly, Asn, and His in blocks I, II, III, and V, respectively (Fig. 1.3 B). Each of the four residues plays a key role in the catalytic function of the enzyme. The catalytic Ser in block I serves as the nucleophile and a proton donor to the oxyanion hole. The Gly residue in block II and the Asn in block III serve as two other proton donors to the oxyanion hole. The histidine residue in block V serves as a base to make active site Ser more nucleophilic by deprotonating the hydroxyl group (Molgaard *et al*., 2000, Li *et al*., 2000). Another feature in block V is the presence of Asp located at the third amino acid preceding His (i.e., DxxH serves as the third member of the catalytic triad). As a result, the GDSL family was further classified as the SGNH-hydrolase. These enzymes have little homology to true lipases. Another important differentiating feature of the GDSL subfamily of lipolytic enzymes is that the serine containing motif is located closer to the N-terminus unlike other lipases were the G-X-S-X-G motif is near the center (Akoh *et al*., 2004).

In 1994, Schmidt-Dannert and co-workers reported on the purification of the smallest lipase (16 kDa) known to date from *G. thermocatenulatus*. This lipase displayed biochemical properties characteristic to lipases from thermophilic *Bacillus* species (optimally active at elevated temperatures and alkaline pH) (Schmidt-Dannert *et al*., 1994). These results were supported by data reported by Lee *et al*., (2001) and Castro-Ochoa *et al*., (2005) who respectively reported on the purification of an 18 and 11 kDa lipolytic enzyme from related *G. thermoleovorans* strains (ID1 and CCR11). Both Schmidt-Dannert and Lee published identical N-terminal amino acid sequences for the 16 and 18 kDa lipase, respectively (Fig 1.3 C). However, sequence analysis and similarity searches revealed that the enzyme purified by both authors did not display any significant identity to known *Bacillus* lipases (Lee *et al*., 2001). Moreover, further similarity searches revealed that the N-terminal sequence of the protein displayed very high sequence identity to a hypothetically conserved protein from *G. kaustophilus* HTA426 (Takami *et al*., 2004), which did not contain conserved sequence blocks characteristic to most known lipases (this study). As a result, this lipolytic enzyme is yet to be classified under any known family of bacterial lipolytic enzymes. Moreover, these observations could suggest a novel family of bacterial lipases.

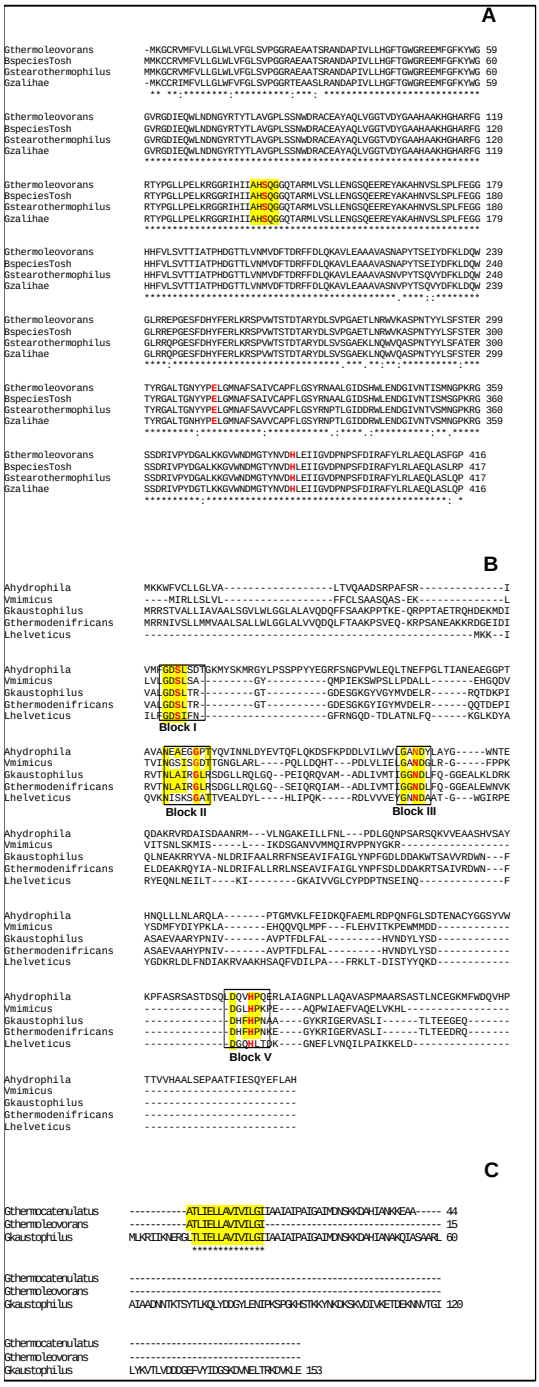


Figure 1.3: **A**, Alignment of amino acid sequences encoding selected members of Subfamily I.5. The conserved pentapeptide is shaded in yellow while the residues making up the catalytic residues are highlighted in red. **B**, Alignment of amino acid sequences enconding selected members of the GDSL lipase subfamily (SGNH-hydrolase family). Four consensus blocks I, II, III and V are boxed in black. Conserved amino acid residues Ser (S), Gly (G), Asn (N) and His (H) in blocks I, II, III and V, respectively are highlighted in red. **C**, Alignment of

amino acid sequences encoding the smallest *Geobacillus* lipase known to date. A sequence block that appears to be conserved in these proteins is shaded in yellow.

1.4 Physiological roles of microbial lipases

Most lipase producers harbor more than one lipase encoding gene on their genome and, in most cases, the genes are differentially expressed. The conditions under which each lipase gene is expressed could be a possible indication of the gene product's primary role. However, due to the broad substrate specificity characteristic of lipases it becomes difficult to assign these enzymes to one specific role.

The most prominent role of extracellular lipases from a microorganism is the digestion of lipids for nutrient acquisition (Fig. 1.4). These enzymes might help microorganisms to grow in a carbohydrate-restricted area or environment where lipids are the sole carbon source (Stehr *et al*., 2003). However, in pathogenic microorganisms, lipases may function to assist the cells in adhering to host cells and/or neighboring cells (Fig. 1.4). For *Staphylococcus aureus* it has been postulated that its lipase enhances adhesion by degrading host surface molecules and thereby liberating new receptors. Additionally, free fatty acids might increase unspecific hydrophobic interactions, as it is assumed for *Propionibacterium acnes* (Miskin *et al*., 1997). In some cases, the development of infection has been attributed to synergism, where a lipase might act hand in hand with another enzyme or it might optimize condition for other enzymes (Fig 1.4). König and co-workers (1996) have shown that phospholipases and lipases may act in concert. The combined action of lipases and phospholipases may occur also during *C. albicans* infection, as both activities have been detected in this fungus.

In microorganisms that produce lipase isozymes, these enzymes may perform more general functions by acting as survival factors, optimizing conditions for other enzymes (Stehr *et al*., 2003). The use of a combination of various inhibitors and the generation of multi-gene knock-out strains will be helpful in exploring this hypothesis. Besides the lipolytic activity, microbial lipases might have ancillary enzyme activity. In *Staphylococcus warneri*, it has been shown that lipase 2 has additional phospholipolytic activity (van Kampen *et al*., 2001) which adds another putative pathogenic trait to lipases. Through the cleavage of phospholipids, microorganisms may have

the ability to actively degrade host tissue and lyse cells, since phospholipids are major components of cell membranes.

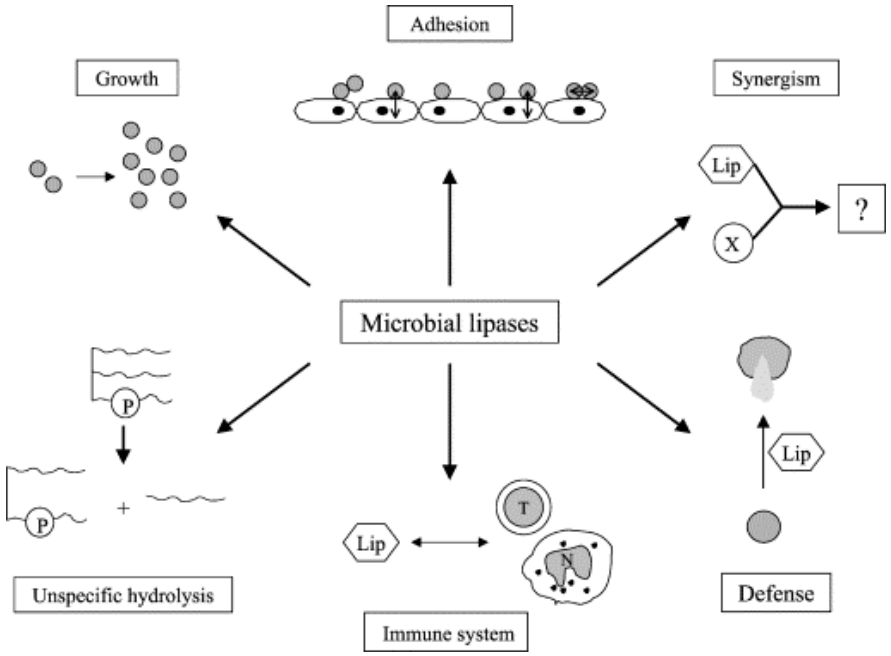


Figure 1.4: Putative roles of microbial extracellular lipases. Growth: lipolysis might provide carbon sources that the microorganism could use for growth; adhesion: the release of free fatty acids due to lipolytic activity could support cell to cell and/or cell to host tissue adhesion; synergism: a lipase might work hand in hand with another enzyme or it might optimize conditions for other enzymes; unspecific hydrolysis: lipases might posses additional phospholipolytic activity; immune system: lipases and their catalytic end products may have an effect on different immune cells and might initiate inflammatory processes; defense: microorganisms that secrete lipolytic enzymes might have a selection advantage by lysing competing microflora (Stehr *et al.*, 2003).

1.5 Growth and lipase production in bacteria

Microbial lipases are mostly extracellular and their production is greatly influenced by medium composition besides physicochemical factors such as temperature, pH and dissolved oxygen. The major factor for the expression of lipase activity has always been reported as the carbon source since most lipases are inducible enzymes. These enzymes are generally produced in the presence of a lipid such as oil or any other inducer such as triacylglycerols, fatty acids, hydrolysable esters, Tweens, bile salts and glycerol (Gupta *et al.*, 2004; Sharma *et al.*,

2001). Lipidic carbon sources seem to be essential for obtaining high lipase yields. However, nitrogen sources and essential micronutriens should also be carefully considered for growth and production optimization.

1.5.1 Enhancing lipase production

Literature suggests that every microorganism capable of the production and secretion of lipases requires a very distinct set of environmental conditions for optimum production. Lipase production has been shown to be directly affected by cultivation temperature, pH, agitation and oxygenation. Furthermore, nitrogen and carbon sources, their ratios, the inducer type and salt concentration all had a notable effect on production. These influences of culture conditions and other factors on lipase production have been studied extensively (Tan *et al*., 1984; Nesbit *et al*., 1993; Dharmsthiti and Luchai, 1999). Since the aim is to get lipase overproduction, the concentration of each compound that constitutes a cultivation media has to be optimized. This is usually a time consuming procedure. The classical procedure of changing one variable at a time, while keeping others constant, was found to be inefficient since it does not explain the interaction effects among variables and their effects on the fermentation process (Rodrigues and Iemma, 2005). An efficient and widely used approach is the application of Plackett-Burman (PB) designs that allow efficient screening of key variables for further optimization in a rational way (Rodrigues and Iemma, 2005).

An alkaline lipase from *Bacillus multivorans* was produced after 15 h of cultivation in a 14-L bioreactor. The medium optimization was carried out to lead to an increase of 12-fold in lipase production. Initially, the effect of nine factors, namely, concentrations of glucose, dextran, olive oil, NH_4Cl, trace metals, K_2HPO_4, $MgCl_2$, and $CaCl_2$ and inoculum density were studied using the PB experimental designs. These components were varied in the basal medium containing olive oil as an inducer and yeast extract as a nitrogen source. After the screening of the most significant factors by the PB design, the optimization was carried out in terms of the olive oil, inoculum density and fermentation time. The optimal medium composition for the lipase production was shown to be (Percentage *w*/*v*): glucose 0.1, olive oil 3.0, NH_4Cl 0.5, yeast extract 0.36, K_2HPO_4 0.1, $MgCl_2$ 0.01 and $CaCl_2$ 0.4 mM (Gupta *et al*., 2007).

Takaç and Murai (2008) improved the lipase production by *B. subtilis* using different concentrations of lipidic carbon sources such as vegetable oils, fatty acids, and triglycerides. One percent of sesame oil afforded the highest activity with 80% and 98% enhancement with respect to 1% concentrations of linoleic acid and triolein as the favored fatty acid and triglyceride, respectively. The same authors tested the use of glucose as carbon source and verified that it presented a positive effect on lipase production. Abada (2008) produced lipase from *B. stearothermophilus* AB-1. The authors observed that the use of xylose, tryptophan, alanine, phenylalanine, and potassium nitrate as supplements led to the highest lipase production.

Lin *et al*., (2006) investigated the influence of different culture conditions, temperature, pH, carbon, nitrogen, mineral sources and vitamins on the production of lipase by *Antrodia cinnamomea* in submerged cultures (shake flask cultivation). Nine carbon sources, 14 nitrogen sources, six mineral sources, and vitamins were investigated. The authors found that 5% glycerol, 0.5% sodium nitrate, and 0.1% thiamine provided the best results. The lipase production reached 54 U ml^{-1}.

The above-mentioned studies suggest that although a lipid substrate is in most cases an important factor in the induction of lipase production in most microorganisms, it is however, not the only factor required for optimum lipase production. Moreover, it appears that media constituents and culture conditions that promote lipase production are organism specific. Hence, a consensus on factors that enhance microbial lipase over-production is yet to be reached.

1.6 Lipase gene regulation in bacteria

The production of lipases by microorganisms has been studied preferentially from a biotechnological point of view. With respect to maximum lipase production, much effort has been put into studies describing several kinds of growth media and culture conditions which are suitable for induction or improvement of lipase production (Jaeger *et al*., 1994). In addition, chemical agents such as certain lipids (Tanaka *et al*., 1999) polysaccharides or detergents (Schulte *et al*., 1982) can increase the production levels of lipases. However, the molecular mechanisms regulating lipase gene expression remain widely unknown. In general, the release of an enzymatically active lipase into the extracellular medium requires a concerted action of

various cellular processes, starting at the level of transcription of the structural gene, proceeding with the translation of the particular mRNA and the subsequent translocation of the protein through both the inner and the outer membrane. Thus, regulation can generally affect each step involved in this maturation process ending with the secreted and enzymatically active enzyme (Rosenau and Jaeger, 2000).

According to the research on the regulation of lipase encoded genes in *Candia rugosa* by Lotti *et al*. (1998) it has been hypothesized that the lipase genes maybe grouped into two classes, encoding for a constitutive and an inducible form respectively. These two sets of genes are controlled through different regulatory pathways because of the inhibitory effect of glucose on the lipase production of cells grown in olive oil as inducer. The synthesis of the inducible enzymes is inhibited at the level of transcription by the addition of glucose and conversely, oleic acid (major product of lipid hydrolysis) appears to hinder the synthesis of the constitutive lipase.

Enzymes involved in the metabolism of complex carbon and energy source are unnecessary under conditions of abundant, readily metabolized alternative such as glucose. The glucose-mediated reduction in the rates of transcription of operons that encode enzymes involved in the catabolic pathways (catabolite repression) has been found to be a general feature for degradative enzymes in *Bacillus* while, other authors have suggested that it could be global regulatory mechanism for Gram-positive bacteria (Hueck and Hillen, 1995). Catabolite repression in bacteria is regulated by *cis*-acting elements, the so-called catabolic responsive elements (CRE-box) and the DNA binding proteins Ccpa (catabolite control protein) (Ludwig and Stülke, 2001). Weickert and Chambliss (1995) reported that, in *Bacillus subtilis*, genes encoding several degradative enzymes (including *amyE* and β-glucanase genes) were regulated through the catabolite repression mechanism. However, Eggert *et al*. (2003) identified a CRE-box in the *lip*A gene expressed by *Bacillus subtilis* but expression of the *lip*A gene proved to be independent of the glucose in the culture media.

Regulation of lipase production by fatty acids produced by the hydrolysis of lipase substrates such as olive oil and triolein have been reported in literature (Jaeger *et al*., 1994; Rosenau and Jaeger 2000). This mode of regulation (often referred to as feedback inhibition) is not unique to only bacterial lipases but prevalent in the regulation of lipases from various sources including mammalian tissues (Sessler and Ntambi, 2008). In 1996, Kok and co-workers who were investigating the effects of various physiological factors on lipase production in *Acinetobacter*

calcoaceticus reported that fatty acids produced as a result of triolein hydrolysis, strongly repressed lipase production. These findings were explained by proposing the existence of an as yet unidentified regulatory protein which is believed to repress lipase production upon binding of a fatty acid (Kok *et al*., 1996). However, the exact regulation mechanism is as yet not well understood.

It is known that lipases from a number of *Pseudomonas and Burkholderia* are expressed from a unique type of operon, where the structural gene for lipase (*lipA)* is followed by a gene coding for a helper protein (*lipB*). In the past, this lipase-helper-protein (LipB) has been investigated for its suspected role in regulation of gene expression (Frenken *et al*., 1993). It is now generally accepted that the lipase helper protein plays a role in periplasmic lipase folding and not in transcriptional regulation (Jaeger *et al*., 1996). However, a similar type of operon (*lipAR*) has been identified in some *Streptomyces* species (Servin-Gonzalez *et al*., 1997; Valdez *et al*., 1999). Unlike the lipase helper protein, the product of *lipR* mediates the expression of *lipA.*

Lipases synthesized by pathogenic bacteria play an important role as virulence factors (Stehr *et al*., 2003). A few examples are known where regulation of lipase gene expression has been studied. Many Gram-negative bacteria produce extracellular signaling molecules called autoinducers, which belong to the class acylated homoserine lactones. They can bind to the transcription regulator proteins (name 'R' proteins) which in turn induce or cease to repress specific target genes. The insect pathogen *Xhenorhabdus nematophilus* that exists in the intestines of the parasitic nematode *Steinernema carpocapsae* produces a lipase. Its biosynthesis is stimulated by *N-β*-hydroxybutanoyl homoserine lactone (HBHL) known as the autoinducer of the luminescent system of *Vibrio harveyi* (Dunphy *et al*., 1997) suggesting a quorum sensing type of lipase regulation. The term quorum sensing describes the control of gene expression in response to cell density (Rosenau and Jaeger, 2000). In *Pseudomonas aeruginosa*, at least two different LuxR/I-homologous systems were described (Pesci *et al*., 1999). The transcriptional activator RhlR (also named VsmR) using the autoinducer N-butyryl-homoserine lactone (BHL) synthesized by the corresponding synthetase RhlI (VsmI), controls the cell density dependent production of *P. aeruginonasa* rhamnolipid (Ochsner *et al.*, 1994) as well as several extracellular lipids. Studies with a *lipA*::*lacZ* reporter gene fusion revealed that the expression of the lipase gene is regulated by the Rh1R/I system, although it seems that the Rh1R system does not activate *lipA* expression directly (Ochsner and Reisner, 1995). For *B. cepacia* and *P. aeruginosa,* which both cause severe pulmonary infections in cystic fibrosis

patients; an autoinducer-mediated 'interspecies communication' was suggested to regulate the expression of genes encoding potential virulence factors (McKenney *et al*., 1995). Such a coordinated regulation of gene expression for the lipase and other extracellular virulence factors by quorum sensing would facilitate pathogenic bacteria of different species to invade and persist in a given host.

1.7 Secretion-dependent lipase regulation in bacteria

All living cells are subdivided into distinct compartments, most of which are confined by membranes. As protein synthesis takes place primarily in the cytoplasm, proteins that are functional in other compartments need to be transported from the cytoplasm to their destination compartment. All known bacterial lipases are extracellular enzymes requiring their translocation through the cytoplasmic membrane in Gram-positive bacteria and, in addition through the periplasm and the outer membrane in Gram-negative bacteria.

At least five distinct pathways for protein transport have been identified in bacteria. In *Bacillus subtilis*, the majority of ~ 300 potentially secretory proteins appear to be translocated by the "Sec" pathway for protein secretion (Antelmann *et al*., 2001; Jongbloed *et al*., 2002). Typical proteins of this type include degradative enzymes (e.g. carbohydrases, DNAses, lipases, phosphatases, and RNAses), proteins involved in cell wall biogenesis, substrate binding proteins and even pheromones involved in cell population density for onset of developmental processes such as natural competence and sporulation. Other pathways for protein transport, such as the *t*win-*a*rginine *t*ranslocation "Tat" pathway, a pseudopilin export (Com) pathway involved in natural *com*petence development , phage like holins, and certain *A*TP-*b*inding *c*assette (ABC transporters, are "special purpose" transporters, limited to the export of a small number of specific proteins (Sarvas *et al*., 2003).

1.7.1 The Sec pathway

Most of the known lipases from Gram-positive and Gram-negative bacteria posses N-terminal signal peptides (Fig. 1.5B) qualifying them as potential substrates for Sec-homologous protein exporters. As a result, the Sec dependent pathway is a major mechanism of secretion of lipases into the extracytoplasmic space (Rosenau and Jaeger, 2000). The Sec pathway can be

divided into three distinct but sequential and interdependent stages: targeting, translocation and release (Fig. 1.5Aa).

The targeting stage requires a cytosolic chaperone, SecB (Fig. 1.5Ab). SecB keeps newly synthesized proteins in the 'translocation-competent state' and targets them to the SecA subunit of the membrane bound translocase (Driesden *et al*., 1998). The association between SecB and model proteins is readily reversible and diffusion limited (Fekkes *et al*., 1995). Both the folding and the aggregation of the proteins can be very rapid; thus the rate at which a chaperone binds must be high, if the chaperone is to intercept those processes, which are essentially irreversible and facilitate proper localization (Randall and Hardy, 1995). SecB targets the preprotein by associating to the membrane-embedded SecYEG bound to the SecA (Fig. 1.5Ab) to stimulate the interaction between SecA and SecB. The SecB-SecA interaction causes the release of mature domains of the preprotein with SecB. The preprotein is thus transferred from SecB to SecA by synchronous 'hand-shake' mechanism (Sariya and Hortaçsu, 2004). Binding of the SecB to the SecA triggers the event and the tight binding of the SecB to the carboxy terminus of SecA dissociates the preprotein from its SecB bound state (Sariya and Hortaçsu, 2004). An important implication of this mechanism is that SecB bound to the membrane will be able to accept new proteins destined for translocation in its bound state to SecA. Only after the initiation of translocation by binding of ATP to SecA, is SecB released from the membrane to bind a new protein in the cytosol (Fekkes *et al*., 1997).

The translocation stage of the secretion reaction takes place wholly within the membrane (Economou, 1999). The reaction at this stage is catalyzed by the translocase that comprises a dissociable SecA and two integral heterotrimeric protein complexes, SecYEG and SecDFYajc (Fig. 1.5Ab) (Wickner and Leonard, 1996). SecA is an ATPase that exists both in the cytosolic and membrane bound forms (den Blaauwen and Driessen, 1996). The SecYGE-bound forms serve as a receptor for SecB binding (Hartl *et al*., 1990) whereas the cytosolic form functions as a translational repressor for its own synthesis (Wickner and Leonard, 1996).

The Sec pathway uses two kinds of energy to drive the passage through the membrane, the universal energy unit ATP and the proton motive force (PMF). There is an absolute requirement for ATP to initiate the translocation reaction, whereas the PMF may affect the rate only after the preprotein is halfway through the membrane. The translocation reaction is initiated by the binding of ATP to the translocase (den Blaauwen and Driessen, 1996). This brings

conformational changes to SecA by which it moves into the membrane with a 20-30 amino acid segment of the preprotein. When ATP is hydrolyzed, SecA changes its conformation to move back out of the membrane releasing the bound protein (Schiebel *et al*., 1991). Multiple cycles of these steps allows the translocation of the entire preprotein.

1.7.2 Post-translocational folding

While, the Tat pathway has the potential to transport fully folded proteins (Berks *et al.,* 2003), the Sec pathway exclusively transports proteins in an unfolded or loosely folded state (de Keyzer *et al*., 2003). Upon exit from the channel of the Sec translocase, proteins must fold rapidly into their native conformation. This is not only required for the activity of the exported proteins but also for their stability, since the membrane-cell wall interface is a highly proteolytic environment that monitors and maintains the quality of secreted proteins (Bolhuis *et al*., 1999; Meens *et al*., 1997). As the vast majority of secretory proteins are exported from the cytoplasm by the Sec-dependent translocase, the mechanism of post-translocational protein folding has attracted considerable interest. The analysis of these mechanisms is of scientific relevance because of the unique characteristics of Gram-positive cell envelopes that are not found in their Gram-negative counterparts. Moreover, translocation and post-translocation folding appear to be amongst the most important regulating factors in the production of functional extracellular enzymes by the cell.

Various general or protein specific chaperones and foldases have been found to be involved in the folding of periplasmic or outer membrane folding in bacteria. Moreover, some lipases from Gram-negative bacteria were found to be expressed from an operon where the structural lipase gene (*lip*A) is followed by a gene (*lip*B) encoding a helper protein that facilitates its post-translocational folding (Frenken *et al*., 1993). It is interesting, especially in the context of the unique microenvironment at the cytoplasmic membrane-wall interface, that relatively few proteins have been shown to assist the post-translocational folding of Gram-positive bacterial secretory proteins (Sarvas *et al*., 2004). One such protein, found ubiquitously in all Gram-positive species but not at all in Gram-negative species, is PrsA (Sarvas *et al*., 2004).

The PrsA from *B. subtilis* is a membrane-associated lipoprotein of 270 amino acid residues (Kontinen and Sarvas, 1993). It is a hydrophilic, protein with no membrane spanning regions and a calculated p*I* of about 10 (Kontinen and Sarvas, 1993). It is accessible to trypsin in the

protoplasts (Leskela *et al*., 1999) and anchored to the outer surface of the cytoplasmic membrane by the two fatty acid moieties attached to its N-terminal cysteine, in a manner typical of bacterial lipoproteins.

PrsA is an abundant protein with about 20,000 molecules per cell and thus in obviously high excess over the number of translocase complexes (Vitikainen *et al*., 2001). The biological activity of PrsA was initially addressed through mutation studies (Kontinen and Sarvas, 1988; Jacobs *et al*., 1993). Point mutations in the PrsA gene (Kontinen and Sarvas, 1988), or depletion of the protein by placing the *prsA* gene under an inducible promoter (Vitikainen *et al*., 2001), resulted in the decreased secretion of a heterologous model protein, the α-amylase (AmyQ) of *B. amyloliquefaciens* expressed at high levels in *B. subtilis.* There was a linear relationship between the rate of secretion and the cellular levels of PrsA (Vitikanen *et al*., 2001). More recent proteomic studies have demonstrated decreased amounts of several endogenous exoproteins in the growth media of bacteria partially depleted of the PrsA protein (Vitikanen *et al*., 2004). In contrast, the opposite effect was observed when PrsA was overproduced by expressing the *prsA* gene from a multi-copy plasmid or a strong inducible promoter; dramatic increases in the production of heterologous model proteins such as AmyQ or SubC protease of *B. lichenoformis* indicated that the level of PrsA is a potential bottleneck for secretion (Kontinen and Sarvas, 1993).

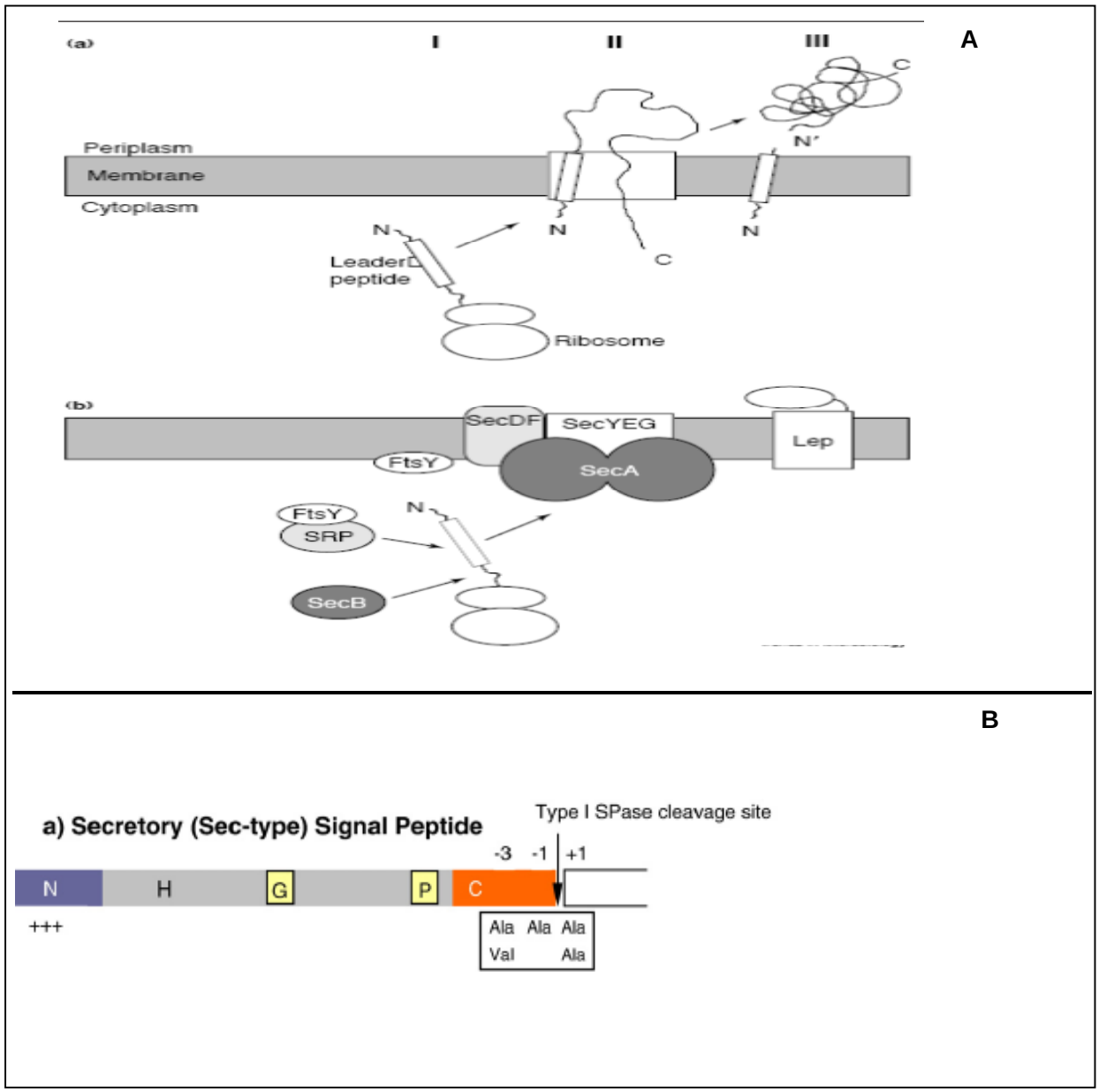


Figure 1.6.

A: Bacterial preprotein secretion. (**a**) Secretion occurs in three distinct statges, targeting (I), translocation (II) and release (III). The preprotein crosses the membrane through the protein translocase (white box). N' symbolizes the new amino terminus of the secretory protein after cleavage of the leader peptide has taken place. (**b**) The targeting chaperones, preprotein ttranslocase and the leader peptidase. The celluar machinery intimately involved in the three stages of protein secretion in *Escherichia coli* is shown. Several house-keeping chaperones (not shown) can also contribute efficient membrane-targeting. SecA and SecYEG compose the core of the preprotein translocase, wherease SecD and SecF are regulatory subunits (Economou, 1998). **B**: Schematic representation of the Sec-type signal peptide characterized by a tripartite structure: a positively charged

N-terminal domain (N), a hydrophobic H-domain (H) and a C-domain (C) containing a cleavage site. Moreover, about 60% of the predicted Sec-type signals contain a helix-breaking praline (P) of glycine (G) residues in the H-domain (Ling *et al*., 2007).

1.8 Conclusions

The number of patents on *Geobacillus* products and processes is indicative of the current and the potential importance of this genus in the biotechnology industry. Lipolytic enzymes are amongst some of the most valuable products from this genus. The importance of these enzymes stems from the added advantage (thermostability) they have over their mesophilic counterparts, in addition to properties such as enantioselectivity and stability in organic solvents which makes them suitable for industrial applications.

It is clear from the genome sequencing projects of related *Geobacillus* species that these organisms harbor more than one lipase/esterase encoding gene on their genomes. These genes are usually differentially expressed by the cell. Most true lipases are produced by the cell when the organism is grown on a lipid substrate; in this case, the lipase affords the bacterium the ability to use the lipid as a carbon source. However, these lipases have the potential to catalyze other reactions due to broad substrate specificity. Moreover, GDSL lipases (often referred to as rhamnogalacturonan esterases, thioesterases etc.) have been identified in *Geobacilli.* Some of these enzymes have been found to display protease, arylesterase and lysophospholipase activity in addition to lipolysis (Akoh *et al*., 2004) which makes it difficult to predict the culture conditions that would promote their production by the cell.

It is reported in the literature that lipolytic enzymes belong to α/β-hydrolase fold superfamily, members of which are believed to have evolved from one common ancestor into a number of hydrolytic enzymes. Apart from the structural similarities, most members of this family share in common the catalytic triad residues (Ser, His, Asp or Glu) with the active site serine being the most conserved feature of the fold. As a result, there is a possibility of an overlap in the reaction that the different members of this family catalyze. As an example, Nakajima *et al*. (2005) reported on an isoenzyme of earthworm serine proteases that displayed hydrolytic activity on triacylglycerols. Since they could not identify any other lipolytic proteins in the earthworm cells.

They suggested that the isozyme might act on the hydrolysis of triacylglycerols as well as protein decomposition.

G. thermoleovorans GE-7 was found to display high lipase activity. Given that GE-7 could be producing more than one enzyme capable of hydrolyzing triacylglycerols, relating the lipase activity back to a particular enzyme becomes challenging. This observation necessitates that the lipolytic enzymes responsible for the organism's activity profile be identified and the activity characterized using both biochemical and molecular techniques.

Chapter 2

Introduction to the present study

2.1 Introduction

A thermophilic bacterium, *G. thermoleovorans* ID-1, isolated from a hot-spring in Indonesia, showed extracellular lipase activity and high growth rates on lipid substrates at elevated temperatures (Lee *et al*., 1999). On olive oil (1.5% w/v), the isolate ID-1 grew very rapidly at 65˚C with its specific growth rate (2.50 h^{-1}) while its lipase activity reached 520 UI^{-1} during the late exponential phase and then decreased (Lee *et al*., 1999). An inducible extracellular lipase with a molecular mass of ~45 kDa was purified from this strain. Moreover, growth of the organism in media containing a variety of lipid substrates led to a hypothesis by Lee and Co-workers (1999) that the bacterium could be expressing different lipases in addition to the inducible lipase. However, attempts by the authors to identify different lipases from the culture supernatant were unsuccessful. Furthermore, Lee and co-workers (2001) found that ID-1 could produce a thermostable lipase in the absence of a lipid substrate as an inducer. As a result they purified and sequenced a constitutively expressed extracellular lipase (~16 kDa) from the ID-1 culture supernatant.

The above-mentioned results are in agreement with the data reported by Schmidt-Dannert *et al*., (1994) and Castro-Ochoa *et al*., (2005) who respectively purified a small lipase (less than 20 kDa) from *G. thermocatenulatus* and *G. thermoleovorans* CCR11 in addition to the large lipase (~43 kDa). However, supporting sequence information in this regard is only available for the ~18 kDa lipase (Schmidt-Dannert *et al*., 1994). The lipase production profile of the above-mentioned and numerous other *Geobacillus* species (when grown on media containing a lipid substrate) is characterized by a broad lipase production peak that stretches from the late exponential to the stationary phase. This could be due to the fact that some microorganisms prefer easily utilizable carbon sources (e.g. glucose) over complex carbon sources such as olive oil (Weickert and Chambliss 1995; Banchio and Gramajo, 1997; Ludwig and Stülke, 2001). As a result, the depletion of easily utilizable carbon sources triggers the production of other enzymes such as lipases that afford the microorganisms the ability to utilize complex carbon sources such as olive oil. However, in some cases lipases can be produced independent of the inducer and in some

cases factors such as the age of the culture can influence lipase production (Lee *et al*., 2001; Nawani and Kaur, 2007).

While studying the growth and lipase production by *G. thermoleovorans* GE-7, Knoesen (2004) observed that the thermophilic isolate from a South African gold mine displayed a peculiar lipase production profile (Fig. 2.1 A). The lipase production profile was characterized by two activity peaks during the stationary phase of growth (under shake flask and batch culture conditions). The activity corresponding to the first peak appeared to be present from the initial stages of growth and was coupled to an accumulation of the free fatty acids (FFA) in the culture media which was as a result of olive oil hydrolysis by the lipase. The drop in the FFA concentration (the uptake) in the culture media preceded the drop in the lipase activity corresponding to the first peak (Fig. 2.1 A). The uptake of the free fatty acids and the drop in activity corresponding to the first peak was found to be followed by a second lipase activity peak (Fig. 2.1 A). Barnard (2005) purified a ~45 kDa lipase at the phase of growth corresponding to the second activity peak. Moreover, lipase activity staining using the culture supernatant corresponding to the second activity peak revealed two lipase active protein bands (Fig. 2.1 B). As a result, Barnard (2005), hypothesized that GE-7 produces two distinct lipases, a "scout enzyme" (activity peak 1, Fig. 2.1 A) that is constitutively expressed by the bacterium from the early stages of growth, activity of which results in the accumulation of FFA in the culture media. The uptake of the FFA by the cells inhibits further production of the "scout enzyme" (hence the drop in activity) while inducing the production of the ~45 kDa lipase (LipA) represented by the second activity peak (Fig. 2.1 A).

The present study is therefore aimed at characterizing the GE-7 lipase production profile using molecular and proteomic techniques by: (1) identifying the lipase represented by the first activity peak, (2) profiling the transcription of the GE-7 lipases using reverse-transcription mediated PCR and (3) understanding the physiology of lipase production in GE-7.

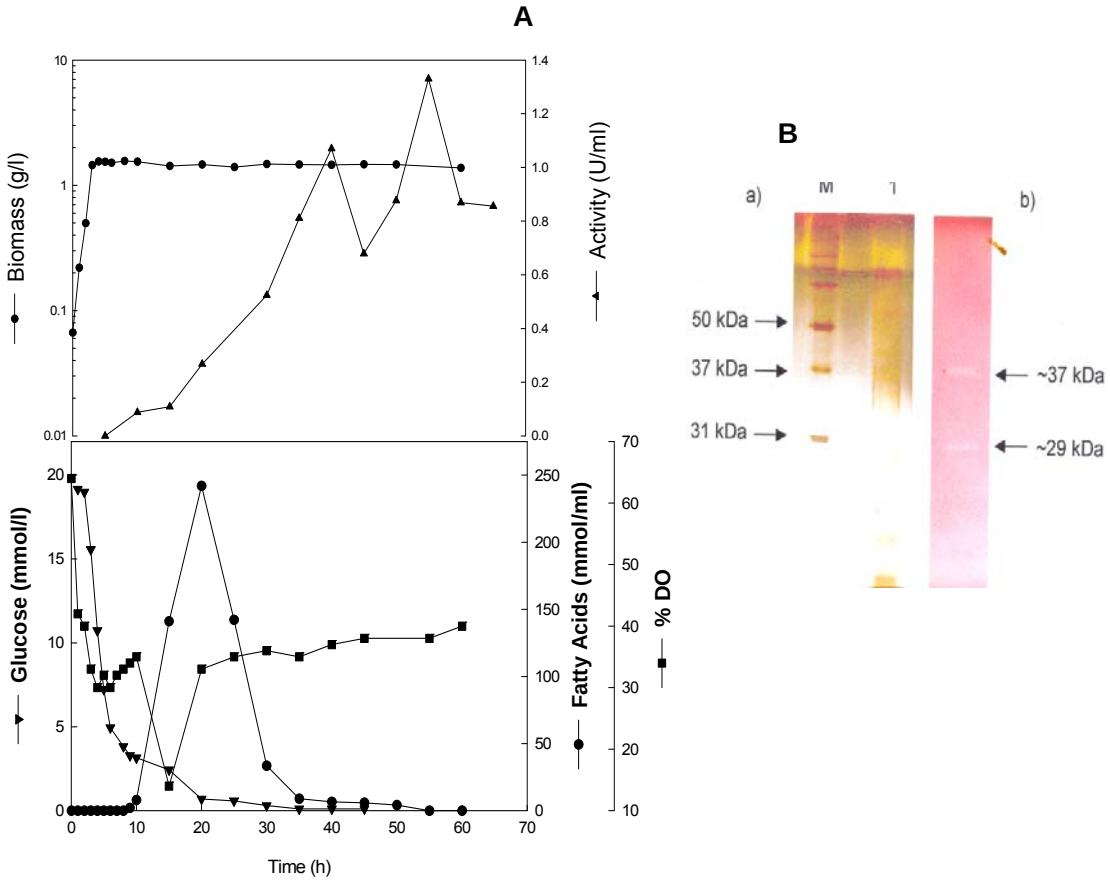


Figure 2.1: **A**, Graph showing the relationship between growth, glucose consumption, % dissolved oxygen (%DO), extracellular lipase activity and the subsequent release and utilization of fatty acids. Cultivation was performed in lipase production media, 2.5g/l olive oil and pH 7 (Knoesen, 2004). **B**, SDS-PAGE gel of the GE-7 culture supernanant applied to the Pheny-Toyopearl column in lane 1 (a) and the corresponding olive oil zymogram showing two lipase active bands (b) (taken from Barnard, 2005)

.

Chapter 3

Characterization of the extracellular lipolytic activity of *Geobacillus thermoleovorans* GE-7

3.1 Introduction

In 1999, Arpigny and Jaeger reported on the classification of bacterial lipolytic enzymes based mainly on the alignment of their amino acid sequences and some fundamental biochemical properties. These enzymes were classified into 8 different families with the largest being further divided into 6 subfamilies. Most of the families are characterized by unique conserved sequence blocks. Moreover, there appears to be a very high intra-family sequence similarity especially with amino acid sequences encoding enzymes from related bacterial species. The conserved sequence blocks and the high sequence similarity characteristic to the families facilitate the characterization of lipolytic activity from a given bacterium. This is due to the fact that the conserved sequence blocks can be used as templates in the design of family specific primers, for PCR detections of genes encoding members of the family from related or various bacterial species (Akoh *et al*., 2004a; Nthangeni *et al*., 2005). The high intra-family sequence similarity can aid in the design of primers that would result in the amplification of complete genes encoding members of the families which are yet to be cloned (Soliman *et al*., 2007). Moreover, this high sequence similarity that is characteristic to most lipases complements other gene cloning strategies such as genomic DNA library construction because it aids in the sequence based identification of genes of interest from the libraries.

Characterization of enzyme/lipase activity from a given organism can also be achieved through protein purification studies. The most commonly employed enzyme purification techniques involve the modification of genes to express active enzymes fused to a “tag” to rapidly purify the recombinant protein. However, such techniques are limited by the fact that gene sequence information is a prerequisite. In cases where the primary amino acid sequence of an enzyme is not known, several steps are employed to purify the enzyme of interest to homogeneity (Sharma *et al*., 2001). Most lipase purification strategies are usually time consuming and deliver low enzyme yields. However, in 2008, Fernandez-Lorente and co-workers reported on an improved method for the purification of lipases. The method entails the immobilization of lipases onto hydrophobic supports at low ionic strength and permits one-step purification, hyperactivation,

and stabilization of most lipases. This selective adsorption occurs because the hydrophobic surface of the support is able to promote the interfacial activation of the lipases, yielding enzyme preparations having the open form of the lipases very strongly adsorbed onto these hydrophobic supports. At low ionic strength, only proteins having large hydrophobic pockets may become adsorbed onto the hydrophobic support, and the only soluble proteins are lipases, which in the closed form are fairly hydrophilic, but in open form expose a very hydrophobic pocket. Desorption of the proteins from the hydrophobic support is achieved through the progressive addition of a detergent (Triton X-100 or CTBAB) and usually results in relatively pure enzyme preparations (a function of the hydrophobicity of the enzyme's active site and the binding efficiency of the hydrophobic support). This purification method can be used in combination with protein N-terminal amino acid sequencing or peptide mass spectrometry in order to determine the primary sequence of the enzyme preparation.

Over the years, *lip*A/LipA has been cloned/purified and characterized from a number of *Geobacillus* species (Lee *et al*., 2001; Castro-Ochoa *et al*., 2005; Soliman *et al.,* 2007). However, purification of a lipase different from LipA has only been described by three authors (Schmidt-Dannert *et al*., 1994; Lee *et al*., 2001; Castro-Ochoa; *et al*., 2005). These three authors reported on the purification of an 18, 16 and 11 kDa lipase respectively. However, only Schmidt-Dannert *et al*. (1994) and Lee *et al*. (2001) published N-terminal amino acid sequences encoding the 18 and 16 kDa lipase, which shared a very high sequence similarity which suggested that the two proteins were homologs. Since Lee and co-workers (2001) reported that the *G. thermoleovorans* ID-1 small lipase was purified from an uninduced culture, and since Barnard (2005) hypothesized that the lipase responsible for the first lipase activity peak in GE-7 could be constitutively expressed by the bacterium, we propose in this study that, the lipase responsible for the first activity peak in GE-7 could be a homolog of the ID-1 and *G. thermocatenulatus* small lipase.

However, as discussed in Section 1.3.2.1, genome sequencing projects by Takami *et al*., (2003) and Feng *et al*., (2007) on *G. kaustophilus* HTA426 and *G. thermodenifricans* NG80-2, respectively, revealed by sequence annotation the presence of a lipase/esterase (~25 kDa) with a GDSL motif instead of the common lipase motif encoded on the genomes of both organisms. GDSL hydrolases have been reported to posses flexible active sites that appear to change conformation in the presence and binding of different substrates (Akoh *et al*., 2004a), much like the induced fit mechanism proposed by Koshland (1958). We therefore suggest that, the broad

substrate specificity and the flexible catalytic site characteristic to the GDSL lipases increases the likelihood that they could be active against complex lipid substrates such as olive oil and as a result, if encoded on the genome of GE-7, could play a role in the observed activity profile.

Therefore, we present in this study attempts to identify the enzyme(s) associated with the first lipase activity peak (Chapter 2, Fig. 2.1 A), using PCR and enzyme purification techniques. This will be followed by relating the GE-7 lipase gene(s) transcription profiles to that of the observed activity profiles using reverse transcription PCR (RT-PCR).

3.2 Methods and material

3.2.1 Materials

Oligonucleotides (Table 3.1) were purchased from Integrated DNA Technologies (Iowa, USA) or Inqaba Biotech. The Bio-Spin gel and plasmid DNA extraction kits were purchased from Bioer Technology Co., Ltd. (Hangzhou, China). The cloning (pGEM-T Easy) and expression (pET28a) vectors were purchased from Promega and Novagen (Madison, WI USA), respectively. Tryptone, yeast extract and bacteriological agar were purchased from Biolabs (Johannesburg, RSA). Tributyrin, olive oil and Gum arabic were purchased from Sigma (Steinheim, Germany). Kanamycin and ampicillin were respectively purchased from Roche (Manheim, Germany) and Sigma (Steinheim, Germany). IPTG , X-gal, restriction and modification enzymes were purchased from MBI Fermentas (Burlington Ontario, Canada), Promega, Madison, WI USA) and Roche (Maiheim, Germany). Super-Therm and Pfu DNA polymerases for PCR were respectively purchased from Southern Cross Biotechnology (Cape Town, RSA) and MBI Fermentas (Burlington Ontario, Canada).

3.2.2 Growth of bacterial strains and media

3.2.2.1 Bacterial strains

Geobacillus thermoleovorans GE-7, isolated from a West Driefontein mine (South Africa), was kindly supplied by Dr. Mary de Flaun (Geosyntec Consultants Inc). Cloning and expression hosts, *E. coli* JM109 and *E. coli* JM109 (DE3) were purchased from Promega (Madison, WI USA).

3.2.2.2 Growth and lipase production

Geobacillus 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 glucose; 0.5 g/l casamino acids; 0.5 g/l starch; 0.3 g/l sodium pyruvate; 0.3 g/l potassium phosphate; 0.05 g/l magnesium phosphate; and 16 g/l bacteriological agar, pH 7.5 at 55˚C.

Lipase production was performed in media defined by Knoesen (2004), termed the lipase production media (LPM). The media consisted of 5 g/l glucose; 2.5 g/l proteose 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 was added consisting of $FeSO_4.7H_20$ 35 g/l, $MnSO_4.7H_2O$ 7 g/l, $ZnSO_4.7H_20$ 11 g/l, $CuSO_4.5H_20$ 5 g/l, $CoCl_2.5H_2O$ g/l, $Na_2MoO_4.2H_2O$ 1.3 g/l, H_3BO_3 2 g/l, KI 0.35 g/l, $Al_2(SO4)_3$ 0.5g/l dissolved in 20X dilution of concentrated HCl. Lipase activity was induced with 2.5 g/l olive oil unless stated otherwise.

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.

Thermal cycling was performed using an Eppendorf Mastercycler Temperature Gradient Personal with the following cycling program: Initial denaturing (hot start) of 5 min at 94ºC, 35 cycles of denaturing (94ºC for 15 sec), annealing (30 sec at 50ºC) and elongation (72ºC for 90

sec). After 35 cycles, a final elongation step of 20 min at 72ºC was added to ensure complete elongation of the amplified product.

3.2.3 Lipase activity assays

3.2.3.1 Lipase activity plate assays

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

When a differentiation between esterolytic and lipolytic acitivity was required, Rhodamine-olive oil agar plates were prepared as described by Kouker and Jaeger (1987).

3.2.3.2 Olive oil assay

Unless otherwise stated the GE-7 lipase activity was quantified using the olive oil assay. An olive oil emulsion (enzyme substrate) was freshly prepared by sonicating a solution containing 10% (v/v) olive oil and 1% (w/v) Gum Arabic with a Branson Sonifier cell disruptor B-30 until the mixture was milky. Centrifuged culture supernatant was then added to the assay mixture containing 500 μl substrate and 500 μl assay buffer consisting of 1 mM Tris-HCl and 5 mM $CaCl_2$ (pH8). The assay mixture was then incubated at 55ºC in a shaking waterbath for 1 hour. The reaction was stopped by the addition of 2.5 ml copper reagent which was prepared by separately dissolving 16.125 g $Cu(NO_3)_2.3H_2O$ and 32.5 ml tri-ethanolamine in 200 ml distilled water. These solutions were mixed, the pH set at 7.5 with HNO_3 and the final volume adjusted to 500 ml (Duncombe, 1963).

The complex formed between the released fatty acid and the copper was extracted by the addition of 5 ml chloroform, vortexing for 30 seconds and centrifugation at 4000 rpm for 2 min. The aqueous phase was carefully removed by aspiration and 2 ml of the chloroform phase was transferred to new test tubes. To these test tubes a freshly prepared 0.25% (w/v) solution of

sodium-diethyldithiocarbamate dissolved in 2-Butanol was added, and the absorbance was measured at 440 nm in a quartz cuvette against a reagent blank containing chloroform. A substrate blank was prepared and subjected to the same treatment but no culture supernatant (enzyme) was added to the reaction mixture.

A set of fatty acid standards was prepared from a stock solution of 1 mmole stearic acid dissolved in chloroform. From this stock solution appropriate amounts were transferred to clean test tubes and the chloroform was allowed to evaporate. The standards were subjected to the same treatment as described above. A standard curve of known fatty acid (stearic acid) amount *versus* absorbance measured at 440 nm was constructed (Fig. 3.1).

The 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 the chloroform analyzed (ml)
v = Volume of the enzyme assayed (ml)
E = Gradient of the standard curve
t = Incubation time of the assay (min)
d = light path of cuvette (1 cm)
A_{440} = Absorbance wavelength

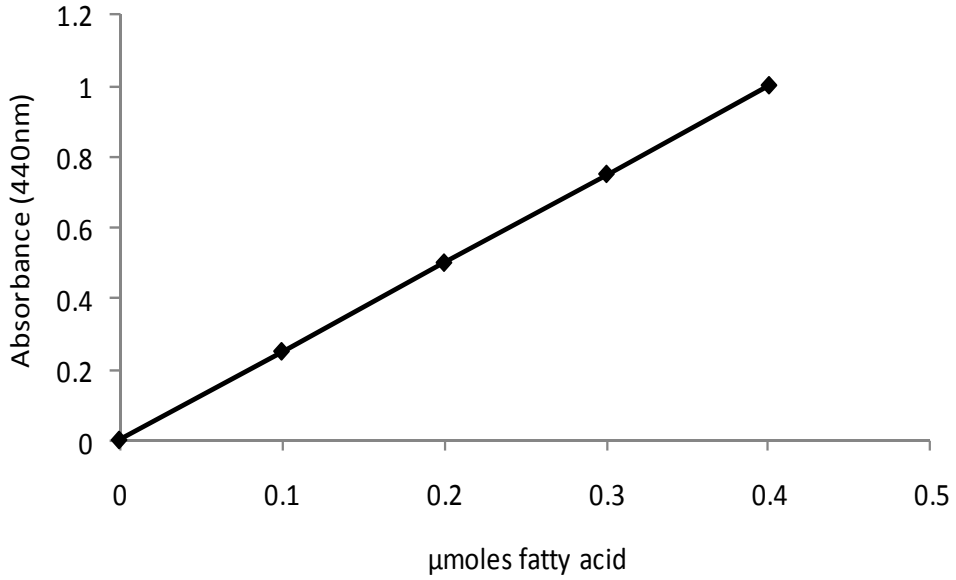


Figure 3.1: Standard curve for determining fatty acids released during the olive oil assay and for quantification of the free fatty acids present in the medium due to lipase activity. Stearic acid was used as the standard. The averages of triplicate determinations are shown.

3.2.4 PCR cloning of the GE-7 small lipase (ID-1 lipase homolog)

Similarity searches using the N-terminal amino acid sequence of the ID-1 small lipase revealed that this protein shared very high sequence similarity with the *G. thermocatenulatus* small lipase (N-terminal sequence) and a hypothetically conserved protein (~18 kDa) from *G. kaustophilus* HTA426. The N-terminal amino acid sequences encoding the ID-1 and *G. thermocatenulatus* lipase were aligned to the HTA426 hypothetically conserved protein sequence (Fig. 3.2 A) and the region of the latter that aligned best to the published N-terminal sequences was used to design primers GTL2N-1F and GTL2N-2R (Fig. 3.2 A) for the PCR detection of the small lipase in the GE-7 genome. The primers were designed such that they specified the amplification of an ~80 bp PCR product (the size of the published ID-1 N-terminal sequence in base pairs). A second set of primers (GTL2-1F and GTL2-400R, Fig. 3.2 A) was designed based on the hypothetical protein's nucleotide sequence in order to facilitate amplification of the GE-7 small lipase open reading frame (ORF). These primers were designed

such that they specified the amplification of a ~ 400 bp product. However, PCR with this primer set was unsuccessful.

Since the complete genome sequence of *G. kaustophilus* HTA426 is available in the nucleotide database, a new set of primers was designed based on the genes flanking the gene encoding the hypothetically conserved protein (hcp) in order to clone the regions up and down stream of the ~80 bp GE-7 small lipase gene (Fig 3.2 B). Primers Pep-1F and Twi-1R were designed based on the peptidase A24 enconding gene upstream of the hcp gene sequence and twitch-motility protein encoding gene downstream the hcp sequence such that they could be used in a PCR with primers GTL2N-2R and GTL2N-1F, respectively (Fig. 3.2 B). All PCR reactions were performed under amplification conditions that complemented each primer set.

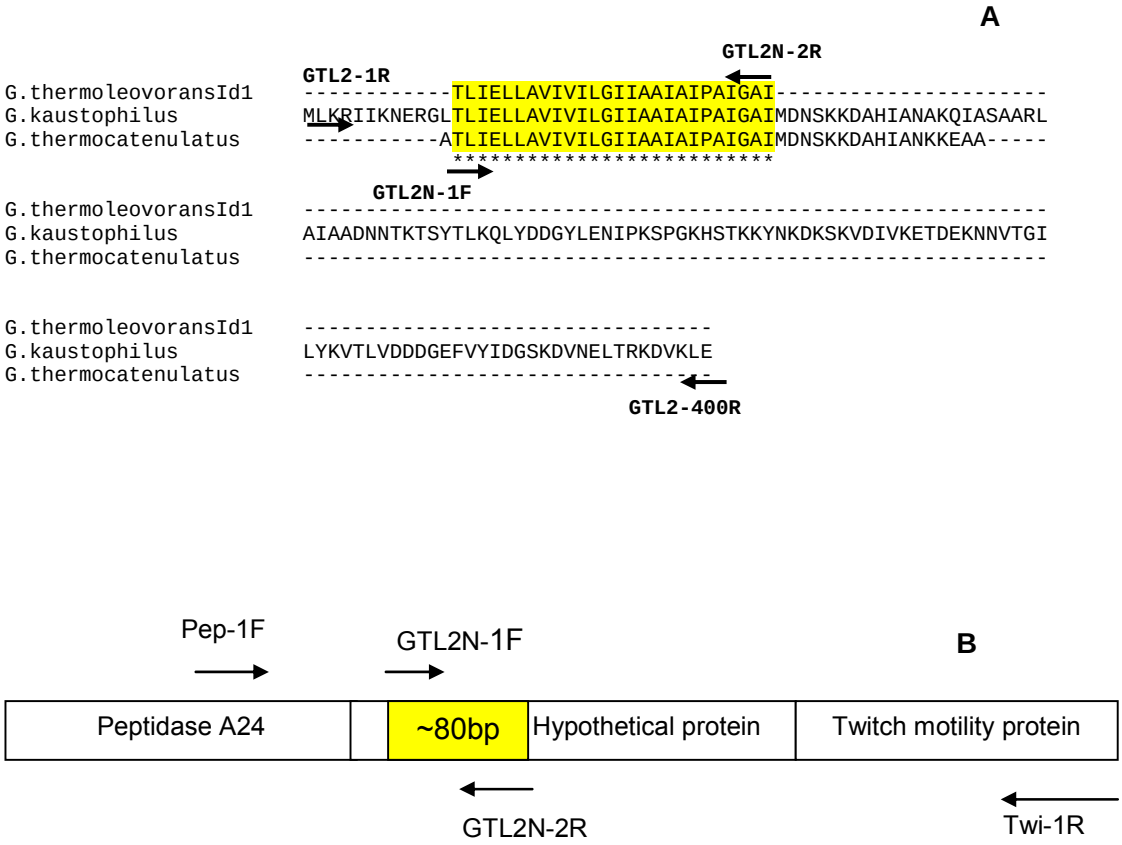


Figure 3.2: **A**, Alignment of amino acid sequences encoding lipases from *G. thermocatenulatus*, *G. thermoleovorans* ID-1 and a hypothetically conserved protein from *G. kaustophilus*. The arrows indicate the position of the primers for the detection and cloning of the GE-7 small lipase. The sequence block in grey represents a comparison of published lipase sequences to the

corresponding sequence in the hypothetical protein. **B**, Schematic representation of the strategy employed in the cloning of the complete GE-7 small lipase. The arrows represent the position of the primers while the yellow block represents the pubslihed small lipase N-terminal amino acid sequence.

3.2.5 PCR cloning of the GE-7 GDSL lipase

As discussed in Section 1.3.2.1, the *G. kaustophilus* HTA426 and *G. thermodenifricans* NG80-2 genome sequencing projects revealed, through annotation, the presence of the GDSL lipase encoding gene in addition to *lipA* in the genomes of both organisms. However, only the nucleotide sequence encoding the GDSL lipase (accession number: NC_006510.1) from *G. kaustophilus* HTA426 was used to design primers for the amplification of the GE-7 GDSL lipase ORF.

Primer set GDSLorf-1F and 700R (Table 3.1) was used in a PCR with GE-7 genomic DNA as a template under the following conditions: 1 denaturation cycle (94˚C, 2 min) and 30 cycles of amplification (94˚C, 30 sec; 55˚C, 30 sec; 72˚C, 2 min).

3.2.6 Sequencing and sequence analysis

The DNA fragments obtained by PCR amplifications were ligated into pGEM-T easy and sequenced at Inqaba Biotech using T7 and Sp6 promoter primers. Sequences were analyzed using tools available on the ExPASy Proteomic Server (http://au.expasy.org/).

3.2.7 Functional expression of the lipolytic genes

Primer sets GDSLorf-1F, -700R and GTLorf-1F, -400R were designed to facilitate the amplification of the GE-7 small lipase and GDSL lipase respectively. Both the primer sets were designed such that the forward and the reverse primers respectively haboured *Nco*I and *Xho*I restriction sites to facilitate directional cloning into expression vector pET28a restricted with the same enzymes. The ligation mixture was used to transform *Escherichia coli* host cells and

selected on LB agar plates containing kanamycin. The transformed colonies were patched on tributyrate agar plates containing kanamycin and IPTG (0.02 mM) and grown at 37˚C for 1-2 days. Colonies that became surrounded by zones of clearance were selected and grown in 5 ml LB kanamycin followed by plasmid isolation and restriction analysis. To distinguish between lipase and esterase activity colonies that became surrounded by zones of clearance on the tributyrate agar were selected and replica plated onto Rhodamine-olive oil agar plates.

3.2.8 *G. thermoleovorans* GE-7 lipase transcription profiling

3.2.8.1 Total RNA extraction and complementary DNA (cDNA) synthesis

G. thermoleovorans GE-7 was cultured under induced and uninduced conditions as described in section 3.2.2.2. The cells were harvested at 5 hour intervals and the culture supernatant assayed for lipase activity using the olive oil assay. Total RNA was isolated from the cells using the RNeasy® kit (Qiagen, USA) and mature RNA (mRNA) isolated from the total RNA using the MICROB*Express*™ bacterial mRNA purification kit (Ambion, USA). Complementary DNA (cDNA) was synthesized from the total or mRNA using the QuantiTect® reverse transcription kit (Qiagen, USA).

3.2.8.2 Reverse transcription PCR (RT-PCR)

Primers set GDSLorf-1F, -700R and *lipA* specific primers GTlipA-1F and GTLlipA-R (designed based on the GE-7 *lipA* sequence, accession number: EF123044.1) were used in a PCR with cDNA synthesized from total RNA extracted from the GE-7 cells (at the 20 and 40 hour interval) as a template.

Table 3.1: The list of primers used in this study.

Primer	Primer sequence (5'-3')	Position
GTL2N-F	**A**CGCTCATCGAACTGCTCGC	58
GTL2N-R	**G**GCGCCGATCGCCGGAATGG	123
GTL2-1F	GGCGCCG**A**TCGCCGGAATGG	1
GTL2-400R	CATCGTCGA**C**TTCCAGCTTCACATCCT	400
GTL2orf-1F	CC**A**TGGTCAAACGGGTGTT	1
GTL2orf-400R	GAGCT**C**CCTGACAGCCCCA	400
GTlipA-F	**A**TCCATATCATCGCCCACAGCCAAG	402
GTlipA-R	**T**TTGAGCTTTTCTACCGAACGGACG	900
GDSLorf-1F	CC**A**TGGGAAGAAAAAAAGCCGCA	1
GDSLorf-700R	CTCGAG**C**GGCGTTTCGTCGCTGA	835
Pep-1F	**G**ATTATCGCGGCGATTGCCATTCCG	-200
Twi-1R	**C**GGAATGGCAATCGCCGCGATAATC	+200

GTL2N and GTL2 respecitvely represent primers designed based on the small lipase N-terminal amino acid sequence and the *G. kaustophilus* HTA426 hypothetically conserved protein sequence (Fig. 3.2 A). GtlipA and GDSLorf respectively represent *lipA* and GDSL lipase gene specific primers. Pep and Twi respectively represent the primers designed based on the genes encoding the peptidaseA and twitch-motility protein flanking the hypothetically conserved protein in *G. kaustophilus* HTA426. Position refers to the corresponding position of the underlined bold nucleotide of the primer within the gene sequence as submitted to the nucleotide sequence database. The – and +

signs represents the position of the primer (in the flanking genes) relative to the position start and stop codon of the hypothetically conserved protein in *G. kaustophilus* HTA426, respectively (Fig. 3.2 B).

3.2.9 Purification of the lipase associated with the first activity peak

Geobacillus thermoleovorans GE-7 was grown on LPM media containing olive oil (2.5 g/l) and lipase production monitored using the olive oil assay at 5 hour intervals until the 20 hour mark (before the onset of the second peak). Since the lipase activity assays indicated that the enzyme is secreted extracellulary, the culture medium supernatant was used after the cells were removed by centrifugation at 10 000 *xg* for 10 min. Olive oil and free fatty acids were extracted from the culture supernatant using hexane:acetic acid (1:1). The supernatant was then applied to hydrophobic chromatographic resins, butyl-toyopearl and octyl-agarose.

3.2.9.1 Batch purification

The culture supernatant (100 ml) was concentrated with the Amicon Ultrafiltration unit fitted with a 10 kDa molecular weight cut-off membrane, dialysed against 5 mM sodium phosphate buffer pH 7 and the protein concentration determined (A_{280}). To the culture supernatant concentrate (~1 mg/ml protein), 5 g of the hydrophobic support (octyl-agarose) was added, and immobilization of the lipase was allowed to occur under mild stirring conditions at 25°C. The enzyme suspension and supernatant were sampled and assayed for lipase activity until enzyme activity could not be detected in the supernatant after which the adsorbed lipase was washed thoroughly with distilled water.

Samples for electrophoresis were prepared by adding 0.1 g of the butyl-sepharose beads (with immobilized enzyme) to 500 µl SDS-PAGE loading buffer (10% mercaptoethanol and 4% SDS). The sample was boiled for 5 min (desorption) and loaded onto 10% SDS-PAGE.

3.2.9.2 Column purification

The culture supernatant concentrate was applied to butyl-toyopearl column (2.5 cm x 10 cm) pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.6 [1 M $(NH_4)_2SO_4$], at a flow rate 2 ml/min and the fractions were collected every 2.5 min. The column was washed with 2 column volumes of 50 mM Tris-HCl buffer, pH 7.6 [1 M $(NH_4)_2SO_4$] following the application of the sample. The bound proteins were eluted with 4 column volumes of a 1 M – 0 $(NH_4)_2SO_4$ gradient in 50 mM Tris-HCl pH 7.6. Furthermore, 20% ethanol was used to elute proteins that could be tightly bound to the resin and the fractions were collected as described above. The A_{280} readings used to measure protein concentration were used for the construction of appropriate elution profiles. Fractions making up significant protein elution peaks were pooled and dialysed against 50 mM Tris-HCl, pH 7.6. After the dialysis, the fractions were assayed for lipase activity using the olive oil assay. For SDS-PAGE analysis, 0.5 ml of the pooled fractions was vacuum dried, resuspended in 0.02 ml SDS-PAGE loading buffer and subjected to electrophoresis. The band(s) of interest were excised from the PAGE gels and sent for peptide mass spectrometry analysis at the Center for Proteomics and Genomics Research (CPGR) (Cape Town, RSA).

3.2.10 Electrophoresis

3.2.10.1 SDS-PAGE

Electrophoresis in 10% polyacrylamide resolving and 4% stacking gels in the presence of the anionic detergent SDS was used to monitor the purification process, to assess the homogeneity of the purified fractions and to estimate the relative molecular mass of the enzymes by comparing their electrophoretic mobility with those of standard proteins of known molecular masses (Laemmli, 1970).

The protein bands in the gel were visualized by either Coomassie Blue R250 staining (Fairbanks *et al*., 1971) and/or silver stained as described by Switzer *et al*. (1979).

3.2.10.2 Native-PAGE

For electrophoresis under non-denaturing conditions, polyacrylamide gels were prepared as described in section 3.2.10.1. However, the anionic detergent SDS was not added.

3.2.11 Lipase zymogram

Geobacillus thermoleovorans GE-7 growth and lipase production was performed (under induced and uninduced conditions) as described in section 3.2.2.2. The culture was centrifuged at 10 000 x*g* and the culture supernatant concentrated using Amicon Ultra-4 Centrifugal Filter Units with a 10 kDa molecular weight cut-off point (Millipore, Billerica, USA). The culture supernatant concentrate was subject to electrophoresis as described in section 3.2.10.2.

For the zymogram, the gel was prepared by first making a suspension of 2.5 g/l triglyceride (olive oil) 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. Rhodamine was added to a final concentration 0.02 % (v/v) and the gel allowed to set. The native-PAGE gel was equilibrated in 50 mM Tris-HCl, pH 8.0 for 30 min and overlaid onto the agarose gel containing the emulsified olive oil and Rhodamine B. The principle used was that hydrolysis of the substrates by the protein band(s) on the native-PAGE gel will result in corresponding fluorescent band(s) on the Rhodamine-olive oil gel which are visible upon UV radiation.

3.3 Results

3.3.1 Confirmation of strain identity

The 16S rDNA PCR performed on the GE-7 whole cells resulted in the amplification of an approximate 1500 bp product as specified by the 16S rDNA primers (Fig. 3.3 A). The PCR product was cloned into pGEM-T easy cloning vector and the cloning host *E. coli* JM109 was transformed. Plasmid isolation was performed and the plasmids restricted with *Eco*RI to screen for positive clones, which were sent for sequencing at Inqaba Biotech. Sequence analysis [Fig. 3.3 B(a) and (b)] revealed that the cloned 16S rDNA shares 96% sequence similarity to that of *G. thermoleovorans* T80.

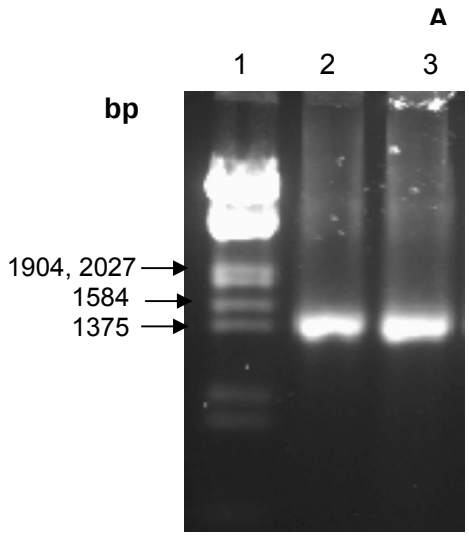


Figure 3.3: **A**, Gel electrophoresis of PCR amplification product of the GE-7 16S rDNA. Lane 1, the shows the λIII marker, lanes 2 and 3 are duplicate experiments.

B

(a)

```
Ge7t7  1    CGGACTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGGATTAGAGTTTGATCA 65
GtT80  1                                                       TAGAGTTTGATCC 13

Ge7t7  66   TGGCTCAGGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCAAATCGGAGCT 130
GtT80  14   TGGCTCAGG-ACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACCAAATCGGAGCT 77

Ge7t7  131  TGCTCTGGTTTGGTCAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCCGCAAGACCGGGA 195
GtT80  78   TGCTCTGGTTTGGTCAGCGGCGGACGGGTGAGTAACAGGTGGGCAACCTGCCCGCAAGACCGGGA 142

Ge7t7  196  TAACTCCGGGAAACCGGAGCTAATACCGGATAACACCGAAGACCGCATGGTCTTTGGTTGAAAGG 260
GtT80  143  TAACTCCGGGAAACCGGAGCTAATACCGGATAACACCGAAGACCGCATGGTCTTTGGTTGAAAGG 207

Ge7t7  261  CGGCCTTTGGCTGTCACTTGCGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTC 325
GtT80  208  CGGCCTTTGGCTGTCACTTGCGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTC 272

Ge7t7  326  ACCAAGGCGACGATGCGTAGCCGGCCTGAAGAGGGTGACCGGCCACACTGGGACTGAGACACGGC 390
GtT80  273  ACCAAGGCGACGATGCGTAGCCGGCCTGA-GAGGGTGACCGGCCACACTGGGACTGAGACACGGC 336

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

Ge7t7  456  AACCCCCCCTGAGC                                                  469
GtT80  399  GACGCCGCGTGAGCGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTGAGGGACGAAGGAGCGCC 463
```

(b)

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

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

GtT80   1149 GTGGGCACTCTAGAGGGACTGCCGGCGACAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATC 1212
Ge7sp6  116  GTGGGCACTCTAGAGGGACTGCCGGCAACAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATC 179

GtT80   1213 ATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCGGTACAAAGGGCTGCGAACCCGC 1276
Ge7sp6  180  ATGCCCCTTATGACCTAGGCTACACACGTGCTACAATGGGCGGTACAAAGGGCTGCGAACCCGC 243

GtT80   1277 GAGGGGGAGCGAATCCCAAAAAGCCGCTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATG 1340
Ge7sp6  244  GAGGGGGAGCGAATCCCAAAAAGCCGCTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATG 307

GtT80   1341 AAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 1404
Ge7sp6  308  AAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTAC 371

GtT80   1405 ACACCGCCCGTCACACCACGAGAGCTTGCAACACCCGAAGTC-GGTGAGGCAACCCTTAAGGGA 1467
Ge7sp6  372  ACACCGCCCGTCACACCACGAGAGCTTGCAACACCCGAAGTCCGGTGAGGCAACCCTTACGGGA 435

GtT80   1468 GCCAGCCGCCGAAGGTGGGGCAAGTGATTGGGGTGAAGTCGTAACAAGGTAACCA          1522
Ge7sp6  436  GCCAGCCGCCGAAGGTGGGGCAAGTGATTGGGGTGAAGTCGTAACAAGGTAACCAATCGAATTC 499
```

Figure 3.3: **B**, Sequence alignments of the 16S rDNA sequences obtained by using T7 (a) and Sp6 (b) (cloning vector promoter primers) with the 16S rDNA sequence of *G. thermoleovorans* T80.

3.3.2 Growth and lipase production

Geobacillus thermoleovorans GE-7 was streaked out onto R_2A plates and grown overnight at 55°C. This was then used as the 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 55°C until an OD_{600nm} reading of ~ 0.6 was reached. The pre-inoculum (5 ml) was used to inoculate the lipase production flask (500 ml Erlenmeyer flask) containing 60 ml LPM with olive oil to a final concentration of 2.5 g/l (induced) and LPM without the olive oil (uninduced). Lipase production was monitored at 5 hour intervals using the olive oil assay.

A lipase production profile characterized by two activity peaks was observed under induced conditions, while a single lipase activity peak was observed under uninduced conditions (Fig. 3.4).

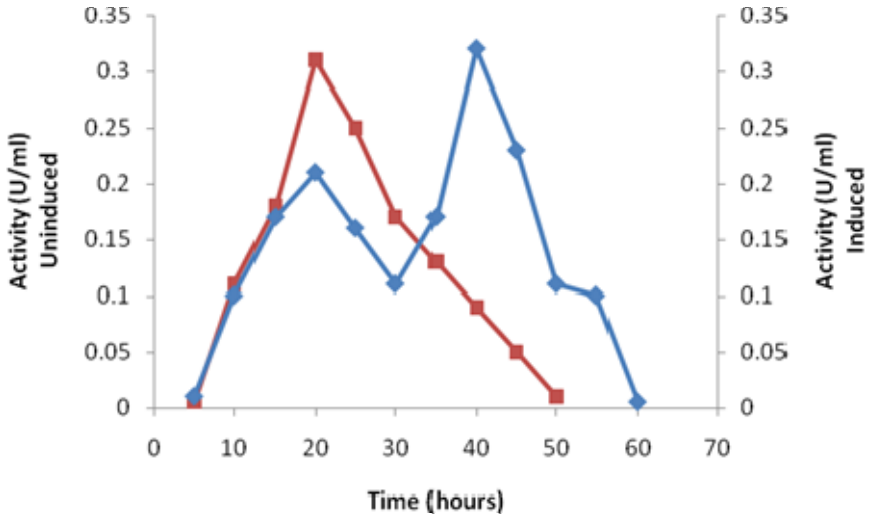


Figure 3.4: *G. thermoleovorans* GE-7 lipase production profile under induced (♦) and uninduced (■) culture conditions.

3.3.3 Lipase activity staining/zymogram

Geobacillus thermoleovorans GE-7 growth and lipase production was performed as described in section 3.3.2. However, the cells were harvested at the 40 hour interval under induced and uninduced culture conditions. Sample preparation, electrophoresis and lipase

activity staining were performed as described in sections 3.2.10.2 and 3.2.11. However, the samples were loaded into 6 wells/lanes of the PAGE gel and prior to overlaying onto the Rhodamine-olive agarose gels; two lanes were excised from the gel, stained using the Fairbanks method (Fairbanks *et al*., 1971) and the remaining 4 lanes were overlaid.

After incubation of the lipase activity staining reaction at 55°C overnight, two fluorescent bands were observed on the Rhodamine-olive oil agarose gel (Fig. 3.5 B). The two fluorescent bands represent two protein bands in the GE-7 culture supernatant (Fig. 3.5 A) capable of hydrolyzing olive oil (lipase substrate).

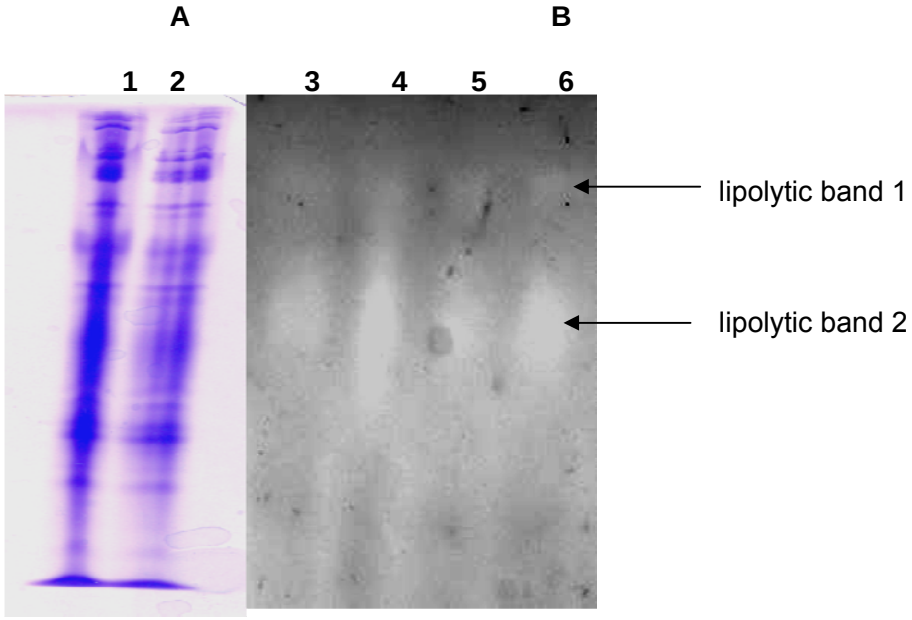


Figure 3.5: **A**, Native-PAGE analysis of the culture supernatant from the induced (1) and uninduced (2) GE-7 culture. **B**, Lipase activity staining of the induced (3, 4) and uninduced (5, 6) GE-7 culture supernatant.

3.3.4 PCR cloning of the GE-7 small lipase (ID-1 homolog)

PCR detection of the small lipase using primers GTL2N-F and R resulted in the amplification of an ~80 bp gene fragment (Fig. 3.6 A) from the GE-7 genomic DNA, as specified by the primer set. PCR amplification of the complete small lipase encoding gene using primer set GTL2-1F and -400R on GE-7 did not yield any product. As a result, a new set of primers was designed based on the genes flanking the gene of interest (using the *G. kaustophilus* HTA424 nucleotide sequence as a template). These primers were designed such that they could be used in a PCR with the primers used for the detection of the small lipase gene in the GE-7 genome (Fig. 3.2 B).

Therefore, a first round of PCR was performed using primer sets Pep-1F, GTL2N-R and Twi-R, GTL2N-F, with genomic DNA from both *G. kaustophilus* HTA426 and GE-7 as a template. These primers respectively resulted in the amplification of 190 and 300 bp gene fragments (Fig. 3.6 B) up- and downstream of the known sequence (~80 bp fragment). A second round of PCR was performed with primers Pep-1F and Twi-1R with genomic DNA from both organisms as template. A 700 bp PCR product (Fig. 3.6 C) was obtained. The 700 bp PCR product was cloned into pGEM-T easy vector and the cloning host *E. coli* JM109 transformed, plasmids isolated and restricted with *Eco*RI to screen for positive clones. Positive clones were selected and sent for sequencing at Inqaba Biotec.

Analysis of the nucleotide (Fig. 3.7 A) and amino acid (Fig. 3.7 B) sequence encoding the GE-7 small lipase revealed that the most conserved feature between this protein and related proteins from *G. thermocatenulatus* and *G. kaustophilus* is the N-terminus, while, the C-terminus appears to be variable. Further analysis of the sequences using SignalP 3.0 (Emmanuelsson *et al*., 2007), which is used to predict a protein's subcellular localization revealed that half of the highly conserved N-terminal region encodes a signal peptide that is cleaved off during the protein's translocation to the extracellular environment (Fig. 3.7 B). Moreover, similarity searches revealed that this protein shares significant similarity to type II secretory pathway pseudopillin protein from *G. thermocatenulatus* (60 %) and *Bacillus* sp. NRRL B-14911 (77 %), and no similarity to other known lipases.

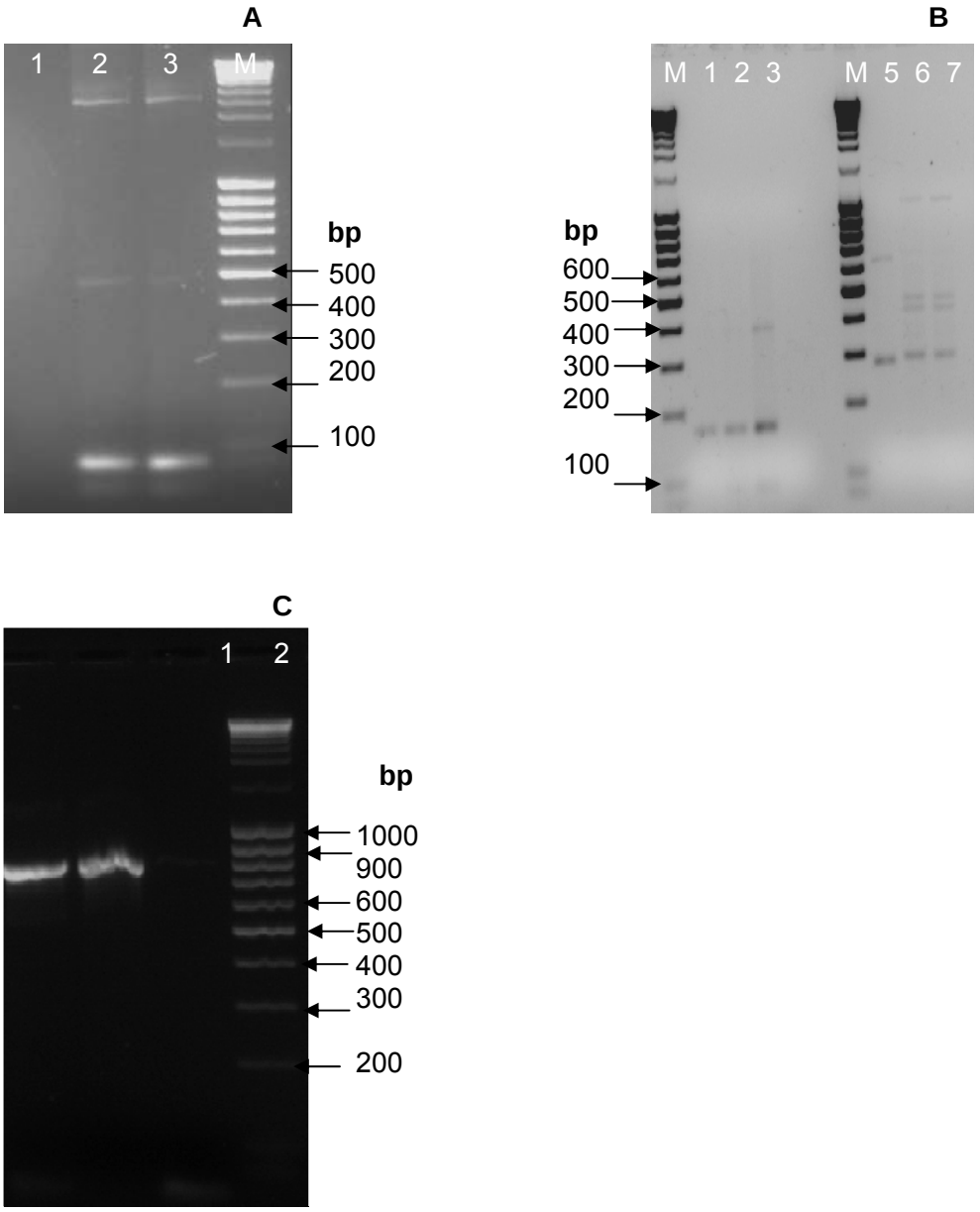


Figure 3.6: **A**, PCR detection of the GE-7 small lipase using primers GTL2N-1F and GTL2N-2R. Lane 1 is the negative control, lanes 2, 3 duplicates of the PCR with GE-7 genomic DNA as a template and M is the molecular weight marker. **B**, PCR amplification of the regions up and downstream the ~ 80 bp GE-7 small lipase gene fragment. Lanes 1, 5, PCR with genomic DNA from *G. kaustophilus* HTA 426 (positive control) as a template. Lanes 2,3 and 6,7 PCR with GE-7 genomic DNA as template (experiment). **C**, PCR amplification of the complete small lipase gene using primers designed based on the genes flanking the gene of interest. Lane 1 represents the positive control (*G. kaustophilus* genomic DNA), lane 2 (experiment), lane 3 (negative control) and lane M (molecular weight marker).

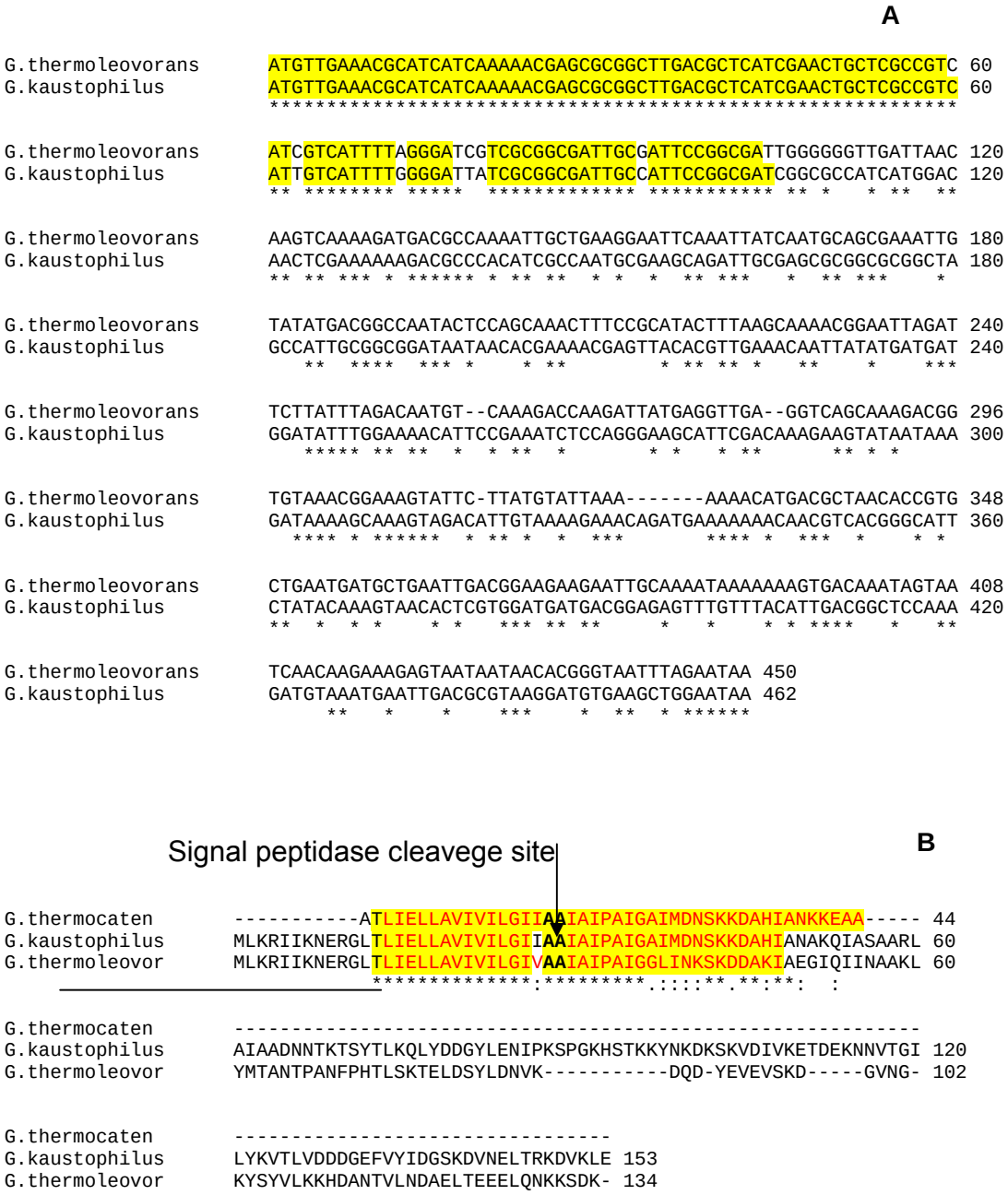


Figure 3.7: **A**, A comparison of the nucleotide sequences encoding the GE-7 small lipase and its homolog from *G. kaustophilus* HTA426, highlighted in yellow, is the most conserved feature of these genes. **B**, A comparison of the *G. thermocatenulatus* small lipase N-terminal amino acid sequence to the sequences of its homologs from GE-7 and *G. kaustophilus* HTA426. The amino acid residues in red and highlighted in yellow represent the highly conserved N-terminus that appears to be characteristic to this group of proteins. The underlined sequence block and the amino acid residues

indicated by the arrow respectively represent the putative signal peptide and peptidase cleavage site.

3.3.5 PCR cloning of the GE-7 GDSL lipase

The primer set GDSLorf-1F; -700R designed based on the nucleotide sequence encoding the *G. kaustophilus* HTA426 GDSL lipase was used in a PCR with GE-7 genomic DNA as a template and a 700 bp PCR product (Fig. 3.8) was obtained. The 700 bp PCR product was cloned into pGEM-T easy cloning vector and the cloning host *E. coli* JM109 transformed. Plasmid isolation was performed and the plasmids were restricted using *Eco*RI to screen for positive clones. Positive clones were selected and sent for sequencing at Inqaba Biotec.

Sequence analysis revealed that the GE-7 GDSL lipase shares very high sequence similarity (~88%) to its homologs from *G. thermodenifricans* and *G. kaustophilus* (Fig. 3.9). Further sequence analysis revealed that the GDSL lipase shares significant sequence similarity to carbonhydrate esterases from several mesophilic *Bacillus* species. Moreover, analysis of the GE-7 GDSL lipase with SignalP 3.0 revealed that this protein has a 100% probability of being translocated extracellularly (signal peptide probability = 1.000) and that the maximum cleavage site probability was found to be between amino acid 30 and 31 (Fig. 3.9). However, the region encoding the putative signal peptide appears to be poorly conserved while the sequence encoding the mature enzyme is highly conserved.

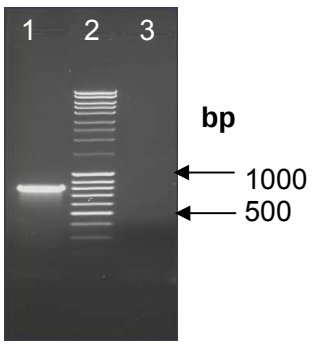


Figure 3.8: PCR amplification of the GDSL lipase from the genomic DNA of GE-7. Lane 1, PCR product obtained with the GE7 genomic DNA, lane 2 (molecular weight marker) and lane 3 (negative control).

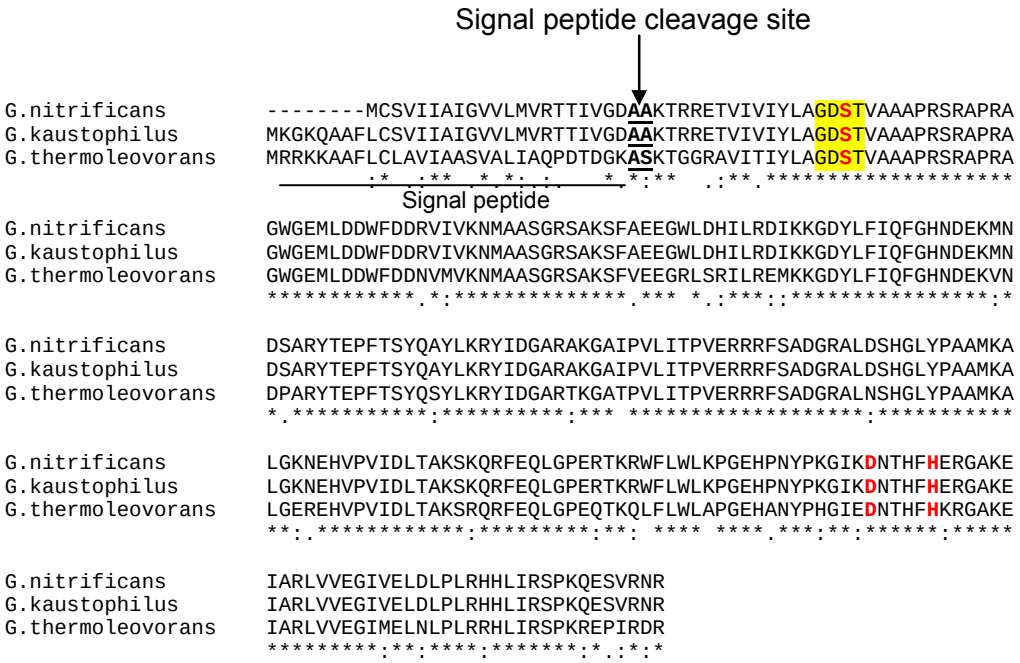


Figure 3.9: A comparison of the GE-7 GDSL lipase to its homologs from selected *Geobacillus* species. The region shaded in yellow represents the conserved motif and residues highlighted in red represent the catalytic triad. The under lined sequences and the amino acids in bold indicated by the arrow respectively represent the putative signal peptide and the peptidase cleavage site.

3.3.5 Functional expression of the cloned GE-7 lipases

The primer set GDSlorf-1F; -700R was used in the amplification of the GDSL lipase ORFs and resulted in the amplification of an ~700 bp (Fig. 3.8) PCR product. The PCR product was digested with *Nco*I and *Xho*I restriction enzymes and ligated into expression vector pET28a restricted with the same enzymes. Ligation of the PCR product to pET 28a resulted in the construct denoted pET-GDSLlip (Figure 3.10 A) which was used to transform the expression host (*E. coli* JM109 DE3). The resultant transformants (carrying the appropriate recombinant plasmid) obtained upon transformation of the host cells were patched on TLB agar plates. Zones of clearance could be observed after 2-4 days incubation at 37 ˚C (Figure 3.10 B). The zones of clearance around the colonies are an indication of the hydrolysis of tributyrin by an esterase or a lipase. In order to distinguish between the esterase and lipase activity colonies

from the TLB agar plates were replica plated onto Rhodamine-olive oil agar plates and allowed to grow at 37˚C for 1 week. No flourescence was observed around the colonies upon exposure to UV radiation. The results are an indication that this enzyme displays estereolytic and not lipolytic activity.

The GE-7 small lipase was also amplified and subcloned into the expression vector as described for the GDSL lipase. Functional expression of the GE-7 small lipase encoding gene was attempted. No positive clones were obtained on either TLB or Rhodamine-olive oil agar plates (data not shown).

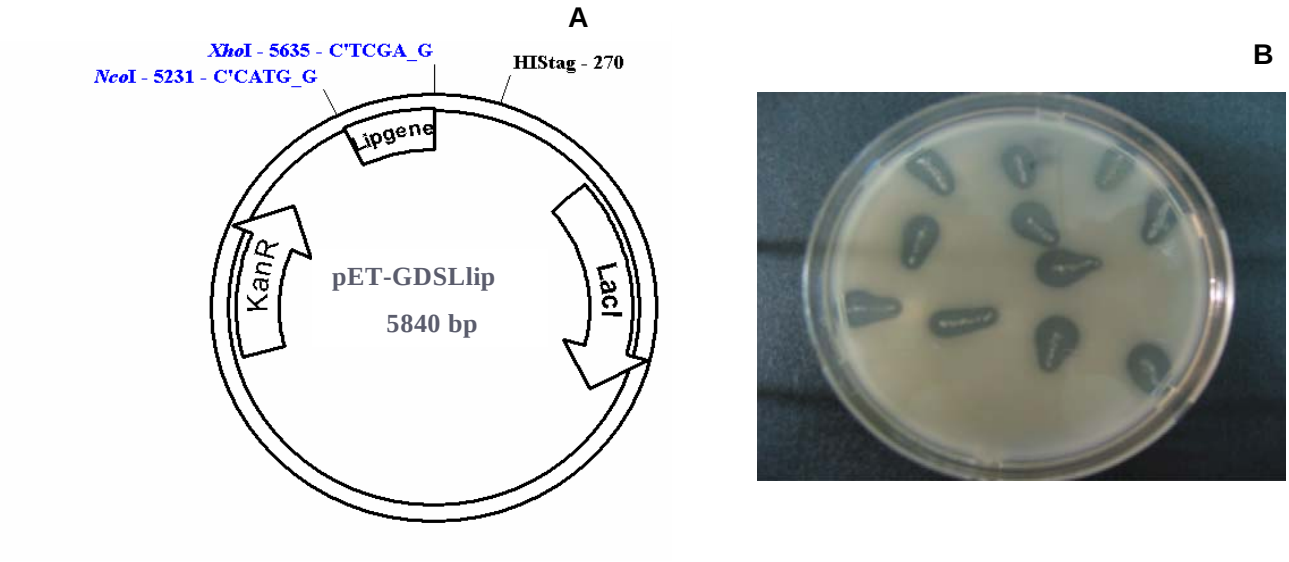


Figure 3.10: **A**, GE-7 GDSL lipase expressin construct. **B**, Colonies carrying expression construct pET-GDSLlip. Zones of clearance around the colonies represent lipolytic activity.

3.3.6 Purification of the lipase associated with the first activity peak

3.3.6.1 Batch purification

Geobacillus thermoleovorans GE-7 was grown on LPM (olive oil) and the cells harvested after incubation at 55°C for 20 hours [before the onset of the second lipase activity peak, (Fig. 3.4)]. The culture supernatant was mixed with octyl-agarose and the enzyme allowed to adsorb onto the support under mild stirring conditions at room temperature. The unbound proteins were washed off several times with distilled water. The bound protein was eluted by mixing the resin with SDS-PAGE loading buffer and boiling for 5 min. The unbound and eluted fraction was analysed using SDS-PAGE. A protein band (~45 kDa) appeared to disappear from the unbound fraction but appeared in the eluted fraction (Fig. 3.11). These results are an indication that the ~45 kDa protein was bound to the resin and was released through the elution process. The protein band was excised from the PAGE gel and sent for sequencing at the Facility for Genomics and Proteomics (University of the Free State, Bloemfontein, RSA). Several attempts to sequence the protein band using mass spectrometry (MS) were unsuccessful.

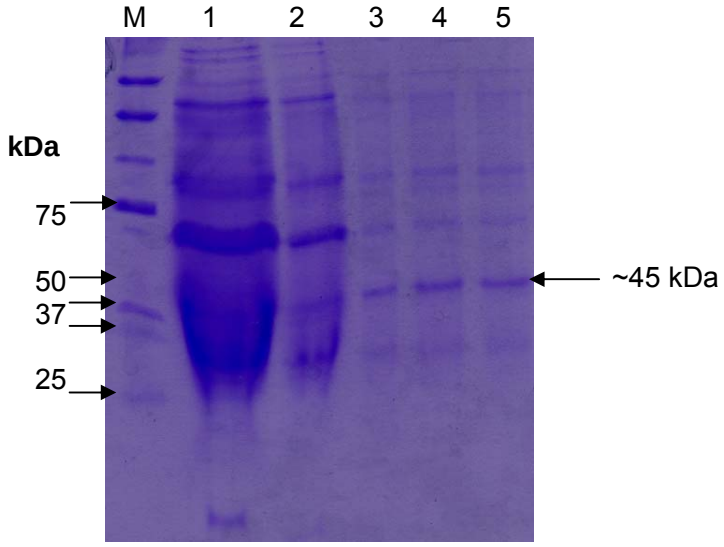


Figure 3.11: Batch purification of the GE-7 lipase. Lane M represents the molecular weight marker. Lanes 1 and 2 represent the unbound fraction ,while lanes 3-5 represent the eluted fractions.

3.3.6.2 Column purification

Geoabacillus thermoleovorans GE-7 was grown as discussed in section 3.3.6.1. The culture supernatant concentrate was loaded onto a butyl-toyopearl column equilibrated with 50 mM Tris-HCl pH 7.6 [1 M $(NH_4)_2SO_4$] buffer. The unbound proteins were washed off with the same buffer. The bound proteins were eluted using a decreasing $(NH_4)_2SO_4$ (1 - 0 M) gradient. A second elution step was carried out using 20% ethanol in order to elute proteins that could be tightly bound to the resin. An elution profile characterized by two peaks was observed (Fig. 3.12 A and B) with the decreasing salt gradient. Elution with 20% ethanol resulted in the observation one broad peak (Fig. 3.12.C). The fractions corresponding to each of the peaks were pooled and dialysed against 50 mM Tris-HCl pH 7.6. The pooled fractions were assayed for lipase activity using the olive oil assay and were all found to be active against the olive oil (P 1, 1.4 U/ml, P2, 0.6 U/ml, and P3, 1 U/ml). The ultrafiltration concentrate of the pooled fractions were analysed using SDS-PAGE (Fig. 3.13). The first two elution peaks (Fig. 3.12 A and B) were found to be comprised of a mixture of protein bands (Fig. 3.13) while the third protein peak (Fig. 3.14 C) was comprised of a single protein band of ~45 kDa (Fig. 3.15 B). The protein band was excised from the PAGE gel and sent for sequencing at the CPGR (Cape Town, RSA).

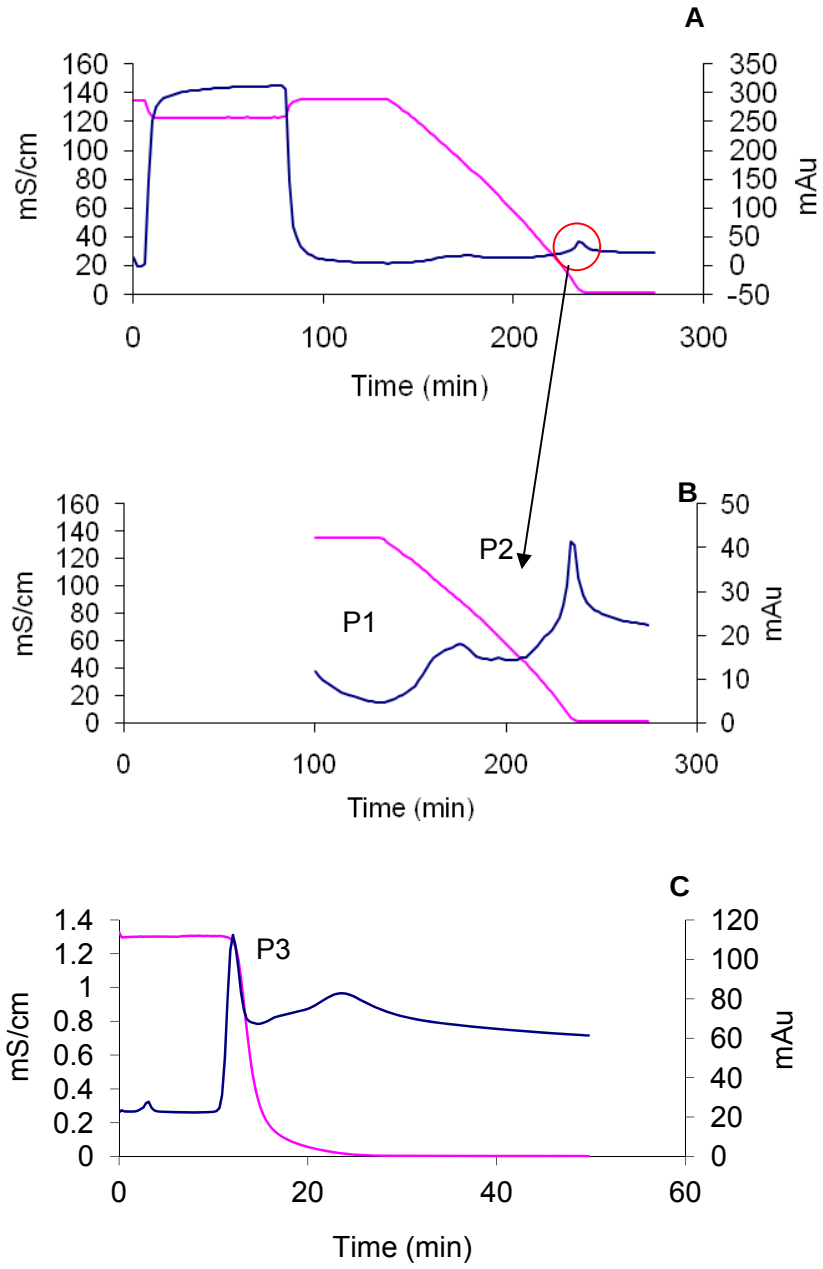


Figure 3.12: **A**, The elution profile obtained for the GE-7 culture supernatant run on butyl-toyopearl resin (decreasing salt gradient). **B**, A magnification of the elution peak observed in elution profile A. **C**, The elution profile obtained using ethanol as the eluent.

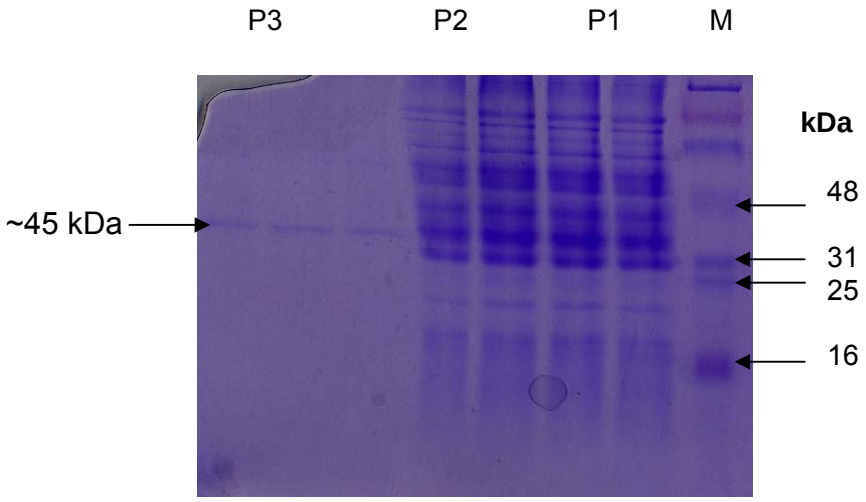


Figure 3.13: SDS-PAGE analysis of the pooled fractions corresponding to the above mentioned protein peaks.

3.3.7 Protein sequencing and identification (peptide mass spectrometry)

3.3.7.1 The sequencing process

All mass spectra were recorded on the ESI-Q-TOF type mass spectometer [ABI 4800 (Applied Biosystems, Framingham, MA, USA)] operating in the positive ion and reflectron mode. The excised protein band (Fig. 3.13 B) was destained, dehydrated, reduced with DTT, alkylated with iodoacetimide and digested with trypsin. The resulting tryptic digests were concentrated and desalted using C_{18} ZipTips (Millipore Corporation, Bedford, MA, USA) according to the manufacture's instructions. The sample was introduced into the machine, ionized [electron spray ionization (ESI)] and subjected to mass analysis in the quadropole ion trap (Q) and mass spectra [mass to charge (M/Z) ratio] of the peptide ions (precursor ions) corresponding to the tryptic digest of the protein band were obtained (Fig. 3.14 A). The precursor ions were fragmented in the quadropole ion trap and subjected to mass analysis using TOF (time-of-flight) and the spectra corresponding to the product ions generated from the fragmentation of the precursor ions were obtained (Fig. 3.14 B).

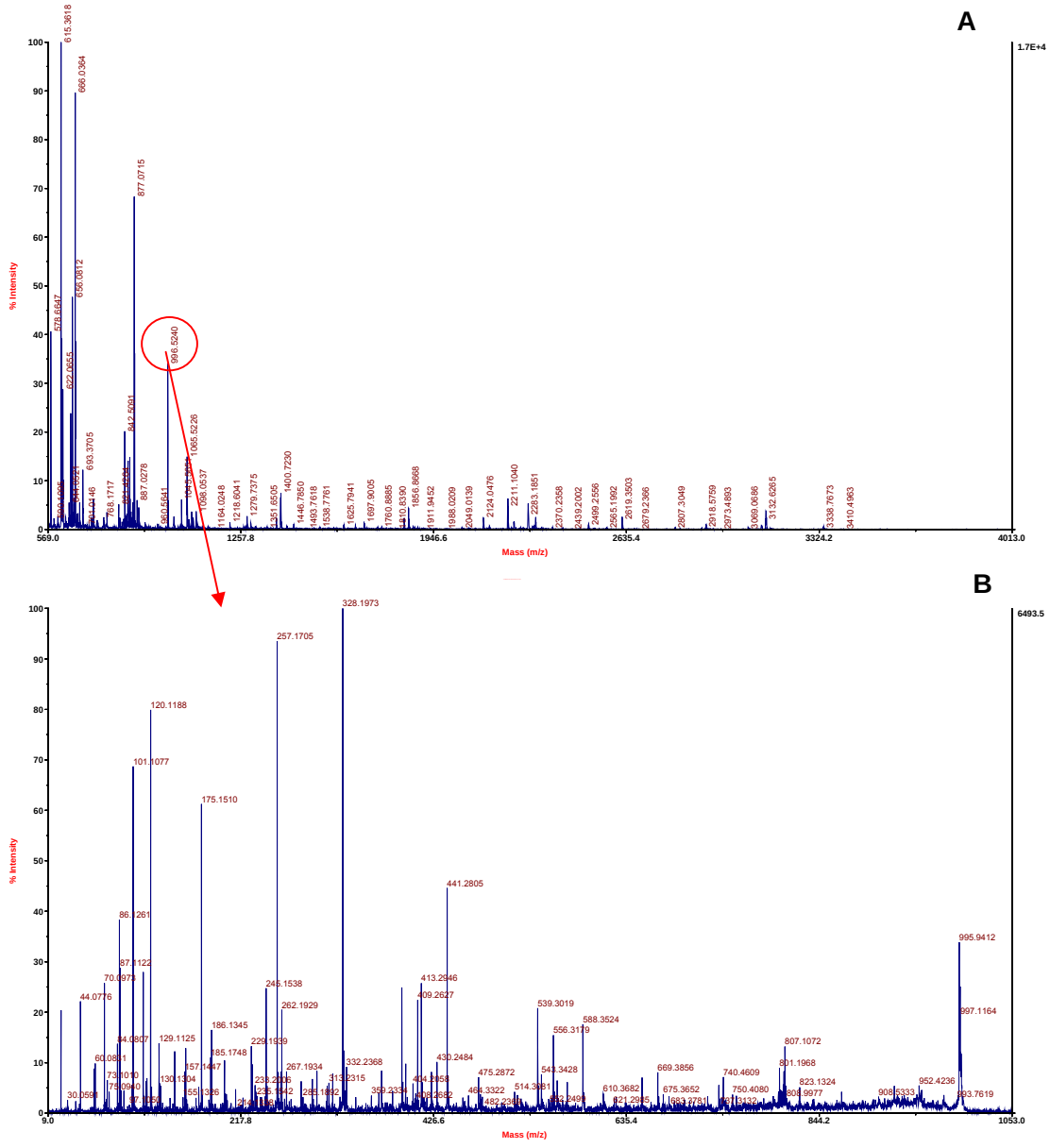


Figure 3.14: **A**, Mass spectrum of the tryptic digest of the ~45 kDa GE-7 protein (precusor ions). **B**, Mass spectrum of one of the product ions that resulted from the fragmentation of one of the precusor ions (996.520 M/Z) circled in red.

3.3.7.2 Sequence determination/protein identification

The mass spectra that were obtained with the product ions were analysed using the Mascot search engine (Perkins *et al*., 1999). This search engine allows the use of mass spectrometry data to identify proteins from the primary sequence databases. No exact matches were made with the spectra on the databases (NCBI, etc.). As a result, a *de novo* sequencing program, Lutefisk (Johnson and Taylor, 2002) was used to analyze the peptide spectra.

Lutefisk is a software that allows the conversion of peptide mass spectra into possible amino acid sequences. Interpretation of the spectra with Lutefisk always yields multiple sequence candidates, where it is often difficult to distinguish the correct sequence from the rest. The variations between the sequence candidates are often minor and typically involve dipeptide inversions, swapping of dipeptides of the same mass, replacements of dipeptides with single amino acids of the same mass, and the replacement of amino acids by dipeptides of the same mass. Lutefisk identifies significant ions from the spectrum, determines the N- and C-terminal evidence list, determines the sequence spectrum, generates completed sequences, and scores and ranks the completed sequences.

Analysis of the ~45 kDa protein spectra using Lutefisk generated a number of sequence candidates (Table 3.2). The amino acid sequences that ranked the highest amongst the sequence candidates from the different spectra were used for similarity searches on the NCBI database. The similarity searches revealed that these sequence candidates shared significant similarity to the thermostable lipase (LipA) from *G. thermoleovorans* (Fig. 3.15 A and B).

Table 3.2: Candidate sequences generated from the ~45 kDa protein band peptide spectrum using Lutefisk.

Sequence	Rank	Pr(c)	PevzScr	Quality	IntScr	X-corr
NPLVRFQEHR	1	0.911	0.354	1.000	0.758	0.473
NPLV[GV]FKEHR	2	0.901	0.335	1.000	0.745	0.473
NPLVRFQHER	3	0.888	0.354	1.000	0.708	0.466
NPLVRFKEHR	4	0.883	0.335	1.000	0.702	0.473
NPLV[GV]FQHER	5	0.882	0.326	1.000	0.709	0.466
NPLV[GV]FKHER	6	0.879	0.335	1.000	0.698	0.466
NPLVRFHQER	7	0.876	0.300	1.000	0.721	0.439
NPLV[GV]FHQER	8	0.869	0.279	1.000	0.718	0.439
NPLVRFHKER	9	0.866	0.284	1.000	0.709	0.439
NPLVRFKHER	10	0.863	0.335	1.000	0.664	0.466

The candidate sequences are ranked according to Pr(C) which is the estimated probability of being correct (values over 0.5 are worth submitting to a homology-based sequence database search program, and anything over 0.8 is particularly worthy of serious consideration). Pr(C) is calculated from an empirically-derived 2-order polynomial fit to a weighted average of the four remaining scores (PevzScr, Quality, IntsScr, and X-corr). PevzScr is an adaptation of the ideas presented by Dancik *et al*., (1999), which is a score that penalizes for the absence of expected ions and accounts for the possibility of random matches. Quality is the percentage of the peptide mass that can be accounted for by a contiguous ion series. IntScr is the percentage of the fragment ion intensity that can be accounted for as b, y, internal fragment, etc, ions. X-corr is the cross-correlation score that has been normalized by its auto-correlation score.

A

```
Sequences producing significant alignments:                    (Bits) E Value

gb|AAD30278.1|AF134840_1  lipase [Geobacillus thermoleovorans]...  18.1    2.1
gb|ABC48693.1|  thermostable lipase [Geobacillus thermoleovorans]  18.1    2.3
emb|CAL36912.1|  lipase [Geobacillus thermoleovorans]              18.1    2.3
gb|AAM21774.1|  thermostable lipase; LipA [Geobacillus thermol...  18.1    2.3
gb|ABD48560.1|  alpha-L-arabinofuranosidase [Geobacillus therm...  17.3    4.1
gb|ACF57670.1|  EstC [Geobacillus thermoleovorans]                 16.5    5.8
gb|ACB12767.1|  DNA-directed RNA polymerase [Geobacillus therm...  16.5    6.9
```

B

```
Lut     ------------------------------------------------------------
LipA    MKGCRVMFVLLGLWLVFGLSVPGGRAEAATSRANDAPIVLLHGFTGWGREEMFGFKYWGG


Lut     ------------------------------------------------------------
LipA    VRGDIEQWLNDNGYRTYTLAVGPLSSNWDRACEAYAQLVGGTVDYGAAHAAKHGHARFGR


Lut     --------------------EQGTLASLLQTSLLNN------------------------
LipA    TYPGLLPELKRGGRIHIIAHSQGGQTARMLVSLLENGSQEEREYAKAHNVSLSPLFEGGH
                         .**  :: : .***:*

Lut     ------------------------------------------------------EAHAL
LipA    HFVLSVTTIATPHDGTTLVNMVDFTDRFFDLQKAVLEAAAVASNAPYTSEIYDFKLDQWG
                                                                 :

Lut     LKREQG------------------------------------------------------
LipA    LRREPGESFDHYFERLKRSPVWTSTDTARYDLSVPGAETLNRWVKASPNTYYLSFSTERT
        *:** *

Lut     TLASLLQTSYPEM-----------------NQLLGPD--PLDND----------PKRGS
LipA    YRGALTGNYYPELGMNAFSAIVCAPFLGSYRNAALGIDSHWLENDGIVNTISMNGPKRGS
          .:*  . ***:                  *  ** *   *:**          *****

Lut     ----------LTNGNFNDHV-----------------------------------
Lipa    SDRIVPYDGALKKGVWNDMGTYNVDHLEIIGVDPNPSFDIRAFYLRLAEQLASFGP
                 *.:* :**
```

Figure 3.15: **A**, Similarity searches with the amino acid sequence candidates that ranked number 1 from all the spectra analysed using Lutefisk. **B**, Alignment of some of the sequence candidates with the *G. thermoleovorans* LipA amino acid sequence.

3.3.8 GE-7 lipase transcription profile

Geobacillus thermoleovorans GE-7 was cultured under induced and uninduced conditions (with and without olive oil). The cells were havested at 5 hour intervals and the culture supernatant assayed for lipase activity using the olive oil assay (Fig. 3.4, section 3.3.2). Total RNA was extracted from the cells at the 20 and 40 hour intervals (Fig. 3.17 A) and used as a template in the reverse transcription reaction (cDNA synthesis). The cDNA was used as a template in a PCR with primers specific for the GDSL lipase and LipA. The PCR with the GDSL lipase specific primers was only successful with cDNA synthesized from total RNA extracted at the 20 hour interval under uninduced conditions (Fig. 3.17 B). The GDSL lipase could not be detected under induced conditions. The PCR with the LipA specific primers was successful with cDNA synthesized from total RNA extracted at the 20 and 40 hour interval under both culture conditions [Fig. 3.17 C(a) and (b)]

.

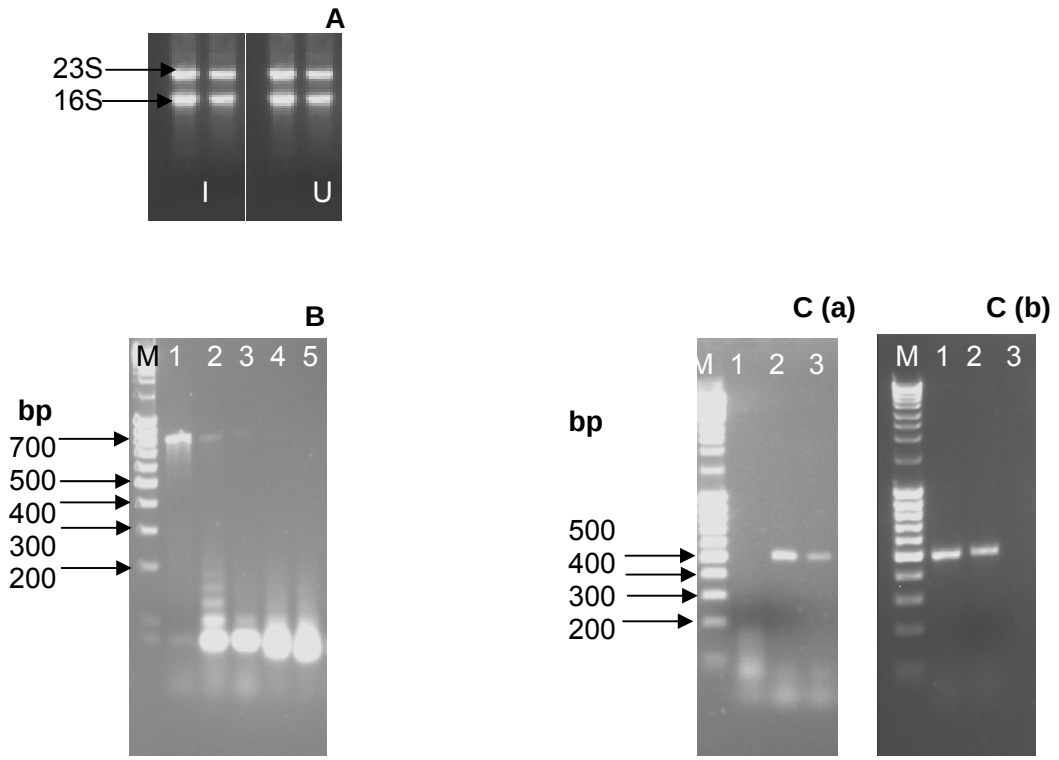


Figure 3.17: **A**, Total RNA extracted from GE-7 cells harvested at the 20 and 40 hour interval under induced (I) and uninduced (U) conditions. **B**, PCR amplification of the GDSL lipase gene from cDNA synthesized from the RNA extracted as in A. Lane M, represents the molecular weight marker, lane 1, represents the positive control (GE-7 genomic DNA), lanes 2 (20 h) and 3 (40 h), PCR performed on cDNA from RNA extracted under uninduced conditions and lanes 4 (20 h) and 5 (40 h), PCR with cDNA from RNA harvested under induced conditions. **C (a)**, PCR amplification of LipA gene from cDNA snthesized from RNA extracted under uninduced conditions (Lanes 2 and 3, 20 and 40 hour interval, respectively). **C (b)**, PCR amplification of the LipA gene under induced conditions (Lanes 1 and 2, 20 and 40 hour interval, respectively). Lanes 1 and 3 [C(a) and C(b), respectively], PCR with total RNA that was not reverse transcribed as a template (negative control).

3.4 Discussion and conclusions

As discussed in chapter 2, *G. thermoleovorans* ID-1, a strain closely related to GE-7, had been shown to produce a lipase (18 kDa) under uninduced culture conditions and LipA when the bacterium was grown in media supplemented with olive oil (Lee *et al*., 1999; 2001).

Knoesen (2004) observed that our strain of interest, GE-7, displayed a peculiar lipase production profile characterized by two activity peaks (Fig 2.1 A, Chapter 2). As a result, Barnard (2005) hypothesized that the two activity peaks represent two distinct lipases differentially expressed by the bacterium. He further hypothesized that the first activity peak represents a "scout" enzyme (a homolog to the ID-1 18kDa lipase) that is constitutively expressed by the bacterium and that the activity of this enzyme results in the accumulation of free fatty acid (products of olive oil hydrolysis) in the culture media that are taken up by the cells and result in the production/induction of LipA (~45 kDa). In 2005, Barnard purified LipA (~45 kDa) from the GE-7 culture supernatant after growth on LPM for 50 hours (second activity peak, Fig 2.1 A). This suggested that LipA was the enzyme associated with this activity peak. However, lipase activity staining with the culture supernatant harvested at this activity peak also showed two lipase active bands which indicated the presence of a second smaller lipase (Chapter 2, Fig. 2.1 B).

The presence of a lipase different from LipA was further supported by the work reported by Schmidt-Dannert *et al*. (1994) and recently by Castro-Ochoa *et al*. (2005), who purified a 16 and ~11 kDa lipase from *G. thermocatenulatus* and *G. thermoleovorans* CCR11 respectively.

In this study, we observed that GE-7 displayed different lipase production profiles characterized by two and one activity peak(s) when cultured under induced and uninduced conditions respectively (Fig 3.4). Moreover, reverse transcription PCR with cDNA from cells cultured under both conditions using *lipA* specific primers revealed that LipA is expressed under both conditions. This suggests that LipA expression occurs independent of the inducer (olive oil), contrary to what was proposed by Barnard (2005). However, the lipase activity staining experiments (Fig. 3.5) with the culture supernatant from the induced and uninduced conditions also revealed the presence of two lipolytic protein bands. This necessitated that the identity of the second lipolytic enzyme be determined.

As a result, we used the available partial amino acid sequence of the small lipase as our reference point to identifying its homolog in the GE-7 genome. The N-terminal amino acid sequences encoding the small lipase from ID-1 and *G. thermocatenulatus* were found to share very high sequence similarity. Since high sequence similarity is a characteristic feature of bacterial lipase gene families, these sequences were used in similarity searches on the NCBI database and were found to share very high sequence similarity to a hypothetically conserved

protein (HCP) from *G. kaustophilus* HTA426 and no similarity to other known lipases. The nucleotide sequence of the HCP (corresponding to the ID-1 lipase N-terminal sequence) was used to design primers for the PCR detection of a homolog to the ID-1 lipase in the genome of GE-7. The ID-1 lipase homolog was successfully detected in the GE-7 genome. However, when the HCP coding sequence (CDS) was used as template for designing primers that would facilitate the amplification of the GE-7 small lipase CDS, the PCR with these primers was unsuccessful. As a result, new primer sets were designed based on the genes flanking the HCP in *G. kaustophilus*, and they were successfully used in the amplification of the GE-7 small lipase CDS (section 3.3.4). A comparison of the nucleotide sequences encoding the HCP and the GE-7 lipase CDS revealed that the N-terminus was highly conserved while the sequence encoding the mature proteins was poorly conserved, which explained why the primers designed based on the HCP CDS did not work. A comparison of the amino acid sequences encoding the above mentioned proteins revealed that the published N-terminal amino acid sequence was the only conserved feature in this group of proteins (Fig 3.7 B). Moreover, half the sequence encoded the protein's signal peptide that is cleaved off during translocation to the extracellular environment. Furthermore, secondary structure prediction (Prosite, http://au.expasy.org/tools/scanprosite/) using the GE-7 small lipase amino acid sequence revealed that the sequence shared significant identity to the type II secretory pathway pseudopilin protein from related *Bacillus* species. Functional expression of this protein using tributyrin/olive oil as a substrate was not successful, indicating that this protein sequence does not encode a lipase, contrary to what was reported by Schmidt-Dannert *et al*., 1994 and Lee *et al*., 2001).

The GDSL lipase (section 3.3.5) was successfully cloned from the GE-7 genome, however, it was found to be active against short chain esters only. The data that had been gathered thus far ruled out the GDSL lipase and "small lipase" as role players in the observed GE-7 lipase activity/production profile. Moreover, GE-7 genomic DNA library construction and screening studies that were performed in parallel to this study by Abbai (2009) resulted in the re-cloning of *lipA* and several genes encoding known esterases. No other lipase encoding sequence was identified. As a result, the sequence based strategy to identify a lipase different from LipA was abandoned.

The lipase activity staining (section 3.3.3) suggests that the two GE-7 protein bands capable of hydrolyzing olive oil co-exist within the culture supernatant suggesting that they are not

differentially expressed. The reverse transcription PCR indicated that LipA is expressed at this point which suggests that one of the active bands represents this enzyme. The identity of the second smaller lipase active band remained a mystery.

In order to identify the smaller lipolytic protein band we employed a property that is inherent to most lipases termed interfacial activation. Lipases have been found to present a specific catalytic mechanism of action, existing in two structural forms, a closed one, where a polypeptide chain (lid) isolates the active center from the medium, and the open form, where the lid moves and the active centre is exposed (Derewenda *et al*., 1992). This equilibrium is shifted towards the open form in the presence of hydrophobic surfaces (eg. droplets of oils), where the lipase becomes adsorbed by the large hydrophobic pocket around their active centre and the internal face of the lid, a phenomenon termed interfacial activation (Verger, 1997).

Olive oil forms droplets/micelles in aqueous medium, as a result, enzymes capable of hydrolyzing olive oil should have a large hydrophobic pocket around their active centre, to promote enzyme-substrate interactions. Fernandez-Lorente *et al*., (2008) reported on a method for selective immobilization of lipases onto hydrophobic supports (using interfacial activation) that permits one-step purification of these enzymes from the support. Since LipA had been purified in a previous study from the second activity peak, GE-7 was cultured in media containing olive oil and the cells harvested at the first activity peak. The olive oil was extracted from the culture supernatant and the enzyme purification was performed using octyl-agarose and butyl-toyopearl hydrophobic supports. In each case, a protein band equivalent to LipA in size was purified. The purified protein was sent for MS/MS analysis (peptide mass fingerprinting) at the CPGR (Cape Town, S.A). Sequence data interpretation and analysis revealed that a protein sharing significant similarity to a thermostable (LipA) from *G. thermoleovorans* was purified. This data serves as a possible indication that LipA was re-purified from the GE-7 culture supernatant.

The overall data obtained in this study suggest that LipA is the only lipase that is produced by the bacterium under these culture conditions. However, Nawani and Kaur (2007) reported on the purification of two lipase isozymes (Lip1 and Lip2) that were separated by hydrophobic interaction chromatography (HIC) into two lipase activity peaks (a function of the different affinity levels of the proteins to the HIC). When the purified fractions were analyzed using SDS-PAGE

the proteins bands migrated to the same position (~60 kDa). Western blot analysis revealed that there is also cross-reactivity of antiserum against Lip1 with Lip2.

The expression of the lipase isozymes is governed by culture or fermentation conditions, pH of and the nature of the production media (Akoh *et al*., 2004b). Lipase isozymes share over 80% sequence identity but diverge in the sequence of the lid (Brocca *et al*., 2003). Therefore, the question is whether the GE-7 lipase activity profile is due to isoforms that share very high sequence similarity and are co-expressed at different levels due to the changes in the culture media or is it due to *lipA* that is up- or down-regulated by the bacterium in response to changes in the culture medium? Some of these questions will be answered in the next chapter. However, we propose in this study that LipA could be the only enzyme that is being produced by the bacterium under these conditions and that the appearance of the second active protein band on the zymogram could be due to proteolytic activity on LipA, which results in two active forms of the enzyme (a smaller and an intact form). This could also explain why Castro-Ochoa and co-workers (2005) reported on the purification of the smallest lipase known to date.

Chapter 4

The physiology of lipase production in *Geobacillus thermoleovorans* GE-7

4.1 Introduction

Lipases are defined as glycerol ester hydrolases (EC 3.1.1.3) that catalyze the cleavage of the bonds in tri-, di- and monoacylglycerols present at an oil-water interface. Lipases are produced by many microorganism and higher eukaryotes. These enzymes play a vast array of physiological roles in the different organisms, i.e., pathogen defense in some plants (Lee *et al*., 2009) and allow microorganism to grow in carbohydrate-restricted areas or environments where lipids are the sole carbon source (Stehr *et al*., 2003).

Over the years, a number of thermophilic microorganisms have been identified as important sources of industrially important enzymes. Based on total sales volume, lipases are considered to be the third largest group of industrial enzymes, subsequent to proteases and amylases. Lipases are frequently used because they catalyze various useful reactions (Miyawaki and Nakamura, 2002). Moreover, the industrial processes in which lipases are employed normally function at temperatures exceeding 45°C, thus, the enzymes employed are required to at least have an optimum temperature of around 50°C (Sharma *et al*., 2001). As a result, a number of studies have concentrated on isolating thermophilic lipase producers, identifying the lipase genes, understanding and optimizing the production of lipases from these thermophilic hosts (Ginalska *et al*., 2003; Ertuğrul *et al*., 2007; Shariff *et al*., 2007).

Microbial lipases are mostly extracellular and their production is greatly influenced by medium composition apart from physicochemical factors such as temperature, pH and dissolved oxygen (Sharma *et al*., 2001, Gupta *et al*., 2004). The addition of lipid carbon sources to the culture medium seems to be essential for obtaining high lipase yields. However, nitrogen sources and essential micronutrients should also be carefully considered for growth and optimization of lipase production (Nesbit and Gunasekaran 1993; Dharmsthiti and Luchai, 1999). In order to optimize lipase production, the concentration of each compound that constitutes a cultivation medium has to be evaluated. The classical procedure of changing one variable at a time was found to be inefficient since it does not explain the interaction among variables and their effects on the fermentation process (Rodrigues and Iemma, 2005). To circumvent this, factorial designs

that allow the efficient screening of key variables for further optimization in a rational manner have been successfully employed (Abdel-Fatta, 2002; Rodrigues and Iemma, 2005).
In 2004, Knoesen reported on the factorial-type design of the *G. thermoleovorans* GE-7 lipase production media (LPM). The factorial design focused on different combinations of carbon, nitrogen and types of inducers that were individually varied and their effect on growth and lipase production monitored. When GE-7 was cultured in the optimized LPM supplemented with olive oil (inducer), a peculiar production profile characterized by two lipase activity peaks was observed (Fig. 2.1 A). Moreover, the lipase activity staining experiment indicated the presence of two lipase active bands in the culture supernatant (Fig. 2.1 B), which served as a possible indication that the two activity peaks could be representing two lipases.

We have however, shown in the previous chapter that lipase production in GE-7 occurs independent of the inducer and that the zymogram (lipase activity stain) from both the induced and uniduced culture showed the presence of two lipolytic bands. Moreover, the lipase expression profiling PCR revealed that LipA is produced under both culture conditions and at both peaks under induced conditions. However, the differences in the lipase production and the similarity in the lipase activity stain profile observed under both culture conditions are not understood. As a result, we present in this study attempts to uncovering factors that could contribute to the perculiar lipase profile that is observed when GE-7 is cultured in the presence of olive oil (Inducer).

4.2 Methods and materials

4.2.1 Growth and lipase production

GE-7 growth and lipase production was performed as described in section 3.2.2.2. However, when required the media was supplemented with stearic acid as the inducer instead of olive oil.

4.2.2 Induction studies

4.2.2.1 Shake flask cultivation

Unless otherwise stated, shake flask cultivations were performed in 500 ml shake flasks containing 60 ml of the appropriate induction media. These were inoculated from the same source, receiving 5 ml of an inoculum measuring 65 Klett units (using a Klett-Summerson colorimeter). Pre-inocula were all grown on the standard lipase production media (excluding inducer).

4.2.2.2 Bioreactor cultivation

A Scientific Multigen F-2000 benchtop bioreactor (Edison, N.J, U.S.A) was used for batch cultivations. The temperature was thermostatically controlled at the optimum temperature for production and it was necessary, at the elevated experimental temperatures, to insulate the glass reactor vessel by using a space blanket (Cape Union Mart). The pH was monitored with a pH probe (Mettler Toledo, Halstead, U.K) and controlled at the specific set pH by the automated addition of 3 M KOH and 3 M H_2SO_4. A polarographic oxygen probe (Ingold AG, Urdorf, Switzerland) was used to measure dissolved oxygen tension. The dissolved oxygen tension was kept above 40% saturation using manually increased aeration rate and stirrer speed during exponential growth.

4.2.3 Analytical methods

4.2.3.1 Biomass determination

Cyclohexane (2 ml) and 5M NaOH (400 µl) were added to samples (4ml) of broth in test tubes, vortexed for 5 min and then filtered under vacuum through dried and pre-weighed glass fibre filters (GF52 47MM BX200; Schleicher and Schuell). The biomass on the filter was washed with a mixture of distilled water (4 ml), cyclohexane (2 ml) and 5 M NaOH (400 µl) followed by washing with distilled water (26 ml). Biomass on filters was dried overnight in an oven (100°C), and then cooled in a desiccator before weighing.

4.2.3.2 Glucose utilization

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

4.2.3.3 Lipase activity assay, fatty acid liberation and utilization

The lipase activity was monitored using the lipase specific olive oil assay as described in section 3.2.3.2. The method used to monitor the free fatty acids present in the culture medium was a further modification of the lipase specific assay (olive oil assay). The cells were harvested at specific time intervals, cells centrifuged at 10 000 *xg*, residual enzyme activity in the supernatant was inhibited by the addition of the copper reagent [$Cu(NO_3)_2.3H_2O$ and tri-ethanolamine], the free fatty acids extracted from the supernatant and quantified.

4.2.4 PCR cloning and sequence analysis of the GE-7 *lipA* promoter region

Primer set, lipA-Pro1F and lipA-Pro1R was designed for the amplification of the GE-7 *lipA* promoter region. The forward primer (lipA-Pro1F) was designed based on a sequence 200 bp upstream of the *lipA* transcription start site in *G. kaustophilus* HTA426 while the reverse primer was designed using the region in the GE-7 *lipA* sequence that was 10 bp downstream the transcription start site. The primer set was used in a PCR with genomic DNA from *G. kaustophilus* (control) and GE-7 (experiment) as a template. The PCR product obtained with the experiment was subcloned into pGEM-T Easy and used to transform *E. coli* cells. Positive clones were selected on LB media (IPTG, X-Gal and ampicillin), plasmid isolated and restricted using *Eco*RI. Plasmids carrying the correct insert were selected and sent for sequencing at Inqaba Biotech. The sequences were analyzed using the SoftBerry BPROM algorithm for prediction of bacterial promoter regions (http://linux1.softberry.com) and searched manually for consensus sequences that could be indicative of possible regulatory mechanisms.

4.2.5 Semi-quantitative GE-7 *lipA* expression profiling

Geobacillus thermoleovorans GE-7 was cultured as described in section 4.2.3.2 and the cells harvested at 10 hour intervals. Total RNA and mRNA was isolated from the cells using the RNeasy (Qiagen, Valencia, CA, USA) and MICROBExpress Bacterial mRNA Enrichment Kit (Ambion Inc., Austin, Texas, USA), respectively. Complementary DNA (cDNA) was synthesized from the mRNA using the Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA USA). The cDNA and RNA were quantified using the NanoDrop spectrophotometer (Thermo Scientific, Wilmington USA). The cDNA from the different time intervals was diluted to equal concentrations and used in a PCR using equal concentrations of primers GTlipA-F and GTlipA-R (Table 3.1). The PCR products were analyzed electrophoretically on a 1.5% agarose and the differences in the band intensities under UV light were interpreted as indicative of the levels of *lipA* expression.

4.3 Results

4.3.1 Shake flask induction studies

Geobacillus thermoleovorans GE-7 was cultured in LPM supplemented with olive oil, LPM (olive oil as the sole carbon source) and LPM supplemented with stearic (Fig. 4.1 A, B and C, respectively). The lipase production and the amount of free fatty acids in the culture media were quantified as described in section 4.2.4.3. Two lipase activity peaks were observed when GE-7 was cultured in LPM supplemented with olive oil. However, the appearance of the second activity peak was preceded by the uptake of the fatty acids by the cells (Fig. 4.1 A). A lipase production profile characterized by multiple activity peaks was observed when GE-7 was cultured in the presence of stearic acid however; in this case the appearance of the activity peaks was concurrent with the uptake of the stearic acid by the cells (Fig. 4.1 C). When glucose was omitted from the medium and olive oil was used as the sole carbon source, the two activity peaks were observed (Fig. 4.1 B). However, significant activity and the appearance of the peaks could be observed earlier than in Figure 4.1 A.

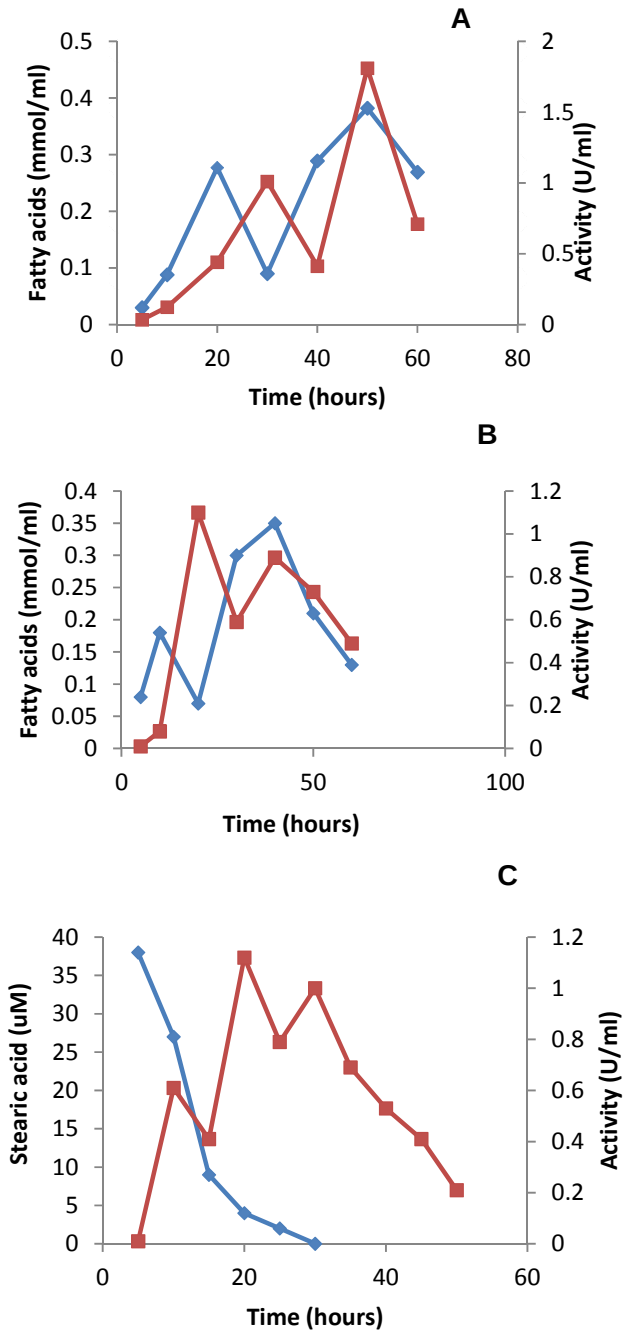


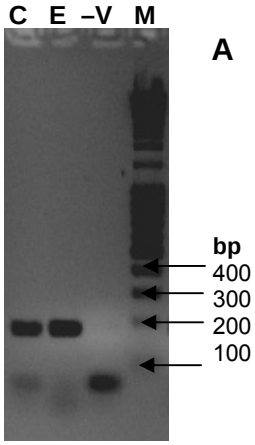
Figure 4.1: **A**, Lipase production profile (■) and the amount of free fatty acid (♦) when GE-7 was cultured in LPM (olive oil). **B**, Lipase production profile (■) and the amount of free fatty acids when GE-7 was cultured (♦) in LPM with olive oil as the sole carbon source. **C**,GE-7 Lipase production profile (■) in LPM supplemented with stearic stearic acid [(40 uM, stearic acid consumption (♦)]. The avarages of triplicate repeats are shown on each graph.

4.3.2 PCR cloning and sequence analysis of the GE-7 *lipA* promoter region

Primers for the amplification of the *lipA* promoter region were designed as described in section 4.2.5. The PCR resulted in the amplification of a ~200 bp product from both the GE-7 (experiment) and *G. kaustophilus* HTA426 (control) genomic DNA (Fig. 4.2 A). The PCR product was sequenced and sequence analysis revealed that it shares very high sequence similarity with the *lipA* promoter regions from *G. thermocatenulatus* and *G. kaustophilus* HTA426 (Fig. 4.2 B). The *lipA* promoter regions from the three bacteria were analyzed using the SoftBerry BPROM algorithm and the putative -35 and -10 conserved sequences were identified with a ~10 bp spacer region separating them (Fig. 4.2 B).

A number of *Bacillus subtilis* genes encoding enzymes involved in the degradative pathways (e.g. *lipA*, *araB* and *amyE*) (Eggert *et al*., 2003; Baerends *et al*., 2004) have been found to harbor the catabolite responsive element (CRE) box on their promoter regions. The CRE-box serves as a docking site for proteins that control catabolite utilization. The CRE-box is a highly conserved sequence [ATG(A/T)AA(A/G)CGTTA(A/T)CA] that has been identified in most genes to be close or overlapping with the -10 consensus sequence (Henstra *et al*., 1999). As a result, the promoter regions of the 3 genes were searched manually for the CRE-box which was identified and found to be overlapping with the -10 sequence (Fig. 4.2 B).

Most genes that harbor the CRE-box on their promoter regions are repressed in the abundance of simple sugars (e.g. glucose). As a result, GE-7 was cultured in LPM supplemented with olive oil and growth monitored until the onset of the stationary phase of growth (10 hour interval, indicative of the depletion of glucose). The cells were then spiked with additional glucose (5 g/l), lipase production, fatty acid release and utilization was monitored using the olive oil assay from the 10 hour interval. Contrary to Fig. 4.1 A, a sharp drop in the lipase activity was observed at the 20 hour interval and the uptake of the free fatty acids was delayed to after the 30 hour interval (Fig. 4.2 C).



B

```
G.stearothermophilus      TCACAGAAAAACCC-GACT--TGCCGGGATTGTCAG--TT-GGTTTGATATATATAGTAT 54
G.thermoleovorans         TCACAGAAAAACCCAGACTATTGCCGGGATTGTCAG--TTTGGTTTGATATATAGTAT 58
G.kaustophilus            TCATATAGGAACCCCGACAATTGCAGGGGTTGAAGGGCCTTGGTCTTATGTATAAAATAT 60
                          *** * *  ***** ***   *** *** ***   *   * *** * ** **** * ***

                                   CRE-box                          -35
G.stearothermophilus      TCAGGTTATATGCA---AAAAGACTCCGGTTTATGTGAGGGGAGGAGGGATAGGATGATG 110
G.thermoleovorans         TCAGGTTATATGCATACAAAAGACTCCGGTTTATGTGAGGGGAGGAGGGATAGGATGATG 117
G.kaustophilus            TCTGGTAATTCAGAATGAAAAGATTTCGGCCTATTGAAGGAAAGGAGAGGGAGAAC-ATG 119
                          ** *** ***    *   ****** * ***  ***   ***  ***** *  ** *  **
                             -10                                              Start codon
```

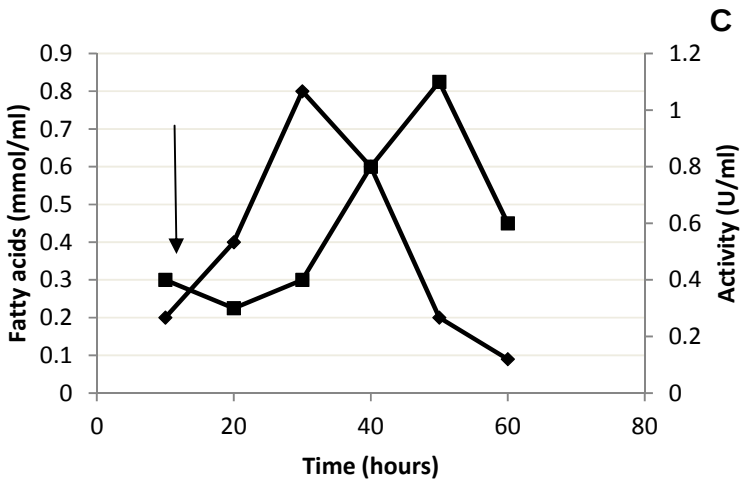


Figure 4.2: **A**, Agarose gel electrophoresis of the PCR amplified *lipA* promoter region from *G. kaustophilus* HTA426 (C, control) and GE-7 (E, experiment). Lane M and –V represent the molecular weight marker and the negative control, respectively. **B**, Alignment of the *lip*A promoter regions from *G. thermoleovorans, G. thermocatenulatus* and *G. kaustophilus*. The sequence in red represents the putative CRE-box consensus sequence. The -10 and -35, respectively represents the putative conserved promoter regions. **C**, Lipase production (■) and free fatty acids (♦) profile observed when

GE-7 was cultured in LPM (olive oil) supplemented with additional glucose at the 10 hour interval (indicated by the arrow).

4.3.4 Bioreactor cultivation

The optimum temperature and pH for lipase production were reported to be 55°C and pH 6.5, respectively (Knoesen, 2004). As a result, GE-7 was cultured in the Multigen F-2000 bioreactor maintained at the above-mentioned temperature and pH in LPM supplemented with 2.5 g/l olive oil. These experiments show the consumption of glucose together with the appearance of fatty acids and lipase during cultivation. The initial exponential growth of the organism led to a rapid decline in glucose, accompanied by an expected rapid consumption of oxygen (Fig. 4.3). In the late exponential to stationary phase, the rate of glucose consumption dropped. During stages of decelerated growth, the rate of oxygen consumption also slowed. Low levels of lipase started to appear at this stage. It is unlikely that the culture experienced carbon limitation as the residual glucose levels were 4-5 mM and fatty acids started to appear (Fig. 4.3).

The slow rate of lipase production from time 5 – 15 h produced small amounts of free fatty acids that appeared in the medium. A dramatic increase in lipase activity and free fatty acids, together with a second phase of consumption of the remaining glucose and a second drop in the dissolved oxygen levels (<5%) followed in the early stationary phase. This was followed by a drop in the lipase activity which preceded an uptake of the fatty acids by the cells and a second sharp increase in the activity (two activity peaks).

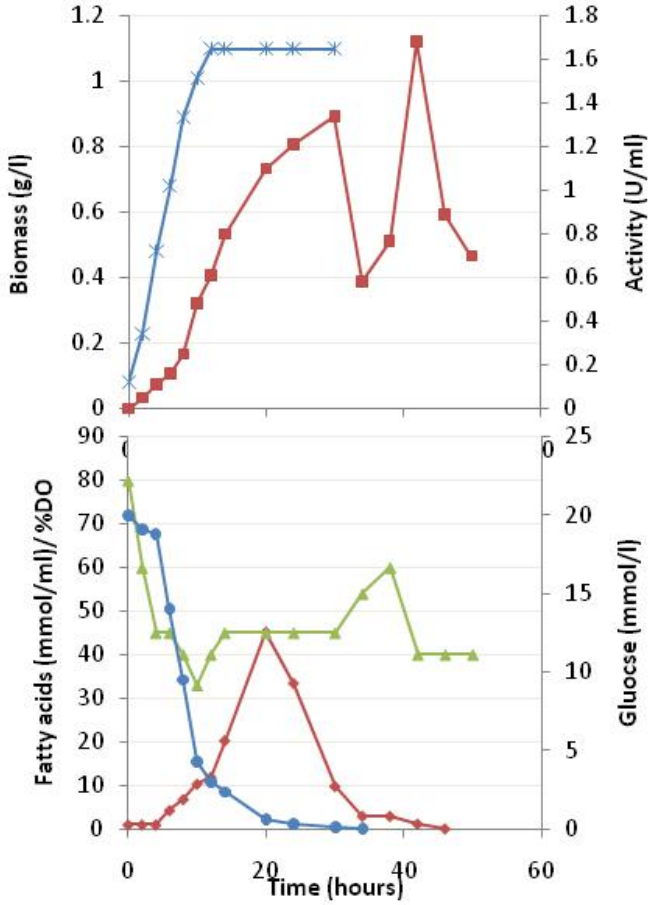


Figure 4.3: Graph showing the relationship between growth (ж), glucose consumption (•), % dissolved oxygen (%DO, ▲), extra cellular lipase activity (■) and the subsequent release and utilization of fatty acids (♦). Cultivation was performed in lipase production media (2.5 g/l olive oil) and pH 6.5.

4.3.5 Semi-quantitative GE-7 *lipA* expression profiling

Geobacillus thermoleovorans GE-7 was cultured as discussed in section 4.3.4. The cells were harvested at the 30, 40, 50 and 60 hour interval (Fig. 4.3) and total RNA extracted from the cells using the RNeasy kit (Fig. 4.4 A). Mature RNA (mRNA) was isolated from total RNA using the MICROBExpress kit and quantified using the NanoDrop spectrophotometer. The mRNA concentration from all the samples was diluted down to 20 ng/µl and reverse transcribed

using the Quantitect Reverse Transcription kit. The cDNA generated from the mRNA was quantified to be 500, 476, 382 and 342 ng/µl from the samples harvested at the 30, 40, 50 and 60 hour interval, respectively. The concentration of the cDNA was diluted down to 30 ng/µl and 5 µl of each dilution was used as a template in PCR with GTlipA-F and GtlipA-R as primers. A 500 bp amplicon was obtained with all the templates (Fig. 4.4 B). The amplicon intensities were compared under UV-light and it was observed that the amplicons from the 30 and 50 hour intervals were more intense while the amplicons from the 40 and 60 hour were less intense (Fig. 4.4 B) which corresponds to the lipase activity levels observed in Figure 4.3.

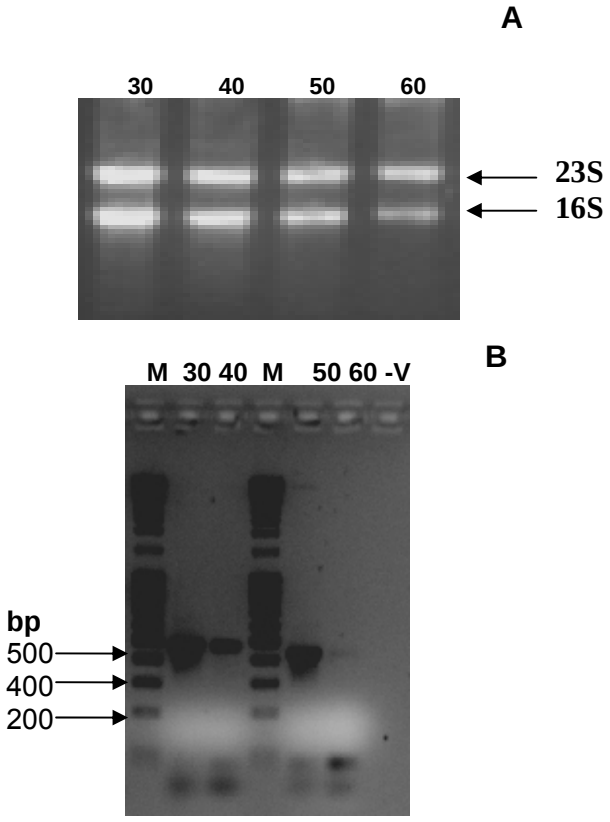


Figure 4.4: **A**, Total RNA extracted from the GE-7 cells cultured as in section 4.3.4 harvested at the 30, 40, 50 and 60 hour interval. **B**, Agarose gel electrophoresis of the PCR products amplified from the cDNA that was synthesized from total RNA harvested as mentioned above. Lane M represents the molecular weight marker and lane –V, represents the PCR with DNase treated RNA as a template (negative control).

4.3.6 Discussions and conclusion

In the previous chapter, we reported on the attempts to isolate from GE-7 a lipase that is distinct to LipA. However, all our attempts resulted in the re-isolation of LipA. As a result, the reason behind the two lipase production peaks observed when GE-7 is cultured in LPM supplemented with olive oil is yet to be answered.

In this chapter, GE-7 was cultured in LPM with olive oil as the sole carbon source and the resultant lipase production profile was compared to that observed when the cells were cultured in LPM (olive oil). In both cases, two lipase production peaks were observed, however, in the former (Fig. 4.1 A), it was observed that the uptake of free fatty acids (FFA) by the cells and the appearance of the second activity peak occurred 10 hour earlier than in the latter (Fig. 4.1 B). This change in production profile that was the result of the exclusion of glucose from the culture medium, suggested that the presence of glucose in the medium suppresses lipase production, an indication of a possible gene regulation mechanism.

In 2003, Eggert and co-workers observed that, a number of *Bacillus subtilis* genes encoding enzymes involved in the degradative pathways (e.g. *lipA*, *araB* and *amyE*) haboured the catabolite responsive element (CRE) box on their promoter regions. The CRE-box is a *cis*-regulatory element that serves as a docking site for *trans*-acting factors (e.g. Ccpa, catabolite control protein) that regulate catabolite utilization. The lipase production profiles observed in section 4.3.1 suggest that the presence of glucose in the culture media resulted in the delay in appearance of significant lipase activity and subsequent FFA uptake by the cells, which served as a possible indication of the catabolite repression regulatory mechanism. In order to investigate this, the promoter region of the GE-7 *lipA* was amplified and sequenced, and the sequence analyzed using the SoftBerry BPROM algorithm. The putative -10 and -35 consensus promoter sequences were identified. The highly conserved CRE-box was identified in close proximity to the -10 consesus sequence as reported by Henstra *et al*. (1999). To investigate whether the CRE-box played a role in the regulation of LipA production, GE-7 was cultured in LPM (olive oil) and allowed to grow until the 10 hour interval (the onset of the stationary phase) and then the cells were spiked with additional glucose to a final concentration of 5 g/L. The lipase activity, fatty acid accumulation and uptake were monitored using the olive oil assay. The results revealed that spiking the cells with additional glucose resulted in a change in the lipase production profile. There was a reduction in the lipase activity after the addition of glucose and the uptake of FFA was delayed to after the 30 hour interval and a single lipase activity peak was

observed at 50 hour interval (Fig. 4.2 B). These results and the presence of the CRE-box in the GE-7 *lipA* promoter region suggest that the gene could be subject to catabolite repression. Moreover, the fact that the production profile was changed from two peaks to one by the addition of glucose suggest that LipA could be the only lipase that is being produced under these culture conditions and that the peaks are only a manifestation of how *lipA* is regulated in response to certain components of the culture medium.

When GE-7 is cultured in LPM without olive oil, one lipase peak (corresponding to the first peak under induced conditions) is observed (Chapter 3, section 3.3.2) and a second peak does not appear. It has been observed that the appearance of the second activity peak (under induced conditions) is preceded by the uptake of the FFA. Which serves as a possible indication that fatty acids could serve as a signal for further lipase production. In 2007, Feng reported on the molecular characterization of a *Fusarium graminearum* lipase gene and its promoter region. The author identified a fatty acid responsive element (FARE) in the promoter region and confirmed it to be responsible for induction of lipase production by fatty acids. In this study, multiple lipase activity peaks were observed concurrent with the FFA uptake when GE-7 was cultured in LPM supplemented with stearic acid (Fig. 4.1 C), which served as a possible indication that fatty acids play a regulatory role in LipA production. Therefore, we hypothesize that the promoter region of the GE-7 *lipA* habours a fatty acid responsive element, as a result the rapid uptake of the fatty acids by the cells result in a momentary down-regulation of *lipA* and a subsequent up-regulation upon fatty acid utilization *via* the β-oxidation pathway and (or) intracellular accumulation as a stored source of energy.

In order to correlate lipase production to the concentrations of certain components of the culture medium, bioreactor cultivation studies were performed (Fig. 4.3). The initial exponential growth led to a rapid decline in glucose accompanied by a slow rate of lipase production and release of free fatty acids. A possible indication that lipase production occurs constitutively and that the presence of glucose represses over-production. Upon the depletion of glucose, lipase production and the FFA concentration in the medium increased. This was followed by a rapid uptake of the fatty acids by the cells and a subsequent momentary drop in the lipase production (first peak) which was followed by the second peak. This observation supports the hypothesis that the fatty acids play a regulatory role in the lipase production. The rapid fatty acid liberation and subsequent consumption (the availability of free carbon) does not cause a secondary exponential phase (Diauxic growth). The possibility still exists that β-oxidation could yield large

amounts of ATP not necessarily utilized for biomass formation. On the other hand, electron microscopy data (DeFlaun *et al*., 2007) showed that fatty deposits do accumulate in the cells in the presence of a lipid substrate. Therefore, the drop in free fatty acid concentration could also be attributed to storage of the fatty acids in these lipid deposits.

The results that have been accumulated thus far suggest that GE-7 only produces one lipase (LipA), and that the peak and drop in the production could be indicative of a possible *lipA* regulatory mechanism. This necessitated that the *lipA* transcript levels be correlated with the observed activity peaks in order to rule out the presence of a second lipase different from LipA. As a result, a semi-quantitative PCR approach was employed. Cells were harvested from the bioreactor cultivation at the 30, 40 and 50, 60 time intervals, respectively for the first and second peak and drop. Total RNA was extracted and from this mRNA was isolated and quantified. The concentration of mRNA from each sample was diluted to equal concentrations and subjected to reverse transcription. The cDNA that was generated from each sample was quantified and diluted to equal amounts and used as a template in a PCR with *lipA* specific primers. A comparison of the PCR products intensities on the agarose gel revealed that the *lipA* transcript level at the 30 and 50 hour intervals (corresponding to the peaks) were higher that the levels at the 40 and 60 hour intervals (corresponding to the drops). Although the technique employed in this study relied on end-point analysis instead of analyzing the amplification reaction in real time, the results indicated a clear difference in the *lipA* transcript levels at the peaks and drops and correlated with the observed activity levels.

Therefore, contrary to previous reports, GE-7 does produce only one lipase (LipA) under the culture conditions investigated in this study. The differences in the production levels and profiles under the various culture conditions indicate that certain medium components (fatty acids etc.) are directly involved in the regulation of lipase production and indicate a possible *lipA* regulation mechanism.

Chapter 5
Summary and concluding remarks

Geobacillus thermoleovorans GE-7 was isolated from the West-Driefontein goldmine in South Africa. It is a Gram-positive rod showing optimal growth at 65°C. This isolate was found to be able to grow on olive oil as the sole carbon source and on a variety of other lipid substrates, a feature which indicated that the bacterium produces lipases.

In 2004, studies aimed at elucidating factors that would improve lipase production by *G. thermoleovorans* revealed that when the bacterium was cultured in medium optimized for lipase production, a production profile characterized by two enzyme activity peaks was observed. In 2005, a lipase (LipA) open reading frame (1251 bp) was amplified from the bacterium's genome. Furthermore, lipase purification studies from the GE-7 culture grown in media containing olive oil resulted in the purification of a lipase (~45 kDa), which corresponded to the LipA ORF that had been amplified from the GE-7 genome. However, lipase activity staining with the supernatant from GE-7 cultured in media supplemented with olive oil revealed the presence of two lipolytic protein bands of different sizes. These observations and reports on the purification of a smaller lipase in addition to LipA from the culture supernatant of related *Geobacilli* led to the hypothesis that the two lipase production peaks observed with GE-7 represent two distinct lipases differentially expressed by the bacterium.

The aim of this study was to characterize the lipolytic activity from this bacterium using molecular and proteomic techniques. The primary objective was to identify from GE-7 a lipase different from LipA and to profile its expression relative to that of LipA. Three authors had already reported on the purification of a smaller lipase (>20 kDa), however only two authors had published sequence information in this regard (Schmidt-Dannert *et al*., 1994; Lee *et al*., 2001). Since bacterial lipolytic enzyme families are characterized by very high intra-family sequence similarity, the published N-terminal amino acid sequences were used for similarity searches on the database to identify homologs of this protein from related *Geobacilli*. Similarity searches revealed that the published sequences shared very high similarity with a hypothetically conserved protein (HCP) from *G. kaustophilus* and no homology to any other known lipase. The nucleotide sequence of the HCP was used to design primers to facilitate PCR amplification of the homolog from GE-7. The "small lipase" ORF (440 bp) was amplified from the GE-7 genome,

however, functional expression using tributyrate/olive oil-Rhodamine plate assays revealed that the protein was not lipolytic. Furthermore, secondary structure predictions using the “small lipase” ORF revealed that the sequence shared significant identity to the type II secretory pathway pseudopilin protein from related *Bacillus* species contrary to what was reported by the two authors.

As a result of the above-mentioned findings a protein purification strategy aimed at isolating a lipase different from LipA was pursued. Since LipA was purified in a previous study from the second lipase peak and given that it was hypothesized that the first peak indicated the production of a lipase different from LipA, GE-7 was cultured, lipase production monitored and the cells harvested prior to the onset of the second peak. Lipase purification experiments from the culture supernatant corresponding to the second peak resulted in the isolation of a ~45 kDa protein. The protein band was analyzed by peptide mass fingerprinting and the amino acid sequence determined. Sequence analysis revealed that the protein that was purified from the first production peak is LipA, which served as an indication that LipA was produced at both peaks.

GE-7 was cultured in media with (induced) and without (uninduced) the lipid substrate (olive oil) and the lipase production profiles compared. It was observed that under induced conditions the two production peaks persisted while only one peak was observed in the uninduced. However, reverse transcription-mediated PCR on RNA isolated from GE-7 cultured as described above with primers specific for *lipA* revealed that LipA is produced under both culture conditions. Moreover, LipA is present at both production peaks under induced conditions which supports the purification results. Detection of LipA from the uninduced culture indicates the GE-7 is constitutively expressed, contrary to the previous reports.

Bioreactor studies aimed at relating the effect of media components on lipase production by GE-7 revealed that, under induced conditions, significant lipase production is only observed after the depletion of glucose and that the second activity peak is only observed upon the uptake of the free fatty acids by the cells. This observation suggested the fatty acids could act as a signal for further lipase production which was supported by the one lipase production peak observed under uninduced conditions (no fatty acids in the culture medium).

The observation that significant lipase production was observed at the stationary phase when the glucose concentration became limiting served as a possible indication of a mechanism of gene expression regulation known as catabolite repression. Which suggests that *lipA* could be down-regulated in the presence of simple sugars (glucose) and up-regulated upon depletion of glucose to afford the bacterium the ability to utilize olive oil as a carbon source. A conserved sequence (CRE-box) has been identified on the promoter regions of a number of genes that are subject to this mode of regulation. As a result, the *lipA* promoter region was amplified and sequenced. Analysis of the sequence revealed the presence of the CRE-box. Experiments were conducted to investigate whether catabolite repression was a possibility for the GE-7 *lipA*. GE-7 was cultured under induced conditions, lipase activity monitored and the cells spiked with additional glucose at the onset of the stationary phase. A significant drop in the lipase production was observed subsequent to the spike.

The above-mentioned observations and experiments where olive oil (used as a sole carbon source) or stearic acid was used as the inducer revealed that changing certain components of the media changed the lipase production profile. All the findings in this study suggest that LipA is the only lipase produced under the culture conditions investigated and that the changes in the production profile could be due to the regulation of the gene in response to changes in the culture medium.

Hoofstuk 5
Opsomming

Geobacillus thermoleovorans GE-7 is geïsoleer vanaf die Wes-Driefontein goudmyn in Suid-Afrika. Die bakterium is 'n gram positiewe stafie wat optimale groei toon by 65˚C. Dit is vasgestel dat hierdie bakterium in staat is om op olyfolie te groei as enigste koolstofbron asook verskeie ander lipied substrate – 'n eienskap wat daarop dui dat hierdie bakterium lipase-ensieme vervaardig.

In 2004 is 'n studie van stapel gestuur om die faktore te bepaal wat lipase vervaardiging in *G. thermoleovorans* GE-7 sou verbeter. Tydens hierdie studie is die bakterium gekweek op medium wat geoptimiseer was vir lipase vervaardiging. Daaropvolgende ensiemsuiweringseksperimente het twee aktiwiteitspieke getoon. In 2005 was 'n lipase (*lipA*) oop leesraam (OLR) van 1251 bp geamplifiseer vanaf die bakterium se genoom. Ensiemsuiwerings eksperimente vanaf die GE-7 kultuur, wat gekweek is op olyfolie, het 'n 45 kDa lipase ensiem opgelewer wat ooreengestem het met die *lipA* OLR van GE-7. Lipase aktiwiteitskleuring met die bovloeistof van die olyfolie-gekweekte GE-7 kultuur het egter twee lipolitiese proteïenbande van verskillende molekulêre groottes opgelewer. Hierdie bevindinge, tesame met ander onafhanklike navorsing rakende die suiwering van 'kleiner' lipase ensieme, *lipA* inkluis, vanaf die bovloeistof van ander naby-verwante *Geobacilli* spesies, het gelei tot die hipotese dat die twee lipase aktiwiteitspieke twee eiesoortige lipase ensieme verteenwoordig wat tydens versillende fases van die bakterium se lewensiklus uitgedruk word.

Die doelwit van hierdie studie was om die lipolitiese aktiwiteit van die GE-7 bakterium te karakteriseer deur gebruik te maak van molekulêre- en proteomikategnieke. Die hoofdoelwit was om 'n lipase ensiem te indentifiseer wat verskillend was van die LipA proteïen en om die uitdrukkingsprofiel te vergelyk met dié van LipA. Drie verskillende outeurs het melding gemaak van die suiwering van 'kleiner' lipase ensieme (>20 kDa), maar slegs twee van die outeurs het die aminosuurvolgorde van hierdie lipase ensieme gepubliseer (Schmidt-Dannert *et al*., 1994; Lee *et al*., 2001). Aangesien bakteriële, lipolitiese ensieme gekenmerk word deur intra-familie aminosuuropeenvolgingooreenkomste, was die N-terminale aminosuurvolgorde gebruik in 'n databasis soektog om soortgelyke proteïene van verwante *Geobacilli* op te spoor. Die databasis

soektog het getoon dat gepubliseerde aminosuurvolgorde data baie ooreenkomste toon met ’n gekonserveerde hipotetiese proteïen (GHP) vanaf *Geobacillus kaustophilus* en dat dit geen ooreenkomste getoon het met bekende lipase ensieme nie. Die nukleïensuurbasispaaropeenvolging van die GHP was gebruik om priemstukke te ontwerp om sodoende die LipA-verwante lipase van GE-7 te PKR-amplifiseer. Die ’kleiner’ lipase (OLR van 440 bp) was geamplifiseer deur gebruik te maak van die GE-7 genoom. Funksionele uitdrukkingseksperimente m.b.v. Tributiraat/olyfolie-Rodamien plate het getoon dat die proteïn geen lipolitiese eienskappe besit nie. Verder het sekondêre struktuurvoorspellings met behulp van die ‘klein’ lipase OLR getoon dat die aminosuurvolgorde beduidende ooreenkomste toon met die tipe II sekretoriese weg pseudopilin proteïen van ‘n verwante *Bacillus* spesie wat in teenstelling is met wat gemeld is deur die twee bogenoemde outeurs.

Na aanleiding van die bogenoemde bevindinge is 'n proteïensuiweringseksperiment ontwikkel wat gemik was op die isolering van ‘n lipase ensiem wat verskillend van LipA is. Aangesien LipA alreeds gesuiwer was uit ‘n vorige studie (vanaf die tweede aktiwiteitspiek) en gegee die feit dat dit vermoed word dat die eerste piek ‘n aanduiding was van die produksie van 'n lipase ensiem verskillend van LipA, was GE-7 gekweek, lipase produksie gemonitor en die selle geoes voor die aanvang van die tweede aktiwiteitspiek. Lipase suiweringseksperimente uit die kultuur bovloeistof, wat ooreenstem met die tweede aktiwiteits piek, het gelei tot die isolasie van 'n ~ 45 kDa proteïen. Die proteïenband was ontleed deur ‘n peptiedmassa vingerafdrukke tegniek en die aminosuurvolgorde was bepaal. Volgorde-analise het getoon dat die proteïen wat uit die eerste aktiwiteits piek gesuiwer was, LipA was. Hierdie was ‘n sterk aanduiding dat LipA vervaardig was in beide pieke.

GE-7 is gekweek in media met (geïnduseerd) en sonder (ongeïnduseerd) die lipiedsubstraat (olyfolie) en die twee lipase produksieprofiele was vergelyk. Dit was waargeneem dat onder geïnduseerde toestande die twee produksiepieke altyd teenwoordig was, terwyl slegs een piek waargeneem kon word tydens ongeïnduseerde toestande. Maar, omgekeerde transkripsie-gefasiliteerde PKR met RNA geïsoleer vanaf GE-7, gekweek soos hierbo beskryf met priemstukke spesifiek vir LipA, het getoon dat LipA geproduseer was onder beide kultuur toestande. Daarbenewens was LipA ook teenwoordig by beide produksie pieke onder geïnduseerde toestande wat gevolglik die suiwerings resultate ondersteun. Die indentifisering van LipA vanaf ‘n ongeïnduseerde kultuur dui op die feit dat GE-7 konstitutief uitgedruk word. Hierdie is in teenstelling met vorige gepubliseerde inligting.

Bioreaktorstudies wat daarop gemik was om die effek van mediakomponente op lipase produksie deur GE-7 te ondersoek, het onthul dat onder geïnduseerde toestande, beduidende lipase produksie slegs waargeneem is na die uitputting van glukose en dat die tweede aktiwiteitspiek slegs waargeneem kon word tydens die opname van vrye vetsure deur die selle. Hierdie waarneming het aangedui dat die vetsure kan optree as 'n chemiese sein vir verdere lipase produksie wat deur die een lipase aktiwiteitspiek ondersteun word soos waargeneem onder ongeïnduseerde toestande (geen vetsure in die groeimedium nie).

Die waarneming van beduidende lipase produksie soos waargeneem tydens die stasionêre groeifase, wanneer die glukose konsentrasie skerp gedaal het, dien as 'n moontlike aanduiding van 'n meganisme van geen regulasie bekend as kataboliet-onderdrukking. Dit kan gevolglik impliseer dat LipA af-gereguleer word in die teenwoordigheid van eenvoudige suikers (glukose) en op-gereguleer word tydens glukose-uitputting. Gevolglik sal die bakterium die olyfolie gebruik as 'n koolstof bron. ‘n Gekonserveerde gebied (CRE-gebied) is geïdentifiseer op die promotorgebiede van 'n aantal gene wat onderhewig is aan hierdie tipe vorm van regulering. As gevolg hiervan, was die LipA promoter geamplifiseer en die nukleïensuurbasispaar opeenvolging daarvan bepaal. Analise van die volgorde het die teenwoordigheid van die CRE-gebied bevestig. Eksperimente is uitgevoer om die moontlikheid te ondersoek dat die GE-7 LipA onderhewig is aan kataboliet-onderdrukking deur glukose. GE-7 was gekweek onder geïnduseerde toestande, die lipase aktiwiteit was gemonitor en die selle aangevul met bykomende glukose by die aanvang van die stasionêre fase van groei. 'n Beduidende daling in lipase produksie was waargeneem na die byvoeging van addisionele/bykomende glukose.

Die bogenoemde waarnemings en eksperimente waar olyfolie (as enigste koolstof bron) of stearіensuur as die induseerder gebruik is, het aan die lig gebring dat die verandering van sekere groeimediumkomponente ‘n verandering in die lipase produksie profiel tot gevolg het. Al die bevindinge in hierdie studie dui daarop dat LipA die enigste lipase is wat geproduseer word onder die kultuur toestande soos vervat in hierdie studie en dat die veranderinge in die lipase produksieprofiel toegeskryf kan word aan die regulering van die gene in reaksie op veranderinge in die groei medium.

Chapter 6
References

Abada EAE (2008). Production and characterization of a mesophilic lipase isolated from *Bacillus stearothermophilus* AB-1. *Pak. J. Biol. Sci.* 11, 1100-1106.

Abbai NS (2009). Mining a South African deep mine metagenome for the discovery of novel biocatalysts. PhD thesis, University of the Free State, Bloemfontein, South Africa.

Abdel-Fattah YR (2002). Optimization of thermostable lipase production from thermophilic *Geobacillus* sp. using Box-Behnken experimental design. *Biotech Lett*. 24, 1217-1222.

(a)Akoh CC, Lee G-C, Liaw Y-C, Huang T-H, Shaw J-F (2004). GDSL family of serine esterases/lipases. *Prog. Lip. Res.* 43, 534-552.

(b)Akoh CC, Lee G-C, Shaw JF (2004). Protein engineering and applications of *Candida rugosa* lipase isoforms. *Lipids* 39, 513-526.

Antelmann, Tjalsma H, Viogt B, Ohlmeier S, Bron S, van Dijl Jm, Hecker M (2001) Aproteomic view on genome-based signal peptide predictions. *Genome Res.* 11, 1484-1502.

Arpigny JL, Jaeger K-E (1999). Bacterial lipolytic enzymes: classification and properties, *Biochem. J.* 343, 177–183.

Ash C, Farrow JAE, Wallbanks S, Collins MD (1991). Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. *Lett. Appl. Microbiol.* 13, 202-206.

Baerands RJS, Smits WK, de Jong A, Hamoen LW, Kok J, Kuipers OP (2004). Genome2D: a visualization tool for the rapid analysis of bacterial transcriptome data. *Genome Biol.* 5, R37.

Banchio C, Gramajo HC (1997). Medium- and long-chain fatty acid uptake and utlilization by *Streptomyces Coelicolor* A3(2): first characterization of a Gram-positive bacterial system. *Microbiol.* 143, 2439-2447.

Barnard TG (2005). A kinetic and molecular study of the lipase from *Geobacillus thermoleovorans* GE-7. PhD Thesis. University of the Free State, Bloemfontein, South Africa.

Berks BC, Palmer T, Sargent F (2003). The Tat protein translocation pathway and its role in microbial physiology. *Adv. Microb. Physiol.* 47, 187-254.

Bollhuis A, Tjalsma H, Stephenson K, Harwood CR (1999). Evaluation of bottlenecks in the late stages of protein secretion in *Bacillus subtilis. Appl. Env. Microbiol.* 65, 2934-2941.

Bornsheuer UT (2002). Microbial carboxyl esterases: classification, properties and application in biocatalysis. *FEMS Microbiol. Rev.* 26, 73-81.

Brocca S, Secundo F, Ossola M, Alberghina L, Carrea G, Lotti M (2003). Sequence of the lid affects activity and specificity of *Candida rugosa* lipase isozymes. *Protein Sci.* 12, 2312-2319.

Brzozowski AM, Savage H, Verma CH, Turkenburg JP, Lawson DM, Svendsen A, Paktar S (2000). Structural origins of the interfacial activation in *Thermomyces (Humicola) lanuginosa* lipase. *Biochem.* 49, 15071–15082.

Bustard MT, Whting S, Cowan DA, Wright PC (2002). Biodegradation of high concentration solvents by a solvent tolerant thermophile, Bacillus pallidus *Extremophiles* 6, 319-323.

Castro-Ochoa LD, Rodríguez-Gómez C, Valerio-Alfaro G, Oliart Ros R (2005). Screening, purification and characterization of the thermoalkalophilic lipase produced by *Bacillus thermoleovorans* CCR11. *Enzyme Microbial Technol.* 37, 648-654.

Claus D, Berkeley RCW (1986). The genus *Bacillus* cohn 1872, p. 1105-1139. *In* Sneath PHA, Mair NS, Sharpe ME, Holt JG (ed.), *Bergey's manual of Systematic bacteriology*, Vol. 2, Williams and Wilkins, Baltimore.

Cygler M, Shrag JD, Sussman JL, Harel M, Silman J, Gentry MK, Doctor BP (1993). Relationship between sequence conservation and three-dimensional structure in a large family of esterases, lipases and related proteins. *Protein Sci.* 2, 336-382.

Dalrymple BP, Swadling Y, Cybinski DH, Xue GP (1996). Cloning of a gene encoding cinnamoyl ester hydrolase from ruminal bacterium *Butyrivibrio fibrisolvens* E14 by a novel method. *FEMS Lett.* 43, 115-120.

Dancik V, Addona T, Clauser K, Vath J, Pevzner PA (1999). De novo peptide sequencing via tandem mass spectrometry. *J. Comp. Biol*. 6, 327–342.

de Keyzer J, van der Does C, Driessen AJM (2003). The bacterial translocase: a dynamic protein channel complex. *Cell Mol. Life Sci.* 60, 20034-22052.

DeFlaun MF, Frederickson JK, Dong H, Pfiffner SM, Onstott TC, Balkwill DL, *et al*. (2007). Isolation and Characterization of a *Geobacillus thermoleovorans* strain from an ultra-deep South African gold mine. *Syst. Appl. Microbiol.* 30, 152-164.

den Blaauwen T, Driesen AJM (1996). Sec-dependent preprotein translocation in bacteria. *Arch. Microbiol.* 165, 1-8.

Derewenda U, Brzozowski AM, Lawson DM, Derewenda ZS (1992). Catalysis at the interface: the anatomy of a conformational change in a triglyceride lipase.*Biochemistry* 31,1532–1541.

Desnuelle P (1961). Pancreatic lipase. *Adv. Enzymol.* 23, 129-161.

Dharmsthiti S, Luchai S (1999). Production, purification and characterization of thermophilic lipase from *Bacillus* sp. THL027. *FEMS Microbiol. Lett.* 179, 241-246.

Driesden AJM, Fekkes P, van der Wolk JPW (1998). The Sec system. *Curr.Opin. Microbiol*. 1, 216-222.

Duncombe, W.G. (1963). The colorimetric micro-determination of long-chain fatty acids. *Biochem. J*. 88, 7-10.

Dunphy G, Miyamoto C, Meighen E (1997). A homoserine lactone autoinducer regulates virulence of an insect-pathogenic bacterium, *Xhenorhabdus nematophilus* (Enterobacteriaceae). *J. Bacteriol.* 179, 5288-5291.

Dutra JCV, Terzi SC, Bevilaqua JV, Damaso MCT, Couri S, Langone MAP et al. (2008). Lipase production in solid state fermentation monitoring biomass growth of *Aspergillus niger* using digital image processing. *Appl. Biochem. Biotechnol.* 147, 63-75.

Economou A (1998). Bacterial preprotein translocase: mechanism and conformational dynamics of a processive enzyme. *Mol. Microbiol.* 27, 511-518.
Economou A (1999). Following the leader: bacterial protein transport through the Sec pathway. *Trends Microbiol.* 7, 315-320.

Eggert T, Brpckmeier U, Dröge MJ, Quax WJ, Jaeger K-E (2003). Extracellular lipases from *Bacillus subtilis*: regulation of gene expression and enzyme activity by amino acid supply and external pH. *FEMS Microbiol. Lett.* 225, 319-324.

Emmanuelson O, Brunak S, von Heijne G, Nielsen H (2007). Locating proteins in the cell, TargetP, SignalP and related tools. *Nature Protocols* 2, 953-971.

Ertuğrul S, Dönmez G, Takaç S (2007). Isolation of lipase producing *Bacillus* sp. from olive mill wastewater and improving its enzyme activity. *J. Hazard Materials* 149, 720-724.

Ewis HE, Abdelal AT, Lu C-D (2004). Molecular cloning and characterization of two thermostable esterases from *Geobacillus stearothermophilus*. *Gene* **329**, 187-195.

Fairbanks VF, Fahey JL, Beutler E (1971). Clinical disorders of iron metabolism. Grune and Stratton, Inc., New York.

Fekkes P, den Blaauwen T, Driessen AJM (1995). Diffussion-limited interactions between unfolded polypeptides and the *Escherichia coli* chaperone SecB. *Biochem.* 34, 10078-10085.

Fekkes P, van der Does C, Driessen AJM (1997). The molecular chaperone SecB is released from the carboxy-terminus of SecA during initiation of precursor protein translocation. *EMBO J.* 16, 6105-6113.

Feng J (2007). Molecular characterization of a *Fusarium graminearum* lipase gene and its promoter . *PhD thesis*, University of Saskatchewan. http://library2.usask.ca/theses/available/etd-02072007-133456/unrestricted/PHDThesisJieFeng.pdf

Feng L, Wang W, Cheng J, Ren Y, Zhao G, Gao C, et al. (2007). Genome and proteome of long-chain alkane degrading *Geobacillus thermodenitrificans* NG80-2 isolated from a deep-subsurface oil reservoir. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5602-5607.

Fernandez-Lorente G, Cabrera Z, Godoy C, Fernandez-Lafuente R, Palomo JM, Guisan MJ (2008). Interfacially activated lipases against hydrophobic supports: Effect of the support nature in the biocatalytic properties. *Process Biochem.* 43, 1061-1067.

Ferreira LMA, Wood TM, Williamson G, Faulds C, Hazlewood GP, Black GW, Gilbert HJ (1993). A modular esterase from *Pseudomonas fluorescens* subsp. *cellulosa* contains a non-catalytic cellulose-binding domain. *Biochem. J.* 294, 349-355.

Fiedler K, Simons K (1995). Protein sequence motifs: a new family of lipolytic enzymes? *TIBS* 20, 178-179.

Franken LPG, Marcon NS, Treichel J, Oliviera D, Freire DMG, Dariva C, *et al*., (2009). Effect of treatment with propane on lipases hydrolytic activity. *Food Bioproc. Technol.* Doi:10.1007/s11947-008-0087-5.

Franken SM, Rozeboom HJ, Kalk KH, Dijkstra BW (1991). Crystal structure of haloalkane dehalogenase: an enzyme to detoxify halogenated alkanes. *EMBO. J.* 10, 1297-1302.

Frenken LGJ, Bos Jw, Visser C, Muller W, Tommassen J, Verrips CT (1993). An accessory gene, *lipB*, required for the production of active *Pseudomonas glumae* lipase. *Mol. Microbiol.* 9, 579-589.

Ginalska G, Bancerz R, Kornillowicz-Kowalska T (2003). A thermostable lipase produced by a newly isolated *Geotrichum*-like strain, R59. *J. Ind. Microbiol. Biotechnol.* 31, 177-182.

Grbavcic SZ, Dimitrijevic-Brankovic SI, Bezbradica DI, Siler-Marinkovic SS, Knezevic ZD (2007). Effect of fermentation conditions on lipase production by *Candida utillis*. *J. Serb. Chem. Soc.* 72, 757-765.

Griebeler N, Polloni AE, Remonatto D, Arbter F, Vardanega R, Cechet JL, et al. (2009) Isolation and screening of lipase producing fungi with hydrolytic activity. *Food Bioproc. Technol.* doi:10.1007/s11947-008-0176-5.

Grochulski P, Li Y, Schrag JD, Bouthillier F, Smith P, Harrison D, Rubin B, Cygler M (1993). Insights into interfacial activation from an 'open' structure of *Candida rugosa* lipase. *J. Biol. Chem.* 268, 12843-12849.

Gupta N, Shai V, Gupta R (2007). Alkaline lipase from a novel strain *Burkholderia multivorans*: Statistical media optimization and production in a bioreactor. *Proc. Biochem.* 42, 518-526.

Gupta R, Gupta N, Rathi P (2004). Bacterial lipases: An overview of production, purification and biochemical properties. *Appl. Microbiol. Biotechnol.* 64, 763-781.

Hartl F-U, Lecker S, Schiebel E, Hendrick JP, Wickner W (1990). The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the *E. coli* plasma membrane. *Cell* 63, 269-279.

Henikoff JG, Pietrokovski S, Henikoff S (1997). Recent enhancements to the Blocks Database servers. *Nucleic Acids Res.* 25, 222-225.

Henstra SA, Tuinhof M, Duurkens RH, Robillardt GT (1999). The *Bacillus stearothermophilus* Mannitol Regulator, MtlR, of the phosphotransferase system. *J. Biol. Chem*. 274, 4754-4763.

Hetch HJ, Sobek H, Haag T, Pfeifer O, van Pee KH (1994). The metal-ion-free oxireductase from *Streptomyces aureofaciens,* has an α/β hydrolase fold. *Nat. Struct. Biol.* 1, 532-537

Hotelier T, Ludovic R, Xavier C, Negre V, Marchot P, Arnaud C (2004). ESTHER, the database for α/β-hydrolase fold superfamily of proteins. *Nucleic Acids Res.* 32, 145-147.

Hueck CJ, Hillen W (1995). Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for Gram-positive bacteria? *Mol. Microbiol.* 15, 395-401.

Jacobs M, Andersen JB, Kontinen V, Sarvas M (1993). *Bacillus subtilis* PrsA is required in vivo as an extracytoplasmic chaperone for secretion for active enzymes synthesized with or without pro-sequences. *Mol. Microbiol.* 8, 957-966.

Jaeger KE, Dijkstra BW, Reetz MT (1999). Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications. *Annu. Rev. Microbiol*. 53,315-351.

Jaeger KE,Scneidinger B, Liebeton K, Haas T, Reetz MT, Phillipou G, *et al*. (1996). Lipase of *Pseudomonas aeruginonasa*: molecular biology and biotechnology application, p. 319-330. *In* T. Nakazawa, K. Fukorawa, Haas D, Silver S (ed.), molecular biology of pseudomonads. ASM Press, Washington, DC.

Jaeger KE, Ransac S, Dijkstra BW, Colson C, Heuvel M, Misset O (1994). Bacterial lipases. *FEMS Microbiol. Rev*. 15, 29-63.

Johnson RS, Taylor JA (2002). Searching sequence databases via de novo peptide sequencing by tandem mass spectrometry. *Mol. Biotechnol*. 22, 301-315.

Jongbloed KDH, Martin U, Antelman H, Hecker M, Tjalsma H, Venemam G *et al*., (2000). TatC is a specificity determinant for proteins secretion via the twin-arginine translocation pathway. *J. Biol. Chem.* 2275, 41350-41357.

Joseph B, Ramteke PW, Thomas G (2008). Cold active microbial lipases: Some hot issues and recent developments. *Biotech Advances* 26, 457-470.

Kim H-K, Park S-Y, Lee J-K, Oh TK (1998). Gene cloning and characterization of thermostable lipase from *Bacillus stearothermophilus* LI. *Biosci. Biotechnol. Biochem.* 62, 66-71.

Knoesen S (2004). *Geobacillus thermoleovorans*: Growth and lipase production. M.Sc Thesis. University of the Free State, Bloemfontein, South Africa.

Kok RG, Nudel CB, Gonzalez RH, Nugteren-Roodzant LM, Hellingwerf KJ (1996). Phyiological factors affecting production of extracellular lipase (LipA) in *Acinetobacter calcoaceticus* BD413: fatty acids repression of *lipA* expression and degradation of LipA. *J. Bacteriol*. 178, 6025-6035.

Köning B, Jaeger KE, Sage AE, Vasil ML, Köning W (1996). Role of *Pseudomonas aeruginosa* lipase in inflammatory mediator release from human inflammatory effector cells (platelets, granulocytes, and monocytes). *Infect. Immun.* 64, 3252-3258.

Kontinen VP, Sarvas M (1988). Mutants of *Bacillus subtilis* defective in protein export. *J. Gen. Microbiol.* 134, 2333-2344.

Kontinen VP, Sarvas M (1993). The PrsA lipoprotein is essential for protein secretion in *Bacillus subtilis* and sets a limit for high-level secretion. *Mol. Microbiol.* 8, 727-737.

Koshland DE (1958). Application of a theory of enzyme specificity to protein synthesis. *Proc. Natl. Acad. Sci.* 44, 98–104.

Kouker G, Jaeger K-E (1987). Specific and sensitive plate assay for bacterial lipases. *Appl. Env. Microbiol*. 53, 211-191.

Kraulis PJ (1991). Molscript: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* 24**,** 946–950.

Laemmli UK (1970). Most commonly used discontinuous buffer system for SDS electrophoresis. *Nature* 227, 680-688.

Lawson DM, Brzozowski AM, Dodson GG, Hubbard GE, Huge-Jensen B, Boel E, Derewenda ZD (1994). The three-dimensional structures of two lipases from filamentous fungi, *in*: P. Wooley, S.B. Petersen (Eds.), Lipases; Their Structure, Biochemistry and Application, Cambridge University Press, pp. 77–94.

Lee DS, Kim BK, Kwon SJ, Jin HC, Park OK (2009). Arabidopsis GDSL lipase 2 plays a role in pathogen defense via negative regulation of auxin signaling. *Biochem Biophys Res Commun* 379, 1038-1042.

Lee DW, Kim H-W, Lee K-W, Kim B-C *et al* (2001). Purification and characterisation of two distinct thermostable lipases from *Bacillus thermoleovorans* ID-1. *Enzyme Microb. Technol.* 29, 363-371.

Lee DW, Koh YS, Kim KJ, Kim BY, Choi HJ, *et al.,* (1999). Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. *FEMS Microbiol. Lett.* 179, 393-400.

Leskela S, Whalstrom E, Kontinen VP, Sarvas M (1999). Lipid modification of prelipoproteins is dispensible for growth but essential for protein secretion in *Bacillus subtilis*. *Mol. Microbiol.* 31, 1075-1085.

Li J, Derewenda U, Dauter Z, Smith S, Derewenda ZS (2000). Crystal structure of the *Escherichia coli* thioesterase II, a homolog of the human Nef binding enzyme. *Nat. Struct. Biol.* 7, 555-559.

Liao D-I, Remington S (1990). Structure of wheat serine carboxipeptidase II at 3.5Å resolution. *J. Biol. Chem.* 265, 6528-6531.

Lin ES, Wang CC, Sung SC (2006). Cultivation conditions influence lipase production by the edible basidomycete *Antrodia cinnamomea* in submerged culture. *Enzyme Microbiol. Technol.* 39, 98-102.

Ling LF, Zi RX, Wei FL, Jiang BS, Ping L, Chun XH (2007). Protein secretion pathway in *Bacillus subtilis*: implication for optimization of heterologous protein secretion. *Biotech. Adv.* 25, 1-12.

Lotti M, Monticelli S, Montesinos JL, Brocca S, Valero F, Lafuente J (1998). Physiological control on the expression and secretion of *Candida rugosa* lipase. *Chem. Phys. Lipids* 93, 143-148.

Ludwig H, Stülke J (2001). The *Bacillus subtilis* catabolite control protein CcpA exerts all its regulatory functions by DNA-binding. *FEMS Microbiol. Lett.* 203, 125-129.

Maugeri TL, Gugliandolo C, Caccamo D, Stackebrandt E (2001).Thermophilic bacilli from shallow, marine vents. *Syst. Appl. Microbiol.* 25, 450-455.

McKenney D, Brown KE, Allison DG (1995). Influence of *Pseudomonas aeruginosa* exoproducts on virulence factor production in *Burkholderia cepacia:* evidence of interspecies communication. *J. Bacteriol.* 177, 6989-6992.

McMullan G, Christie JM, Rahman TJ, Banat IM, Ternan NG, Marchant R (2004). Habitat, applications and genomics of the aerobic, thermus genus *Geobacillus*. *Biochem. Soc. Trans.* 32, 215-217.

McQueen DAR, Schottel JL (1987). Purification and characterization of a novel extracellular esterase from *Streptomyces scabies* that is induced by zinc. *J. Bacteriol.* 169, 1967-1971.

Meens J, Berbort M, Klein M, Freudl R (1997). Use of Pre-pro part of *Staphylococcus hyicus* lipase as a carrier for secretion of *Escherichia coli* outer membrane protein A (ompA) prevents proteolytic degradation of ompA by cell-associated protease(s) in two different gram-possitive bacteria. *Appl. Environ. Microbiol.* 63, 2814-2820.

Menoncin S, Domingues NM, Fierre DMG, Toniazzo G, Cansian RL, Oliviera JV, et al. (2009). Studies of the extraction, concentration and partial characterization of lipases obtained from *Penicillium verrucosum* using solid-state fermentation of soy bran. *Food Bioproc. Thechnol*. doi:10.1007/s11947-008-0104-8.

Miskin JE, Farrell AM, Cunliffe WJ, Holland KT (1997). Propionibacterium acnes, a resident of lipid-rich human skin, produces a 33 kDa extracellular lipase encoded by gehA. *Microbiology* 143 (1745).

Miyawaki O, Nakamura K (2002). Enzyme reaction in supercritical fluids. In: Lipid biotechnology, pp 690-697. Marcel Dekker Inc. USA.

Molgaard A, Kauppinen S, Larsen S (2000). Rhamnogalctorunan acetylesterases elucidates the structure and the function of a new family of hydrolases. *Struct. Fold. Des* 8, 373-383.

Moriello S, Lama V, Poll A, Gugliandolo C, Maugeri TL, Gamacorta A, Nicolas B (2003). *Ind. Microbiol. Biotechnol.* 30, 95-101.

Nakajima N, Sugimoto M, Tsuboi S, Tsuji H, Ishihara K (2005). An isozyme of erthworm serine proteases acts on hydrolysis of triacylglycerols. *Biosci. Biotechnol. Biochem.* 69, 2009-2011.

Nawani N and Kaur J (2007). Studies on lipolytic isoenzymes from a thermophilic *Bacillus* sp.: Production, purification and biochemical characterization. *Enzyme Microbiol. Technol.* 40, 881-887.

Nazina TN, Tourova TP, Poltaraus AB, Novika EV, Ivanova AE, Grigoryan AA, *et al*., (2000). Physiological and phylogenetic diversitiy of thermophilic spore-foreming hydrocarbon oxidizing bacteria from oil fileds. *Microbiol.* 69, 96-102.

Nazina TN, Tourova TP, Poltaraus AB, Novikova EV, Grigoryan AA, Ivanova AE, *et al.,* (2001). Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenesis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus G. thermocatenulatus, G. thermoleovorans, G. kaustophilus, G. thermoglucosidasius* and *G. thermodenitrificans. Int. J. Syst. Evol. Microbiol*. 51, 433-446.

Nesbit, E.A., and Gunasekaran, M. (1993). Influence of cultural conditions on growth and lipolytic activity in *Nocardia asteroids*. *Folia Microbiol*. 38, 451-455.

Novotny JF, Perry JJ (1992). Characterization of bacteriocins from two strains of Bacillus thermoleovorans, a thermophilic hydrocarbon-utilizing species. *Appl. Environ. Microbiol.* 58, 2393-2395.

Nthangeni MB, Ramagoma F, Tlou MG, Litthauer D (2005). Development of a versatile cassette for directional genome walking using cassette ligation-mediated PCR and its application in the cloning of complete lipolytic genes from *Bacillus* species. *J. Microbiol. Methods* **61**, 225-234.

Ochsner UA, Koch AK, Fiechter A, Reiser J (1994). Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* 176, 2044-2054.

Ochsner UA, Reiser J (1995). Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aerugionosa*. *Proc. Natl. Acad. Sci. USA* 92, 6424-6428.

Oefner C, D'Arcy A, Daly JJ, Gubernator K, Charnas RL, Heinze I, Hubschwerlen C, Winkler FK (1990). Refined crystal structure of β-lactamase from *Citrobacter freudii* indicates a mechanism of β-lactam hydrolysis. *Nature* 343, 284-288.

Ollis DL, Cheah E, Cygler M, Dijkstra BW, Frolow F, Franken SM et al., (1992). The α/β hydrolase fold. *Protein Eng.* 5, 197-211.

Park H, Lee K, Chi Y, Jeong S (2005). Effects of methanol on the catalytic properties of porcine pancreatic lipase. *J. Microbiol. Biotechnol.* 15, 296-301.

Pelletier I and Altenbuchner P (1995). A bacterial lipase is homologous with non-haem haloperoxidases and displays brominating activity. *Microbiol.* 141, 459-468.

Perkins DN, Pappin DJ, Creasy DM, Cottrell JS (1999). "Probability-based protein identification by searching sequence databases using mass spectrometry data". *Electrophoresis* 20 (18): 3551–67.

Pesci EC, Iglewski BH, (1999). Quorum sensing in *Pseudomonas aeruginosa*, *in*: Dunny GM, Winans Sc (Eds.), Cell-cell signaling in bacteria, ASM Press Washington DC, pp 147-155.

Pohlenz HD, Boidol W, Schuttke I, Streber W (1992). Purification and properties of an *Arthrobacter oxydans* P52 carbamate hydrolase specific for the herbicide phenmedipham and nucleotide sequence of the corresponding gene. *J. Bacteriol.* 174, 6600-6607.

Prim N, Blanco A, Martinez J, Pastor FIJ, Diaz P (2000). *est*A, a gene coding for a cell-bound esterase from *Peanibacillus* sp. BP-23, is a new member of the bacterial subclass of type B carboxyl esterases. *Res. Microbiol.* 151, 303-312.

Rahman RZ, Leow TC, Salleh A, Basri M (2007). *Geobacillus zalihae* sp. nov., a thermophilic lipolytic bacterium isolated from palm oil mill effluent in Malaysia. *BMC Microbiol.* 7, 77-87.

Rainey FA, Fritze D, Stackebrandt E (1994). The phylogenetic diversity of thermophilic members of the genus *Bacillus* as revealed by 16S rDNA analysis. *FEMS Microbiol. Lett.* 115, 205-211.

Randall LL, Hardy SJS (1995). High selectivity with low specificity: how SecB has solved the paradox of chaperone binding. *Trends Biochem. Sci.* 20, 65–69.

Rodrigues IM, Iemma AF (2005). Planejamento de Experimentos e Otimização de Processoas; Uma estratéga seqüenial de planejamentos. Campinas, BRA : Caso do Pão.

Rosenau F, Jaeger K-E (2000). Bacterial lipases from *Pseudomonas* : regulation of gene expression and mechanism of secretion. *Biochimie* 82, 1023-1032.

Sariya B, Hortaçsu A (2004). Mathematical modeling of Sec pathway mechanism in *Escherichia coli*: a case study of periplasmic translocation of maltose binding protein-glucose isomerase fusion protein. *Chem. Eng. Sci.* 59, 1027-1038.

Sarvas M, Harwood CR, Bron S, van Dijl J (2003). Post-translocational folding of secretory folding in Gram-positive bacteria. *Biochim. Biophys. Acta* 1694, 311-327.

Satoh T, Taylor P, Borson WF, Sonal P, Sanghani P, Hosakawa M, La du B (2002). Current progress on esterases: from molecular structure to function. *Drug Metab. Dispos.* 30, 488-493.

Schiebel E, Driessen AJM, Hartl F-U, Wickner W (1991). $\Delta \mu_{H^+}$ and ATP function at different steps of the catalytic cycle of preprotein translocase. *Cell* 64, 927–939.

Schmidt-Dannert C, Szatjer H, Stoclein W, Menge U, Schmid RD (1994). Screening, purifications and properties of a thermophilic lipase from *Bacillus thermocatenulatus. Biochim. Biophys. Acta* 1214, 44-53.

Schulte G, Bohne L, Winkler U (1982). Glycogen and various other polysaccharides stimulate the formation of exolipase by *Pseudomonas aeruginosa*. *Can. J. Microbiol.* 28, 636–642.

Servin-González L, Castro C, Pérez C, Rubio M, Valdez F (1997). *bldA*-Dependent expression of *Streptomyces exfoliatus* M11 lipase gne (*lipA)* is mediated by the product of a contigous gene, *lipR*, encoding a putative transcriptional activator. *J. Bacteriol.* 179, 7816-7826.
Sessler AM, Ntambi JM (2008). Polyunsaturated fatty acid regulation of gene expression. *J. Nutrition* 128, 923-926.

Shariff FM, Leow TC, Mukred AD, Salleh AB, Basri N, Rahman RNZ (2007). Production of L2 lipase by *Bacillus sp.* Strain L2: nutritional and physical factors. *J. Basic Microbiol.* 47, 406-412.

Sharma R, Christi Y, Banerjee UC (2001). Production, purification, characterization and applications of lipases. *Biotechnol. Adv.* 19, 627-662.

Sharp RJ, Reiley PW, White D (1992). *In* Thermophilic Bacilli (Kristjansson JK ed.), pp. 19-50, CRC Press, Boca Raton, FL.

Soliman NA, Knoll M, Abdell-Fattah YR, Schmid RD, Lange S (2007). Molecular cloning and characterization of thermostable esterase and lipase from *Geobacillus thermoleovorans* YN isolated from a desert soil in Egypt. *Process Biochem.* 42, 1090-1100.

Stehr F, Kretschmar M, Kröger C, Hube B, Schäfer W (2003). Microbial lipases as virulence factors. *J. Mol. Catal.* 22, 347-355.

Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, Toker L, Silman I (1991). Atomic structure of acetyl choline esterase from *Torpedo californica*: A prototypic acetyl-binding protein. *Science* 253, 872-879.

Svendsen A (2000). Lipase protein engineering. *Biochim Biophys. Acta.* 1543, 223–238.

Switzer RC, Merril CR, Shifrin S (1979). A highly selective silver stain for detecting proteins and peptides in polyacrylamide gels. *Anal. Biochem*. 98, 231-237.

Takaç S, Marul B (2008). Effects of lipidic carbon sources on extracellular lipolytic activity of a newly isolated strain of *Bacillus subtilis*. *J. Ind. Micro. Biotech.* 35, 1019-1025.

Takami H, Takaki Y, Chee G-J, Nishi S, Shimamaru S, Suzuki H, *et al*. (2004). Thermoadaptation trait revealed by the genome sequence of thermophilic *Geobacillus kaustophilus*. *Nucleic Acids Res*. 32, 6292–6303.

Tan KH, Gill CO (1984). Effect of cultural conditions on batch growth *Saccharomycopsis lipolytica* on olive oil. *Appl. Microbiol. Biotechnol.* 20, 201-206.

Tanaka T, Sudo F, Ihara T, Nihira (1999). Increase production of lactonizing lipase (LipL) from *Pseudomonas* sp. strain 109 by lipids and detergents. *Biosci. Biotechnol. Biochem.* 63, 900–904.

Touzel JP, O'onohue M, Debeire P, Samain E, Breton C (2000). *Thermobacillus xylanilyticus* gen., nov. sp., a new aerobic thermophilic xylan-degrading bacterium isolated from farm soil. *Int. J. Syst. Evol. Microbiol.* 50, 315-320.

Valdez F, González-Cerón G, Kieser HM, Servin-González L (1999). The *Streptomyces coelicolor* A3(2) *lipAR* operon encodes an extracellular lipase and a new type of transcriptional regulator. *Microbiol.* 145, 2365-2374.

van Kampen MD, Rosenstein R, Gotz F, Egmond MR (2001). Cloning, purification and characterisation of *Staphylococcus warneri* lipase 2. *Biochim. Biophys. Acta* 1544, 229-241.

Verger R (1997). Interfacial activation of lipases: facts and artifacts. *Trends Biotechnol*, 15, 32–8.

Villeneuve P, Muderhwa JM, Graille J, Haas MJ (2000). Customizing lipases for catalysis: a survey of chemical, physical and molecular approaches. *J. Mol. Catal. B: Enzym.* 9, 113-148.

Vitikainen M, Lappalainen I, Seppala H, Antelmann H, Boer H, Taira S, *et al*., (2004). Structure-function analysis of PrsA reveals roles for the parvulin-like and flanking N- and C-terninal domains in protein folding and secretion in *Bacillus subtilis*. *J. Biol. Chem.* 279, 19303-19314.

Vitikainen M, Pummi T, Airaksinen U, Wahlstrom E, Wu H, Sarvas M, *et al*., (2001). Quantification of the capacity of the secretion apparatus and requirement for PrsA in growth and secretion of alpha-amylase in *Bacillus subtilis. J. Bacteriol.* 83, 1881-1890.

Weickert MJ, Chambliss GH (1990). Site-directed mutagenesis of a catabolite repression operator sequence in *Bacillus subtilis*. *Proc. Natl. Acd. Sci. USA* 87, 6238-6242.

Whitaker JR (1972). Principles of enzymology for the food sciences. *Marcel Decker Inc. NY,* 481-501.

Wickner W, Leonard MR (1996). *Escherichia coli* preprotein translocase. *J. Biol. Chem.* 271, 29514–29516.

Wisotzkey JDP, Jurtshuk P, Fox GE, Deinhard G, Poralla K (1992). Comparative sequences analyses on the 16S rRNA (rDNA) of *Bacillus acidoterresteris*, and *Bacillus cucloheptanicus* and proposal for creation of a new genus, *Alicyclobacillus* gen. nov. *Int. J. Syst. Bacteriol.* 42, 263-269.

Zeigler DR (2001). *Bacillus* genetic stock center catalog of strains, seventh edition volume 3: The genus *Geobacillus.* http://www.bgsc.org/.