Molecular and kinetic properties of recombinant *Bacillus* lipase

by

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CHAPTER 1

LITERATURE REVIEW

1.1. General introduction

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

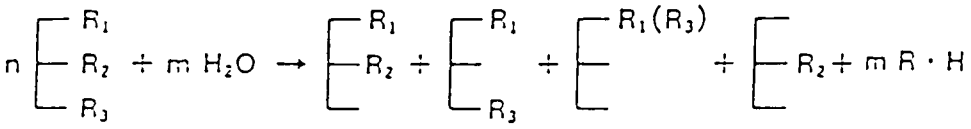
The hydrolysis reaction involves an attack on the ester bond of glycerides in the presence of water molecules to produce both an alcohol functionality and a carboxylic acid (Figure 1.1, reaction 1). The hydrolysis of fats and oils (triacylglycerols) can be reversed by modifying the reaction conditions. The equilibrium between forward and reverse reactions is controlled by the water content of the reaction mixture, so that in a non-aqueous environment lipases catalyse ester synthesis reactions. Different types of ester syntheses can be distinguished: common ester synthesis from glycerol and fatty acids (Figure 1.1, reaction 2) and the biotechnologically more important transesterification reactions in which the acyl

donor is an ester (Figure 1.1, reactions 3). Transesterification involving fats and oils can further be specified depending on the type of acyl acceptor. Acidolysis refers to the exchange of acyl radicals between an ester and an acid (Figure 1.1, reaction 3.1). Alcoholysis and glycerolysis refer to the transfer of an acyl group from a triacylglycerol to either an alcohol or glycerol (Figure 1.1, reaction 3.2). In interesterifications, the acyl group is exchanged between acylglycerols (Figure 1.1, reaction 3.3).

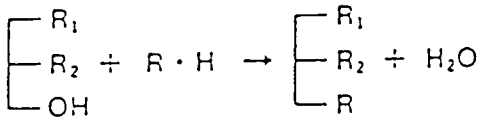
The lipase enzyme has a wide range of properties, depending on its source, with respect to substrate specificity, pH optimum and thermostability. The fact that lipases remain active in organic solvents significantly broadens their biotechnological applications.

The structures of several lipases have been elucidated. The catalytic domains of all lipases whose structures are known have the same α/β hydrolase fold (Ollis *et al.*, 1992). They are all serine esterases and their catalytic triads are almost perfectly superimposible. The active site serine is invariably imbedded in a hydrophobic region and mostly buried under a surface loop or "lid" (Ollis *et al.*, 1994). The topology and length of the lid differs depending on the source of the lipase. The exact position and nature of the lid was thought to be an important modulator of lipase activity (Van Tilbeurgh *et al.*, 1993).

1. Hydrolysis of ester

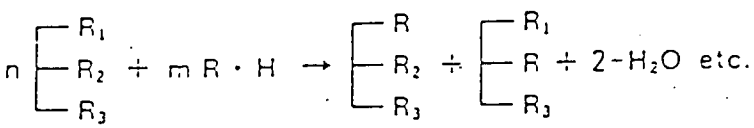


2. Synthesis of ester

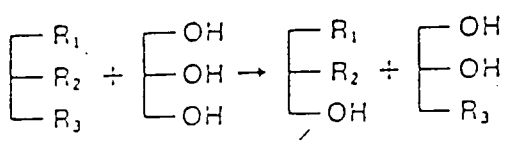


3. Transesterification

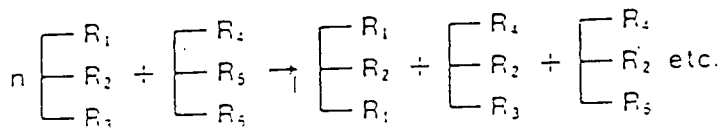
3.1. Acidolysis



3.2. Alcoholysis (Glycerolysis)



3.3. Interesterification



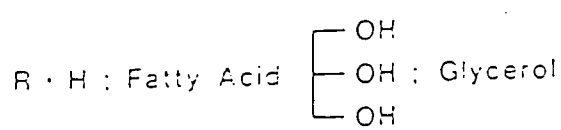


Figure 1.1. Schematic diagrams of lipase-catalysed reactions. (Taken from Kurashige *et al.*, 1989).

1.2. Occurrence and classification of lipases

Lipases are widely distributed in nature, being found in plants, animals and microorganisms. They have been classified according to their sources, kinetic properties and substrate specificities.

1.2.1. Classification according to the source of the lipase

1.2.1.1. Plant lipases

It is known that lipases exist in several plant tissues, but few studies have been done so far on the distribution of lipases in whole plants. Most of the studies that have been done on plant lipases have been devoted to seed lipases. During the initial phase of germination, seeds contain a large amount of triacylglycerols, which serve as a compact source of energy for the newly emerging plant, and a small amount of water (Adlercreutz *et al.*, 1997). The triacylglycerols stores disappear during germination (Ncube *et al.*, 1993). Examples of isolated plant lipases are the lipase from lupin seed (Sanz and Olias, 1990) and *Brassica napus* (Ncube *et al.*, 1993; Adlercreutz *et al.*, 1997).

1.2.1.2. Animal lipases

Animal lipases were originally classified into three groups according to their source organs (tissues) and sites of lipolytic action (Aires-Barros *et al.*, 1994): (i) Digestive lipases included lingual, pharyngeal, gastric and pancreatic lipases, (ii) the tissue lipases included lipases contained in serum, heart, brain, muscle, arteries, kidney, spleen, lung, liver, and adipose tissues, and (iii) the milk lipases are produced by lactating mammary glands and play a major role in neonatal fat digestion. The success achieved in the cloning and sequencing of genes encoding animal lipases has enabled their classification into pancreatic and hormone-sensitive lipase families, based on primary structure analysis and biochemical properties (Carrière *et al.*, 1998; Osterlund, 2001).

1.2.1.2.1. The pancreatic lipase gene family

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

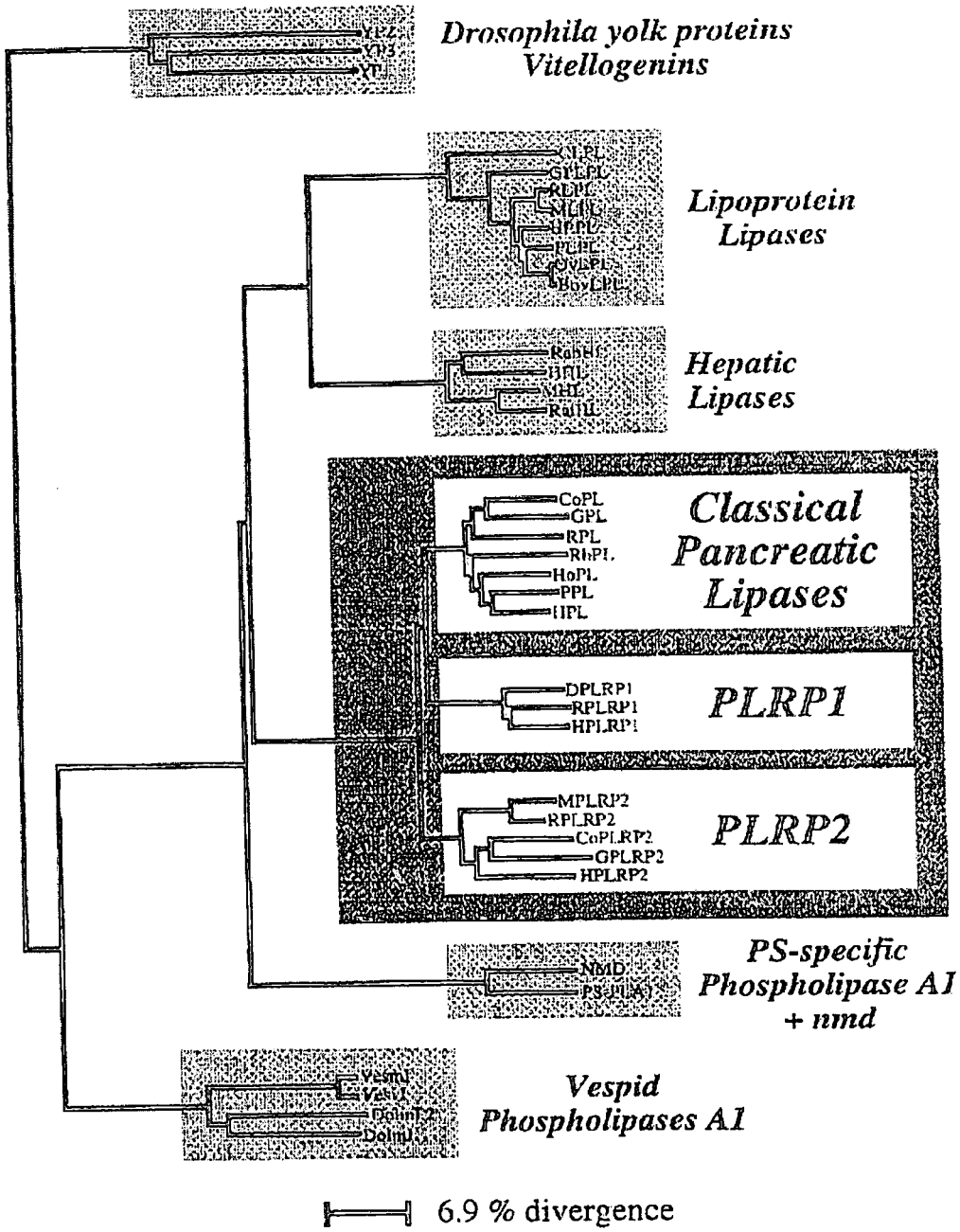


Figure 1.2. Dendrogram of sequence alignment of the eight pancreatic lipase gene family: (1) the yolk proteins from *Drosophila melanogaster* (YP1, YP2, YP3); (2) the lipoprotein lipases, from chicken (CLPL), guinea pig (GPLPL), rat (RLPL), mouse (MLPL), human (HLPL), pig (PLPL), ovine (OvLPL) and bovine (BovLPL); (3) the hepatic lipases from rabbit (RabHL), human (HHL), mouse (MHL) and rat (RatHL); (4) the classical pancreatic lipases, from coypu (CoPL), guinea pig (GPL), rat (RPL), rabbit (RbPL), horse (HoPL), pig (PPL) and human (HPL); (5) the RP1 pancreatic lipases, from dog (DPLRP1), rat (RPLRP1) and human (HPLPRP1); (6) the RP2 pancreatic lipases, from mouse (MPLRP2), rat (RPLPR2), coypu (CoPLRP2), guinea lines; and (8) the vespid phospholipases A1, from the yellow jackets (*Vespula maculifrons*, VesmI, and *Vespula vulgaris*, VesVI) and the white-faced hornet (*Dolichovespula maculata*, DolmI and Dolm.I.2). Taken from Carrière *et al.*, 1998).

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

The lipases secreted by the pancreas have been divided into three subgroups sharing about 70% amino acid identity: (i) the classical pancreatic lipases; (ii) pancreatic lipase-related proteins 1 (PLRP1); (iii) pancreatic lipase-related proteins 2 (PLRP2) (Carrière *et al.*, 1998). The lipases within each subgroup have been biochemically characterized. PLRP1s display no significant activity on triacylglycerols and their physiological role has not yet been explained (Hjorth *et al.*, 1993). The PLRP2 proteins have been investigated in human (Giller *et al.*, 1992) as well as in animal species (Hjorth *et al.*, 1993; Thirstrup *et al.*, 1994). There is a high sequence homology between PLRP1 and PLRP2 but somewhat lower homology with the pancreatic lipases. All the PLRP2s characterized do not exhibit the so-called "interfacial activation" phenomenon. Because of high phospholipase activity of PLRP2, and inhibition by bile salts, (which cannot be overcome by colipase), it has been suggested that they function mainly as phospholipases (Thirstrup *et al.*, 1994).

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

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

A distant amino acid homology relationship was also obtained with non-enzymatic yolk proteins (vitellogenins) from *Drosophila* fruitfly. The vitellogenins do not contain the lipase/esterase catalytic triad and therefore do not display lipase activity. The conserved amino acid residues between yolk proteins and pancreatic lipase surround, however, the active site where interactions with lipids take place (Bownes, 1992). The likely reason for this sequence homology in the yolk proteins is to bind a

steroid hormone and to store it under an inactive form until it is released during embryogenesis of *Drosophila* (Carrière *et al.*, 1998).

1.2.1.2.2. Hormone sensitive lipases

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

1.2.1.3. Microbial lipases

Lipases are found in abundance in bacteria and fungi including yeast. The initial studies on lipases concentrated on animal lipases, but over the last two decades much attention has been focused on microbial lipases due to their biotechnological potential. Many lipases from microbial sources have been purified and sequenced. The number of amino acids range from about 200 in *Bacillus* species to more than

600 in *Staphylococcus* species. Comparison of amino acid sequences between microbial lipases often revealed no detectable similarities beyond the "consensus" pentapeptide Gly_1-X-**Ser**-X-Gly_2, which contains the catalytically active **Ser** residue.

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

Microbial lipases can be subdivided into bacterial and fungal lipases. In the field of biotechnology, much attention has been paid to the use of lipases of fungal or yeast origin (Pandey *et al.*, 1999). This, however, does not imply inferior properties of bacterial lipases, as it has been shown in some reviews that bacterial lipases are as good as, or sometimes to be preferred to their eukaryotic counterparts (Jaeger *et al.*, 1994; 1998). The interest in bacterial lipases has overgrown the initial attempts to classify them (Gilbert, 1993, Jaeger *et al.*, 1994). According to the latest classification, bacterial lipolytic enzymes fall into 8 families based on amino acid sequence similarities and biochemical properties (Table 1.1) (Arpigny and Jaeger, 1999). The number of bacterial lipolytic genes that are cloned is increasing steadily,

and it is hoped that the revised classification would serve as the basis and would evolve into a more complete classification.

Table 1.1. Families of lipolytic enzymes (Taken from Arpigny and Jaeger, 1999)

Family	Subfamily	Enzyme-producing strain	Accession no.	Similarity (%)		Properties
				Family	Subfamily	
I	1	*Pseudomonas aeruginosa**	D50587	100		True lipases
		Pseudomonas fluorescens C9	AF031226	95		
		Vibrio cholerae	X16945	57		
		Acinetobacter calcoaceticus	X80800	43		
		Pseudomonas fragi	X14033	40		
		Pseudomonas wisconsinensis	U88907	39		
		Proteus vulgaris	U33845	38		
	2	*Burkholderia glumae**	X70354	35	100	
		*Chromobacterium viscosum**	Q05489	35	100	
		*Burkholderia cepacia**	M58494	33	78	
		Pseudomonas lutecia	AF050153	33	77	
	3	*Pseudomonas fluorescens SIK W1*	D11455	14	100	
		Serratia marcescens	D13253	15	51	
	4	*Bacillus subtilis*	M74010	16	100	
		Bacillus pumilus	A34992	13	80	
	5	*Bacillus stearothermophilus*	U78785	15	100	
		Bacillus thermocatenulatus	X95309	14	94	
		Staphylococcus hyicus	X02844	15	29	Phospholipase
		Staphylococcus aureus	M12715	14	28	
		Staphylococcus epidermidis	AF090142	13	25	
	6	*Propionibacterium acnes*	X99255	14	100	
		Streptomyces cinnamoneus	U80063	14	50	
II (GDSL)		*Aeromonas hydrophila*	P10480	100		Secreted acyltransferase
		*Streptomyces scabies**	M57297	36		Secreted esterase
		Pseudomonas aeruginosa	AF005091	35		OM-bound esterase
		Salmonella typhimurium	AF047014	28		OM-bound esterase
		Photorhabdus luminescens	X66379	28		Secreted esterase
III		*Streptomyces exfoliatus**	M86351	100		Extracellular lipase
		Streptomyces albus	U03114	82		Extracellular lipase
		Moraxella sp.	X53053	33		Extracellular esterase 1
IV (HSL)		*Alicyclobacillus acidocaldarius*	X62835	100		Esterase
		Pseudomonas sp. B11-1	AF034088	54		Lipase
		Archaeoglobus fulgidus	AE000965	48		Carboxylesterase
		Alcaligenes eutrophus	L36817	40		Putative lipase
		Escherichia coli	AE000153	36		Carboxylesterase
		Moraxella sp.	X53868	25		Extracellular esterase 2
V		*Pseudomonas oleovorans*	M58445	100		PHA-depolymerase
		Haemophilus influenzae	U32704	41		Putative esterase
		Psychrobacter immobilis	X67712	34		Extracellular esterase
		Moraxella sp.	X53869	34		Extracellular esterase 3
		Sulfolobus acidocaldarius	AF071233	32		Esterase
		Acetobacter pasteurianus	AB013096	20		Esterase
VI		*Synechocystis sp.*	D90904	100		Carboxylesterases
		Spirulina platensis	S79419	50		
		*Pseudomonas fluorescens**	S79600	24		
		Rickettsia prowazekii	Y11778	20		
		Chlamydia trachomatis	AE001287	16		
VII		*Arthrobacter oxydans*	Q01470	100		Carbamate hydrolase
		Bacillus subtilis	P37967	48		*p-Nitrobenzyl esterase*
		Streptomyces coelicolor	CAA22794	45		Putative carboxylesterase
VIII		*Arthrobacter globiformis*	AAA99492	100		Stereoselective esterase
		Streptomyces chrysomallus	CAA78342	43		Cell-bound esterase
		Pseudomonas fluorescens SIK W1	AAC60471	40		Esterase III

*Lipolytic enzyme with known 3D structure.

1.2.2. Classification of ester hydrolytic enzymes by kinetic properties

1.2.2.1. Esterases

Enzymes that hydrolyse ester bonds in general are esterases (E.C.3.1.1.1). Esterase enzymes show normal Michaelis-Menten kinetics with respect to substrate concentration (Figure 1.3). The activity of esterase enzymes does not increase at substrate concentrations exceeding solubility.

1.2.2.2. Lipases

Figure 1.1 shows reactions that are catalysed by lipases. Esterase enzymes also catalyse the very same types of reactions. It thus becomes difficult to distinguish between a lipase and an esterase as these two groups of enzymes show considerable overlap in substrate specificities. However, many lipases have been found to possess the unique characteristic of being able to be "activated" by lipid-water interfaces.

Long-chain triacylglycerols, which are the normal substrates of lipase, have hydrophobic properties. In aqueous environments, they form emulsions (lipid-water interfaces) at points of maximum concentration. By contrast, short-chain triacylglycerols posses a distinct solubility due to a higher hydrophilicity. They yield monomers at low concentrations and micelles in more concentrated solutions. It has

been shown that whereas the rate of breakdown of a dilute solution of triacylglycerol by a lipase is very slow, the enzymatic activity increases dramatically once the substrate solubility is exceeded (Verger, 1980). This phenomenon was referred to as "interfacial activation" and was thought to demonstrate a fundamental difference between an esterase and a lipase based upon the presence or absence of "interfacial activation".

In contrast to esterases, which show normal Michaelis-Menten kinetics with respect to substrate concentration, lipases display almost no activity with the substrate present in its monomeric state. Once the solubility limit of triacylglycerol is exceeded, there is however, a sharp increase in lipase activity (Figure 1.3). On the basis of these observations, lipases were defined as a special class of esterase capable of hydrolysing multimolecular aggregates at a high rate. Thus a 'true' lipase was defined as an enzyme which showed "interfacial activation" in the presence of long-chain triacylglycerols as substrates. If an enzyme hydrolysing these substrates did not show interfacial activation it was denoted an esterase.

1.2.2.3. Cutinases

Lipases and esterases have been found to be closely related to cutinases, enzymes that degrade cuticle (the insoluble lipid-polyester matrix covering the surface of plants) and are capable of hydrolysing triacylglycerols. Cutinases differ from classical lipases in that they do not have "lids" covering the active centre of the

enzyme, they do not show "interfacial activation" and they are active on both soluble and emulsified triacylglycerols (Martinez *et al.*, 1992). Cutinases, therefore establish a bridge between esterases and lipases.

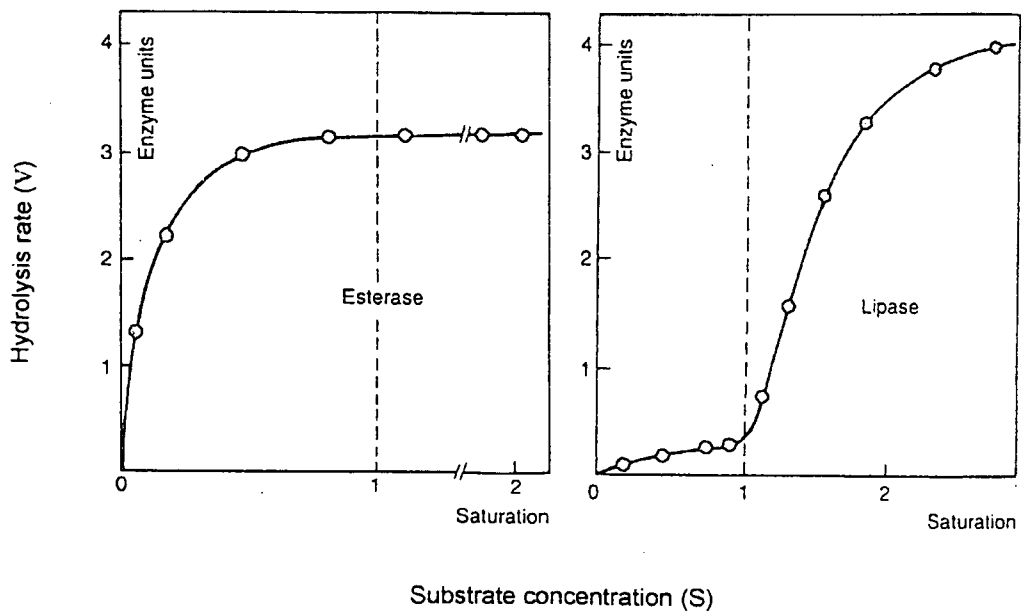


Figure 1. 3. Hydrolysis rates (V) as a function of the amount (S) of partly water-soluble ester. Dashed vertical lines represent the limit of solubility or the critical micellar concentration of the ester used. Such kinetic behaviours have been commonly used to discriminate between esterase (left profile) and lipases (right profile). Taken from Ferrato *et al.*, 1997)

1.3. Views on the "interfacial activation" phenomenon

The "interfacial activation" phenomenon was first observed in 1936 by Holwerda and co-workers and by Schonheyder and Volqvartz, (1945). It amounts to the fact that the activity of lipases is enhanced on insoluble substrates (such as emulsions) rather than on the same substrates in true monomeric solutions. It therefore emerged from the above–mentioned studies that lipases might constitute a special category of esterases which are highly efficient at hydrolysing molecules having a carboxylic ester group and are aggregated in water. This property was used for a long time to distinguish between lipases and esterases. A conceptual shift has , however, occurred, where "interfacial activation" has been taken to mean a hypothetical conformational change occurring as the result of interfacial adsorption (Desnuelle *et al.*, 1960).

The preceeding hypothesis gradually drifted and was then progressively transformed to cover an idealized concept, far away from real experimental facts and artifacts. The first three-dimensional structures to be elucidated (Brady *et al.*; 1990; Winkler *et al.*, 1990) suggested that the "interfacial activation" phenomenon might be due to the presence of the amphiphilic peptidic flap covering the active site of the enzyme in solution, similar to a lid. When contact occurs with a lipid/water interface, this lid must undergo a conformational rearrangement, resulting in the active site becoming accessible. It is worth noting however that the hydrolysis of a substrate having the form of a truly monomeric solution might well also require the

lid to open without any "interfacial activation" being involved. "Interfacial activation" was thought to involve the open and closed forms of lipases.

The results of recent lipase research have nevertheless shown how careful one has to be when extrapolating any kinetics and/or structural characteristics observed to all lipases in general. The catalytic activities of many lipolytic enzymes have been measured using carboxylic esters, which are partly soluble in water, and many differences have been found to exist between the resulting profiles. The greatest caution must be exercised both when performing and interpreting kinetic measurements with lipids. Firstly, it is essential to check that the initial lipase reaction velocity is proportional to the amount of enzyme used, both below and above the solubility limit. Substrate depletion, in the monomeric range of substrate concentration, is sometimes a major experimental limitation. Secondly, it is also essential to check that the same lipase active site, and not other unspecified sites, is responsible for the measured catalytic activity on monomeric substrates. Control experiments with non-enzymatic proteins or inhibited lipase should be performed. Thirdly, since the media is heterogeneous, adding any amphiphilic compound to the system is liable to modify both quantitatively and qualitatively the physicochemical properties of the interface.

In the framework of the European Bridge-T project (1990-1994), some new three-dimensional structures and numerous biochemical data provided new insights into lipases. It emerged from these studies that some lipases do not subscribe to the

phenomenon of "interfacial activation". The main exceptions noted were the lipase from *Pseudomonas glumae* (Noble *et al.*, 1993) and *Candida antartica* B (Uppenberg *et al.*, 1994).

Comparisons between the amino acid sequences of pancreatic lipases have shown that they have a fairly high degree of homology, but they can nevertheless be divided into three subgroups, as explained earlier. Although the kinetic properties of the classical pancreatic lipases, particularly with regards to "interfacial activation", have been fully documented, the PLRP2 lipases of the coypu and the guinea pig were found to show no "interfacial activation". Surprisingly, the coypu lipase has a 23 amino acid lid, which is homologous to that of the classical pancreatic lipases, whereas the guinea pig lipase has a mini-lid consisting of only five amino acid residues (Withers-Martinez *et al.*, 1996).

One can suggest that the molecular explanation for the "interfacial activation" phenomenon had to be investigated not only at the lipase three-dimensional structure, but also in the dynamics of organised multimolecular structures as well as in the interfacial conformations (interfacial quality) of lipids used as lipase substrates. "Interfacial activation" as well as the presence of a lid domain are therefore not in the least appropriate criteria on the basis of which to determine whether such an esterase belongs to the lipase subfamily. "Interfacial activation" is thus sometimes wrongly taken as a criterion for predicting the existence of a lid domain in lipases with an unknown three-dimensional structure.

Because naturally occurring triacylglycerols are totally insoluble in water, in contrast to short-chain triacylglycerols, interfacial activation can be said, in light of the above-mentioned arguments, to be little more than an artifact that has stimulated the imaginations of many biochemists, but which has not turned out to be of any great physiological significance. Lipases are therefore quite pragmatically redefined as carboxyl esterases that catalyse the hydrolysis of long chain acylglycerols (Verger, 1997). In fact, they are simply fat splitting "ferments".

1.4. Lipase assay methods

A number of methods to assay lipolytic activity have been developed. Some of the methods have been adapted to detect lipolytic activity on solid media (plate methods). The plate methods are particularly useful in the screening for lipase producing microbial isolates growing on solid agar medium. A large number of lipase assay methods for the quantitative analysis of lipase enzymes in solutions are available and have been reviewed by Beisson *et al.*, (2000). The methods are based on titration, spectrophotometry, chromatography, radioactivity, interfacial tensiometer, turbidimetry, conductimetry, immunochemistry and microscopy. In this study, only some of the methods based on titration and spectrophotometry will be described.

1.4.1. Plate methods

There are mainly two methods for the lipolytic screening of microorganisms: (i) the plating techniques using visualisation of clear zones in opaque medium, and (ii) the use of dyes to enhance the contrast of the area of lipolysis. The former uses either natural substrates (milk fat, olive oil and beef tallow) or synthetic substrates (tributyrin or triolein) (Fryer *et al.*, 1966). The natural opacity of the medium is due to the presence of micro-droplets of tributyrin and lipolytic organisms convert these into water-soluble butyric acid, so removing opacity. The clearance zone produced on a tributyrin plate is sometimes difficult to see, particularly with low lipase producers. The latter method uses milk fat (Fryer *et al.*, 1966), butterfat (Lawrence *et al.*, 1967), olive oil (Kouker and Jaeger, 1987) or Tween (Samad *et al.*, 1989) together with Nile Blue sulphate (Fryer *et al.*, 1966) or Victoria Blue (Fryer *et al.*, 1966; Samad *et al.*, 1989) as indicators. Hydrolysis in the presence of Victoria Blue is shown as a blue zone against the red background of unchanged dye (Lawrence *et al.*, 1967), while orange fluorescent halos are formed around lipase-producing colonies in the presence of Rhodamine B (Kouker and Jaeger, 1987). Ignjatovic and Dey (1993) described the method of identifying lipase-producing microorganisms on agar plates containing Tween and $CaCl_2$. White opaque halos are formed around the lipase positive isolates as a result of precipitation of Ca^{2+} salts of fatty acids.

1.4.2. Titrimetric assays

Titrimetric methods measure the rate of neutralisation of sodium hydroxide by released fatty acids as a function of time (Shahani, 1975). All the most commonly used titrimetric methods for determining lipase activity, including pH-stat methods suffer from two disadvantages. First, the enzyme concentration in the reaction mixture is gradually diluted by the addition of titrant. This problem can be reduced, but not eliminated, by increasing the concentration of the basic titrant solution. Second, the pH at which the reaction is carried out must be a compromise between the optimum for the enzyme and the significantly higher pH required to complete titration of fatty acids. As a result, complete titration is not achieved and measurement of optimum pH with these methods does not reflect the true properties of the enzyme (Taylor, 1985).

A method which eliminates these two problems while retaining and improving most advantages of the continuous pH-stat methods has been described (Taylor, 1985). This method combines the simplicity, ease of operation, and rapidity of previously described pH-stat methods with flexibility in choice of reaction conditions of manual methods. In this method, enzyme and substrate are pumped into a stirred emulsion reactor where they react and flow to a second stirred vessel for titration of the fatty acid products. Thus, the enzymatic and acid-base reactions are carried out separately.

1.4.3. Spectrophotometric assay

The hydrolysis of carboxylic esters of α-naphthol, *para*-nitrophenol or 2,4-dinitrophenol leads to the release of alcohols that can be monitored continuously and quantitatively using a spectrophotometric method. The appearance of the yellow coloured *para*-nitrophenol can be monitored by reading absorbance at 405-410 nm (Winkler and Stuckman, 1979; Vorderwülbecke *et al.*, 1992; Chemnitius *et al.*, 1992). The formation of 2,4-dinitrophenol is monitored from the increase in absorbance at 360 nm (Mosmuller *et al.*, 1992). *Para*-nitrophenyl esters suffer the drawback that they are not exclusively specific for lipases as they can be hydrolysed by esterases (Stuer *et al*, 1986). The observation that esterases show very low activities towards *p*-nitrophenyl esters of long-chain fatty acids has enabled these substrates to be used as quick assays in the determination of the chain length specificity of microbial lipases (Rangheard *et al.*, 1989).

1.5. Factors affecting microbial lipase production

Although lipases may be obtained naturally from different organisms, their production is influenced by culture conditions. A variety of conditions have been described which stimulate or repress the production of lipases by microorganisms. Lipase production can be induced by the addition of various triacylglycerol substrates to the growth medium. When a *Pseudomonas* strain was cultivated in the medium without oil, which consisted of glucose (1%), peptone (1%), urea (0,2%)

and minerals, lipase activity was scarcely detected in the culture supernatant in spite of the good growth of the microorganism (Narasaki *et al.*, 1968). Addition of olive oil resulted in significant lipase activity indicating a stimulation of lipase production by the olive oil. Since then, olive oil has been used to induce lipase production by various microorganisms (Yamamoto and Fujiwara, 1988; Suzuki *et al.*, 1988; Phillips and Pretorius, 1991; Christakopoulos *et al.*, 1992). Other oils that have been used to stimulate microbial lipases include soybean oil, corn oil, and sunflower oil (Chistakopoulos *et al.*, 1992). The type of oil used is important for lipase production for a given microorganism (Espinosa *et al.*, 1990; Hatzinikolaou *et al.*, 1996).

Addition of compounds containing hydrolysable ester groups such as Tweens and Spans in culture medium was found to increase lipase production by some microorganisms. Of all the carbon sources tested, Tween 80 was by far the best inducer of lipase production by a *Bacillus* sp (Gowland *et al.*, 1987). When Tween 80 was replaced by glycerol, glucose or starch, the lipase level was very low and could only be detected by the most sensitive fluorometric assay. Tween 80 has also been used to induce lipase production by *Bacillus* sp. MC7 (Emanuilova *et al.*, 1993). The presence of Tween 80 in a culture medium for *Rhizopus delemar* increased lipolytic activity by a level of twice that with olive oil or butyric acid (Espinosa *et al.*, 1990.) This effect was postulated to be due to the possible double effect of Tween. It could act as an inducer, as its chemical nature is similar to some substrates of the enzyme, and as a surfactant. When *Trichosporon fermentans* was cultivated in a media containing surfactants such as Tween, Triton and Span,

extracellular lipase activities reached levels of 2-3 times as much as that without surfactants (Chen *et al.*, 1994.)

Long chain fatty acids, as end products of lipase activity, have an inhibitory effect on lipase production (Hegedus and Khachatourians, 1988). Contrary to this, oleic acid was found to be better than olive oil in the induction of lipase production by *Candida rugosa* (Del Rio *et al.*, 1990, Dalmau, *et al.*, 2000). Studies with different soluble short chain fatty acids have demonstrated that caprylic and capric acids could even be better than oleic acid as inducers of lipase production by *Candida rugosa* (Obradors *et al.*, 1993). Addition of light divalent cation Mg^{2+} in culture media showed enhanced production of lipase activity (Hegedus and Khachatourians, 1988). In the study conducted in our department, lipase production by an *Aspergillus niger* strain was increased significantly upon addition of Ca^{2+} in the growth media. It is not clear if the stimulation is due to signal transduction.

Stimulation of lipase production is not only limited to the addition of lipidic substances in the culture media as inducers. Investigations applying one-variable-at-a-time-optimisation procedures showed that extracellular lipase activity from different microorganisms achieved maximal values when carbohydrates (Petrovic *et al.*, 1990) were used as carbon sources and certain ammonium salts (Christakopoulos *et al.*, 1992) served as nitrogenous sources. Sztajer and Maliszewska (1988) demonstrated that while starch induced maximal lipolytic activity in *Bacillus circulans*, *Streptomyces* sp., and *Pseudomonas fluorescens*,

galactose and sucrose exerted an enhanced activity in *Bacillus* sp. The maximal intracellular lipolytic activity of *Nocardia asteroides* was observed in fructose-supplemented cultures (Nesbit and Gunasekaran, 1993). This was followed by cultures grown in glucose, maltose and sucrose; the least activity was observed in media containing starch and citrate. Although the extracellular lipase activity was much lower than the intracellular activity, in culture grown in monossacharides as the primary carbon source, it was significantly higher than that of cultures with maltose, sucrose and starch.

Lipase production in other microorganisms is stimulated by the presence of alkanes in the culture media. The ability of *Acinetobacter Iwoffi* strain to grow on pure alkanes was associated with the formation of cell-bound lipase (Breuil *et al.*, 1978). Chen *et al.*, (1994), reported that *Trichosporon fermentans* Wu-C12 could produce extracellular lipase with petroleum products as carbon sources.

1.6. Molecular regulation of lipase biosynthesis

The studies described above have been conducted from the biotechnological point of view, with the aim of defining conditions for maximum lipase production. The molecular mechanisms regulating the expression of lipase genes have not yet been clearly elucidated. In general, the release of enzymatically active lipase into the extracellular medium requires the interaction of various cellular processes, starting with transcription of the structural lipase genes, proceeding with the translation of

the respective m-RNAs and subsequent secretion of the protein through the cell membranes. In bacterial lipases, most studies on the regulation of the biosynthesis of lipase genes have been on *Pseudomonas* (Rosenau and Jaeger, 2000) and *Staphylococcus* species (Rosenstein and Götz, 1998). This has probably been prompted by the difficulties experienced in the attempts to over-express *Pseudomonas* lipases in heterologous hosts such as *Escherichia coli*, and their role as virulence factors in some *Pseudomonas* strains. *Staphylococcus* lipases have been implicated in the pathogenesis of human diseases, and driven by the quest to understand the molecular basis of *Staphylococcus* pathogenesis, the molecular regulation of their lipases is becoming clear.

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

forming a pore-like structure in the outer membrane through which the lipase protein is released.

The molecular physiology regarding the mechanisms of lipase secretion by *Staphyloccocus* species have been studied (Rosenstein and Götz, 2000). All *Staphylococcus* lipases are translated as a pre-pro-enzyme with a leader signal peptide of 35 to 38 amino acids, followed by a pro-sequence (207-321 amino acids) and the mature form, that is the active lipase that appears in the supernatant of the producing *Staphylococcus* strain (383 to 396 amino acids). The function of the signal peptide is to direct the protein into the secretory pathways. The signal peptide is cleaved before the protein is secreted to the extracelullar medium. The processing of the pro-peptide has been found to occur after the protein has been excreted into the extracellular medium, and is mediated by two extracellular proteases, ShpI and ShpII (Götz *et al.*, 1998). The pro-peptide region turned out to essential as an intramolecular chaperone, required for efficient folding and secretion of the lipase (Götz *et al.*, 1998). Although *Bacillus* species are known for their capabilities in the secretion of extracellular proteins, no data is available on their secretory mechanisms for lipases.

It is evident from these studies that it is difficult to generalize the effect that a given compound would have on different microorganisms with respect to lipase production. Although a number of compounds are known to repress lipase production, the mechanisms of such effects have not yet been explained. For

instance, *Acinetobacter calcoaceticus*, secretes a number of extracellular lipolytic enzymes including *lipA* which is repressed by the presence of fatty acids in the culture medium (Kok *et al.*, 1995). This suggested the existence of an unidentified regulatory protein, which is believed to repress lipase transcription upon binding of a fatty acid (Kok *et al.*, 1996).

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

1.7. Lipase catalytic properties

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

1.7.1. Substrate specificity

The glycerol molecule as the basic building block of the lipase substrate triacylglycerol contains two primary and one secondary hydroxyl groups. Although the molecule has plane symmetry, the two primary groups are sterically distinct. Substitution of these hydroxyl groups with two different substituents leads to optically active derivatives. In a generally adopted nomenclature (IUPAC-IUB Commission on Biochemical Nomenclature), glycerol is written in a Fisher projection with the secondary hydroxyl group to the left, and the carbon atoms numbered *sn*-1,2, and 3 from top to bottom (*sn*- refers to *s*tereospecifically *n*umbered glycerol), thereby allowing the unambiguous description of isomeric glycerides. The substrate specificity of a lipase is defined by its positional specificity, its preference for longer or shorter-chain, saturated or unsaturated acids or by its stereospecificity (Sanz and Olias, 1990). Lipases have also been shown to possess glyceride specificity (Malcata *et al.*, 1992).

1.7.2. Positional specificity and stereospecificity

Several research groups have reported on positional selectivity of microbial lipases. Omar *et al.*, (1987) reported that the lipase of *Humicola lanuginosa* has an *sn*-1,3 positional specificity and Sugihara *et al.*, (1991) reported that the lipase of a *Bacillus* species also has an *sn*-1,3 positional specificity. Several other bacterial lipases were depicted as *sn*-1,3 positional specific (Okeke and Gugnani, 1989; Muderhwa *et al.*, 1986) and it is believed that lipases do not hydrolyse the fatty acid at position *sn*-2 in a triacylglycerol. Sztajer *et al.*, (1992) however, felt that the lipase from the fungus *Penicillium simplissimum* was non-specific, which meant that this lipase hydrolyses any of the three bonds of the triacylglycerol. Sugihara *et al.*, (1993) even suggested that the lipases from *Geotrichum candidum* have some preference for the *sn*-2 position on a triacylglyceride molecule. These positional specificities were all determined with the Thin Layer Chromatography (TLC) technique using a variety of substrates. The problem associated with lipid-water emulsion experiments is that the interphase is ill defined and that acyl migration in aqueous media can make interpretation of the data difficult. Application of pseudolipids containing non-ester linkages in some positions provided an alternative approach (Rogalska *et al.*, 1990). The determination of positional and stereospecific preference of lipase acting on triacylglycerol analogs is however, subject to problems: the non-ester bond could have a distinct effect on the interaction between the lipase and substrate as the exact stereochemical configuration of the linkages are not identical. Stadler *et al.*, (1995) demonstrated that even minor structural differences at *sn*-2 of a

triacylglycerol analog could have strong effects on the stereoselectivity of microbial lipases.

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

The mechanism whereby an enzyme differentiates between two enantiomers of a chiral substrate may be influenced by physicochemical properties such as temperature (Holmberg and Hult, 1991), solvent hydrophobicity (Wu *et al.*, (1990); Matori *et al.*, 1991, Nakamura *et al.*, 1991), hydrostatic pressure (Kamat *et al.*, 1993) or surface pressure (Rogalska *et al.*, 1993), which can affect the lipase reaction

stereoselectivity (Rogalska *et al.*, 1995). Although not much literature is available on the subject of lipase stereoselectivity, a rather large body of literature deals with the preparation of chiral esters and alcohols employing lipase-mediated kinetic resolution of racemic (non-triacylglycerol) substrates (Zaks and Klibanov, 1985; Theil, 1992; Theil and Bjorkling, 1993, Itoh, 1997, Shin *et al.*, 2000). Given the nature of enzymes as chiral catalyts with sophisticated molecular architecture, one might expect selectivity to be the norm, and non-selectivity to be an exception (Sonnet, 1988).

1.7.3. Fatty acid specificity

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

The distribution of activities of some lipases relative to various triacylglycerols changes with temperature; as temperature is increased, the rates of release of long-chain fatty acids increase faster than those of the corresponding short-chain fatty acids. Lipases isolated from *Fusarium heterosporum* and *Bacillus* species showed preference towards fatty acid chain length depending upon the reaction temperature. At 30^{o}C the lipase enzyme from *Fusarium heterosporum* hydrolysed triacylglycerols of short-fatty acids with a much higher velocity than the others (Shimada *et al*, 1993). Elevation of the reaction temperature increased the activity towards the longer fatty acid chain triacylglycerols. The same results were obtained with the studies of the lipase derived from a *Bacillus* sp which showed low activities towards triacylglycerols of long chain length (more than C12) at 30^{o}C, but these substrates were readily subjected to enzymatic hydrolysis at 50^{o}C at which temperature they become liquid (Sugihara *et al.*, 1991).

For the same chain length of the fatty acid residue, the rate of attack by some lipases seems to increase with the number of double bonds in the hydrocarbon backbone (Malcata *et al.*, 1992). Lipolytic activity of lipase from *Pseudomonas aeruginosa* MB 5001 increased as C18-unsaturated fatty acid content of the oils increased (Chartrain *et al.*, 1993). Low activity was obtained with lard oil (C18:0 and C18:1 rich) and olive oil (C18:1 rich), while higher activity was achieved with sunflower oil (C18:2 and C18:3 rich). Similarly, a higher lipolytic activity was obtained with trilinolelin (C18:3) and trilinolenic (C18:2) than with triolein (C18:1) (Chartrain *et al.*, 1993). The rate of triacylglycerol hydrolysis by a lipase from

Pythium ultimum was also found to increase with an increasing number of double bonds per molecule (Mozaffar and Weete, 1993).

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

However, the presence of two, and especially three double bonds in the C18 fatty chains reduced the rate of triacylglycerol hydrolysis by some other lipases. Lipase derived from *Candida deformans* hydrolysed triacyglycerols with C18:2 and especially with C18:3 at a slower rate than those with C18:0 and C18:1 (Muderhwa

et al., 1985). Similarly, *Humicola lanuginosa* No. 3 lipase catalysed polyethylene sorbitan monooleate (Tween 80) to a higher extent than triolein (C18:1) and showed low hydrolytic activity towards esters of a higher degree of unsaturation such as methyl linoleate (D 9,12) and methyl linolenate (D 9,12,15) (Omar *et al.*, 1987).

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

Some other lipases can equally hydrolyse saturated and unsaturated triacylglycerols. For example *Neurospora crassa* lipase preferred tripalmitin (16:0), tristearin (18:0), tripalmitolein (16:1), triolein (18:1) and trilinolein (18:2) and hydrolysed them at the same rates (Kundu *et al.*, 1987). A lipase isolated from lupin seed was found to be more active on saturated than on unsaturated fatty acids (Sanz and Olias, 1990). Lipase enzyme from *Fusarium oxysporum f.sp.lini* exhibited a higher affinity to the ester bond of saturated fatty acids than that of unsaturated fatty acids (Hoshino *et al.*, 1992). This preference was exploited in the concentration of poly-unsaturated fatty acid (n-3 PUFA) content of partially hydrolysed glycerides obtained from fish-oil. The lipase gave increases in n-3 PUFA concentration as the hydrolysis progressed.

1.7.4. Glyceride specificity:

Some enzymes show unusual specificity towards glyceride i.e., the selectivity among mono-, di- and triacylglycerol as substrates. A lipase from *Penicillium cyclopium* MI has been shown to display its highest activity towards monoglycerides, and much lower activities towards di- and triacyglycerols (Okumura *et al.*, 1980). Yamuguchi and Mase (1991) reported a lipase from *Penicillium camemberti* U-150 with absolute specificity towards mono- and diacylglycerol.

1.7.5. pH

Changes in pH profoundly affect the degree of ionisation of the amino, carboxyl and other ionisable residues in protein. Since ionisable amino acid residues may be present in the active site of the enzyme, and other ionisable groups may be responsible for maintaining the protein conformation, it is not surprising that the pH of the solution may markedly affect enzyme activity. Moreover, since many substrates are ionic in character, the active site of an enzyme may require particular ionic species of the substrate for optimum activity. These effects are probably the main determinants of the shape of the curve that represents enzyme catalytic activity as a function of pH (Conn *et al.*, 1987). Usually, the catalytic activity of the lipase changes with pH in a bell-shaped fashion, thus yielding a maximum rate in the stability range (Zaks and Klibanov, 1985). The plateau of the bell-shaped curve usually is small and the rates decrease rapidly with pH on either side of the

maximum. The rate decrease represents changes in the state of ionisation of critical groups of the enzyme or the substrate, or both (Conn *et al.*, 1987).

As with other enzymes each lipase has its own optimal pH. There exists a great diversity in the pH optima of microbial lipases. Development of an alkaline lipase is important, particularly in the use of the enzyme in laundry detergents to enhance cleaning and as a substitute for pancreatic lipase in digestive medicine (Yamane, 1987). Shifts in the pH optimum after immobilisation of various lipases have been observed. After immobilisation, the optimum activity of the lipase from *Candida rugosa* increased to a more alkaline value (Montero *et al.*, 1993). Shifts in pH optima of immobilised lipases have been reviewed by Malcata *et al.*, (1992). The maxima in the rates of the reactions catalysed by immobilised lipases were observed at pH values between 4.0 and 10.0. With very few exceptions, the pH optima for the immobilised lipases are equal to or higher than those for their free counterparts. Hence, the immobilisation procedure seems to render catalytically important amino acid residues more basic. An explanation consistent with these results and with the experimental evidence is that upon immobilisation the active site becomes more exposed to the solvent than it was in the folded soluble, lipase form. Hence, proton transfer to the amino acid residues at the active site becomes less hindered.

The pH also affects the stability of enzymes. Some lipases are stable over a wide pH range. Examples are the lipases from *Pseudomonas cepacia* (which retained 100% activity after incubation over a pH range of 3-11.5 for 24 hours at 30^{o}C)

(Sugihara *et. al.*, 1992) and *Fusarium heterosporum* (stable over a pH range of 4-10 at 30^{o}C for 4 hours) (Shimada *et al.*, 1993).

Studies on the effect of pH on lipases show that lipase activity decreases as the pH is shifted from the enzyme's optimum pH value. In general, shifting the pH of the enzyme solution beyond its pH stability results in the deactivation of the enzyme. This feature can be exploited in inactivating the enzyme after desired changes have been produced (Kilara, 1985).

1.7.6. Temperature

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

$$k=Ae^{-E/RT}$$

where A is a proportionality constant, E is the activation energy, R is the gas constant, and T the absolute temperature. The equation predicts that the rate of the reaction, enzymatically catalysed or not, will increase with increasing temperature. However, since enzymes are proteins and many proteins will be denatured if the temperature is raised sufficiently, enzyme catalysed reactions show an increase in rate with increasing temperature only within relatively small and low temperature range. The optimum temperature of enzyme-catalysed reactions depends on several factors including how long the enzyme is incubated at the test temperature

before the substrate is added and the type of organism from which the enzyme was derived (Conn, *et al.*, 1987).

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

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

and Manolov, 1993), and an optimum temperature of 60 ^{o}C was reported (Sugihara *et al.*, 1991).

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

1.7.7. Effects of metals

Numerous studies have been made concerning the effects of various salts on lipase activity and diverse results have been obtained. Most lipases are inhibited by heavy metals (Co^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+},Fe^{2+}, Sn^{2+}, Ni^{2+} and Ag^{2+}) . However, the lipase isolated from *Penicillium simplicissimum* was found to be resistant to most of the heavy metals tested (Sztajer *et al.*, 1992). It was significantly inhibited by Zn^{2+} and a minor reduction was observed with Ag^{2+}. In most cases monovalent cations, Na^{+}, K^{+} and Li^{+}, have been found to have stimulatory or no effect on the rate of lipase-catalysed reactions. A 50% inhibitory effect by K^{+} was reported on the activity

of a lipase isolated from *Pseudomonas* species (Yamamoto and Fujiwara, 1988). Light divalent cations (Mg^{2+} and Ca^{2+}) appear to stimulate the activity of most of the enzymes studied. A significant inhibitory effect by Mg^{2+} was observed on a lipase isolated from *Aspergillus oryzae* (Ohnishi *et al.*, 1994). Porcine pancreatic lipase has been shown to have an absolute requirement for calcium ions in the presence of bile salts (Benzonana and Denuseulle, 1968). The lipase enzyme isolated from castor bean lipid bodies was stimulated 40-fold by 30 mM free Ca^{2+} (Hills and Beevers, 1987).

It is generally known that free fatty acids tend to inhibit lipase catalysed hydrolysis probably by accumulating at the lipid/water interface, thereby blocking access of the enzyme to the unreacted triacylglycerol molecules (Benzonana and Desnuelle, 1968). The positive effects of metal ions could be due to the formation of complexes with ionised fatty acids which change their solubilities and behaviour at interfaces, whereas negative effects can be attributed to competitive inhibition at the active site. Often the lost activity can be restored via the addition of metal-chelating agents (Malcata *et al.*, 1992).

1.7.8. Effects of bile salts and detergents

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

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

the activity of *Pseudomonas* sp KW I-56 was inhibited by these bile salts (Iuzumi *et al.*, 1990).

The effect of detergents on lipase activity has been widely studied. Different detergents affect lipases differently. In most studies, anionic detergents (SDS, sodium laurylbenzenesulphonate) inhibited lipase activity while non-ionic detergents (Tween 20 and 80, Triton X-100) enhanced activity (Yamamoto and Fujiwara, 1988; Hoshino *et al.*, 1992; Mozaffar and Weete,1993; Lin *et al.*, 1996). Lipases from *Pseudomonas* sp KW I-56 (Iuzumi *et al.*, 1990) and *Brassica napus* (Weselake *et al*, 1989) were inhibited by non-ionic detergents. Cetyltrimethyl-ammonium bromide, which is a cationic detergents inhibited *Brassica napus* lipase (Weselake *et al.*, 1989) and *Pseudomonas* sp lipase (Yamamoto and Fujiwara, 1988). Chaps (3-cholamidopropyldimethyl-ammonio-1-1-propane sulfonate), a zwitterionic detergent, enhanced activity of *Pythium ultimum* lipase (Mozaffar and Weete, 1993) and *Bacillus thermocatenulatus* lipase (Schmidt-Dannert *et al.*, 1994).

1.8. Conclusions

The literature cited in this study clearly demonstrated the huge interest in the lipase enzyme. It is also evident from this review that lipases have a wide range of properties, and one can argue that with careful screening, one can find the lipase enzyme with the desired property. The biotechnological potential of the lipase enzyme is becoming realized. Several applications of lipases have been developed

throughout the last two decades. Some of the industrial applications involving the lipase enzyme have been highlighted by Pandey *et al.*, (1999) and Jaeger *et al.* (1994, 1998).

Lipases are having a significant impact in the food industries where they are used in the production of a variety of products, ranging from fruit juices, baked foods, vegetable fermentation and enhancement of flavours in dairy industries. Because of their excellent capabilities for specific regioselective reactions in a variety of organic solvents with broad substrate recognition, lipases have emerged as important biocatalysts in biomedical applications. They are used in the production of compounds in high enantiomeric excess, which are used as chiral building blocks for the synthesis of compounds of pharmaceutical interest.

Lipases have become indispensable ingredients in the detergent industries where they are used in combination with proteases and cellulases. At present they are in extensive use in household detergents and industrial cleaners. They are also used in the formulations prepared to clean clogged drains. Bioremediation for waste disposal is a new avenue in lipase biotechnology. Oil spills during rigging and refining, oil-wet night soils and shore sand, lipid-tinged wastes in lipid processing factories and restaurants are succesfully handled by the use of lipases of different origins.

The impacts of lipases are also felt in the leather industries where they are used in the removal of subcutaneous fat, de-hairing and stuffing. Enzymatic processes for the production of hides and skins, ready for tanning, involving steps of soaking, washing, de-hairing and bathing in aqueous baths have been described, where each bath had a pH of 8-13 and contained alkaliphilic lipase (Jurgen *et al.*, 1992a). Patents describing the use of alkaliphilic *Bacillus* species producing lipase enzymes with potential applications in the extremely alkaline tanning industries (Jurgen *et al.*, 1992a, 1992b) and as ingredients in washing detergents (Möller *et al.*, 1991) have been granted.

The use of lipases in fields such as cosmetics, bleaching, pulping, lubricants and filtration are under-exploited. In fact, in terms of application, lipases are the most versatile biological catalysts.

Although lipase biotechnology is entering an exponential phase in terms of application, the present industrial deployment has focused mostly on lipases of fungal origin. In the few applications involving bacterial lipases, the majority of them involve lipases from *Pseudomonas* species, and very few on *Bacillus* or other bacterial genera.

Given this background, the aim of this study was to screen bacterial isolates from the genera other than the *Pseudomonas* for production of lipase enzymes with an aim of finding a bacterial lipase with novel properties. Desirable properties would be

high lipase activity, high temperature stability, and the ability to function in extremes of pH. *Bacillus* species isolated mainly from dairy alkaline water samples were screened for their lipase production. Two isolates were identified as best producers of lipase on the basis of relative activity, temperature, pH optima and stability.

In the first chapter of the study, the factors influencing the production of the lipase enzyme by one of the bacterial isolates are described, attempts to purify the enzyme, and the properties of the produced lipases. The gene encoding the lipolytic activity of the second isolate was cloned and compared with the gene sequences from related *Bacillus* species. Chapter 3 describes the over-expression of the gene encoding lipolytic activity of the first isolate. The lipase enzyme is purified as a recombinant protein and its properties are reported. Chapter 4 describes the identification by site-directed mutagenesis of amino acid residues that are essential for *Bacillus licheniformis* lipase activity. Chapter 5 describes the cloning of the previously unreported lipolytic gene encoded by the first isolate.

1.9. REFERENCES

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CHAPTER 2

Production, regulation and some properties of a partially purified *Bacillus licheniformis* lipase and the cloning of a DNA sequence encoding *Bacillus pumilus* lipase activity

2.1. Introduction

Lipolytic enzymes are widely distributed in nature, and are produced by plants, animals and microorganisms (Wooley and Petersen, 1994). Lipolytic enzymes are physiologically important during processes involving fat degradation and metabolism (Connely, 1999; Osterlund, 2001). However, it is for their economic significance that they have attracted much attention (Jaeger and Reetz, 1998). Microbial lipases in particular have been used extensively in the food and dairy industry and have been found to be essential in the production of desirable and characteristic flavours in certain foods (Shahani, 1975; Pandey *et al.*, 1999). However, if the production of microbial lipolytic enzymes is not appropriately controlled, they can hydrolyse lipids and concomitantly produce undesirable rancid flavours in fat-containing food products (Frank, 1997). Contamination of dairy product processing equipment and food products by *Bacillus* species has been reported (Lindsay *et al.*, 2000). Production of lipolytic enzymes by the contaminating *Bacillus* species has been implicated in the post-pasteurisation spoilage of dairy products (Frank, 1997). The mechanisms inducing lipase production by *Bacillus* species have, however, not yet been elucidated.

The other fields were lipases are finding important applications are in the detergent and tanning industries, where they are considered indispensable (Pandey *et al.*, 1999). These applications require enzymes that are stable and active at alkaline (pH 8-12) conditions. Alkaliphilic microorganisms, particularly

Bacillus species, are being explored as potential sources of alkali-stable enzymes. This is evidenced by the number of patents and amount of literature describing temperature and alkali stable lipolytic enzymes from *Bacillus* species with potential industrial applications (Möller *et al.*, 1991; Jurgen *et al*, 1992a, 1992b, Lesuisse *et al.*, 1993, Rua *et al.*, 1997, Kim *et al.*, 1998; Lee *et al.*, 1999).

Although cell-free extracts lipase are mainly used, trends are shifting toward using live microorganisms. Microbial species of the *Bacillus* genus are again finding significant consideration in the formulation of "live" detergents as a result of their capabilities in producing and secreting extracellular enzymes. *B. licheniformis*, owing to its biological safety (de Boer *et al.*, 1994) is at the forefront as an ingredient in "live" detergent formulations. Strains of *B. licheniformis* have been introduced in "Liquid Live" (Ameri-Khem, USA) and in "Ultra Biozyme" (Ultra Bio-Logics Inc, Canada) "live" detergents due to their prowess in the production of lipases. These detergents are marketed as products for biological bioremediation and they contain strains of living microorganisms.

Although the properties of lipase enzymes produced by *Bacillus* species are being described (Lesuisse *et al.*, 1993, Kim *et al.*, 1998, Rua *et al.*, 1997, Lee *et al.*, 1999), very little attention has been given to the physiological factors affecting their production. In the studies where conditions of lipase production have been described, it was with the aim of defining conditions of optimum lipase production

rather than elucidating the actual mechanisms responsible for the observed effects.

A screening programme in our laboratory identified two *Bacillus* species isolated from alkaline dairy water samples as good producers of lipase activity. These isolates were identified as *B. licheniformis* and *B. pumilus*. The aim of this study was to investigate the influence of carbon sources on lipase production by the *B. licheniformis* strain isolated by our laboratory. This would facilitate an insight into the mechanisms of lipase gene regulation. The gene encoding *B. pumilus* lipase was cloned and its nucleotide sequence compared with the sequence of the lipase gene from *B. subtilis*.

2.2. Materials and Methods

2.2.1. Chemicals

Nutrient broth and agar were purchased from Biolab Diagnostics (Johannesburg, South Africa), and Holpro (Midrand, South Africa) supplied D-glucose. Oligonucleotide primers, deoxynucleotides and *Taq* polymerase were purchased from Roche (Germany), T7 and Sp6 sequencing primers, DNA molecular mass markers, restriction and ligase enzymes were purchased from Promega (Madison, USA). The pGem-T plasmid and *Escherichia coli* cells JM 109 were obtained from Promega (Madison, USA), and used for PCR product cloning and as transformation host, respectively. Polyoxyethylenesorbitan monolaurate (Tween 20), Polyoxyethylenesorbitan monooleate (Tween 80), olive oil, triolein, tricaprylin, and tributyrin were supplied by Sigma (St. Louis, USA). Glycerol was obtained from BDH laboratories (England). Pierce Co. (Ilinois, USA) supplied caprylic and caproic acid. Toyopearl 560M, Phenyl-Toyopearl 650M and Toyopearl HW-50F were purchased from Tosohaas. Pharmacia supplied Bio-Gel HPHT hydroxylapatite and Phenyl-Sepharose. The vitamin and mineral (A to Z vitamin and mineral) Supradyn Complete tablets were purchased from Roche Products (Isando, South Africa). All other chemicals were commercially available and of analytical or molecular biology grade.

2.2.2. Bacteria and growth conditions

B. licheniformis and *B. pumilus* were isolated from dairy water samples and are obtainable from the culture collection in our department. Screening for lipase production was done by streaking bacterial isolates on Luria-Bertani (LB) [12g agar, 10g tryptone, 5g yeast extract, 10g NaCl, per liter; pH 7.0] agar plates containing (i) an emulsion of olive oil containing Rhodamine B as indicator, pH 7.0 (Kouker and Jaeger, 1987), (ii) Tween 80 and $CaCl_2$, pH 7.0 (Ignjatiovic and Dey, 1993) or (iii) gum arabic-tributyrate emulsion (Fryer *et al.*, 1966). Growth of microorganisms in liquid cultures was done at 30 °C on a rotary shaker (~160 revolutions per minute) and was monitored by OD measurement at 660 nm.

2.2.3. Growth of B. licheniformis on different carbon sources

Different carbon sources were investigated for their ability to support microbial growth in minimal medium. Production of extracellular lipase was monitored in minimal and rich media. The minimal medium contained per liter, 6.0g Na_2HPO_4, 3.0g KH_2PO_4, 0.5g NaCl, 1.0g NH_4Cl, 1 ml of 0.5g $MgSO_4$, 100 µl of 1.0 M $CaCl_2$, 5 ml of micronutrients and vitamin solution. The Mg_2SO_4 and $CaCl_2$ were prepared and steam-sterilized separately. The vitamin and mineral solution was prepared by dissolving one (A to Z vitamin and mineral) Supradyn Complete tablet in 100 ml distilled water followed by centrifugation at low speed to remove

residues that did not dissolve. The vitamin and mineral solution was microfilter-sterilized before being added to the mineral medium. Different carbon sources (Tween 20, Tween 80, tributyrin, tricaprylin, triolein, olive oil, glycerol, caprylic acid, caproic acid, glucose) were prepared in distilled water, the pH adjusted to 7.0, autoclaved, and added to the mineral media to a final concentration of 2 g/l. The rich medium contained nutrient broth 2 g/l. The production of lipases was also monitored in rich medium in the presence of each of the carbon sources (1 g/l). The microorganism was grown in 50 ml medium in a 250 ml culture flasks for 48 hours.

2.2.4. Repressive and inductive effects of carbon sources

The inductive or repressive effects of different carbon sources on lipase-producing culture were investigated firstly by growing the microorganism in 1 liter rich medium (2 g/l), pH 7.0, in a 5 liter flask until a lipase activity of 0.3 units/ml was reached. The growing culture (48 ml) was divided among 250 ml conical flasks containing 0.1 g carbon source dissolved in 2 ml distilled water, pH 7.0, to give a final concentration of 2 g carbon source per liter. Microbial growth and production of lipase activity were monitored over a period of 15 hours after the addition of the carbon source.

2.2.5. The influence of Tween on lipase biosynthesis

The influence of Tween 20 and 80 on intracellular and extracellular lipase levels was done firstly by growing the microorganisms in 1 liter rich medium until the lipase activity of about 0.2 units/ml was achieved. The growing culture (48 ml) was divided into 250 ml flasks containing 0.1 g Tween 20 or 80 dissolved in 2 ml distilled water, and grown for an additional period of 12 hours. The control culture flasks contained distilled water in place of Tween. Culture flasks were removed from the 30 °C incubator at different times, centrifuged at 4 °C for 15 minutes at 13 000*g* for 20 min. The harvested cells were washed twice by resuspension in 50 ml 50 mM phosphate buffer (pH 7.6) followed by centrifugation. Cell lysis was performed by resuspending the cell pellet in phosphate buffer containing 10 % (w/v) sucrose, 1 mM EDTA and 1mM dithiothreitol. Lysozyme was added to 1 mg/ml final concentration, and cell lysis allowed for at least 2 hours at 4 °C. Cells were disrupted by sonication using the Branson Sonifier Cell Disrupter B-30 (settings: 100% duty cycle at 30 W for 60 pulses). The temperature during sonication was maintained at 4 °C by cooling the cell extract on ice for 5 min for every 10 pulses. Cellular debris was removed by centrifugation and the cleared cell lysate was assayed for intracellular lipase activity.

2.2.6. DNA preparation and transformation

For genomic DNA preparation, *B. pumilus* was grown on nutrient broth at 30 °C until an OD_{660} of about 1. The genomic DNA was isolated essentially as described by Zock *et al.*, 1994. Plasmid DNAs were isolated using a nucleospin plasmid isolation kit (Macherey-Nagel, Germany). Transformation of *E. coli* with the recombinant plasmid was performed using standard $CaCl_2$ procedures (Sambrook *et al.*, 1989).

2.2.7. Cloning and sequencing of the lipase gene

A pair of degenerate primers was designed according to the sequences available of the *B. pumilus* lipase gene (GenBank accession no. A34992). The primers used were forward (5'-CAC GAA TTC CCA AGG TGC TTT TTG A-3') and reverse (5'-ACT GAA TTC CAT ATG CCG CTG TCT T-3'). *Taq* polymerase was used to perform PCR with *B. pumilus* genomic DNA as the template. The PCR conditions were as follows: 1 initial denaturation step at 94 °C for 2 min, 35 cycles at 94 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 2 min, except for the final cycle where extension proceeded for 10 min.

The PCR product was electrophoretically resolved on a 1% (w/v) agarose gel, and the appropriate DNA fragment recovered with an Agarose Gel DNA Extraction kit (Roche, Germany). The purified DNA fragment was ligated into a

pGEM-T vector system, according to the manufacturers' recommendations, transformed into *E. coli* JM109 competent cells, and plated on tributyrin-LB agar plates containing 100 μg ampicillin per ml. The plasmid contained in lipase positive colonies was purified and the sequence of the DNA insert determined (T7-Sequencing kit, Pharmacia).

2.2.8. Protein purification

The crude *B. licheniformis* lipase obtained in the supernatant of Tween 80 induced culture was loaded onto a DEAE-Toyopearl 650M column equilibrated with 10 mM Tris-HCl buffer, pH 8.5. The bound proteins were eluted with a 0-1.5 M KCl gradient in 10 mM Tris-HCl, pH 8.5. The lipase active fractions were pooled together and subjected to size exclusion chromatography, hydroxylapatite or hydrophobic interaction chromatography. Butyl Toyopearl 650M, Phenyl Toyopearl 650M or Phenyl Sepharose were prepared for hydrophobic interaction chromatography by equilibration with 1 M $(NH_4)_2SO_4$ in 10 mM Tris-HCl buffer, pH 8.5. Elution of bound proteins was performed in 10 mM Tris-HCl buffer, pH 8.5 containing 30-60% ethylene glycol. The Bio-Gel HPHT hydroxylapatite chromatography column was prepared by equilibration with 10 mM potassium phosphate buffer, pH 8.0. Elution of bound proteins was performed in 0.5M potassium phosphate buffer, pH 8.0. Size exclusion chromatography on Toyopearl HW-50F was performed by equilibrating and eluting the column with 10 mM phosphate buffer, pH 8.0 containing 0.3 M KCl.

2.2.9. Enzyme assays

2.2.9.1. Spectrophotometric assay

Enzyme activity was measured spectrophotometrically at 410nm with *p*-nitrophenyl palmitate (*p*NPP) as substrate at 37 °C in 50 mM phosphate buffer pH 8.5, 0.1% (w/v) gum arabic and 0.2% (w/v) sodium deoxycholate, according to the method of Winkler and Stuckmann, (1979). One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol from *p*NPP per minute.

2.2.9.2. pH-stat assay

The substrate preference for the crude enzyme was investigated using 10 mM of tributyrin, tricaprylin, olive oil and triolein as substrates. A Metrohm 641 Impulsomat pH stat was used with a thermostatically controlled vessel at 37 °C. Substrates were mixed with the assay buffer (1 mM Tris-HCl buffer, 0.1 M NaCl, pH 8.5), in the titration vessel and the emulsion formed by vigorous stirring using a propeller agitator. The reaction was initiated by adding 100–200 μl enzyme sample. The hydrolysis rates of the emulsified triacyglycerols were measured potentiometrically by titrating the liberated fatty acids with standardized NaOH. One unit was defined as the amount of enzyme which released 1 μmol of fatty acid per min.

2.2.10. The effect of lipase hydrolysis products on lipase activity

The crude lipase enzyme was incubated in the presence of 0.25 mg/ml glycerol, caprylic or caproic acid for 30 min at 22 °C. The activity of the lipase enzyme was assayed using the spectrophotometric assay in the presence of glycerol, caprylic acid or caproic acid in concentrations of up to 2 mg/ml.

2.2.11. Electrophoresis

SDS-PAGE was performed on 12% running gels as described by Laemmli (1970), and the resolved proteins visualised by Coomassie staining following standard procedures.

2.2.12. pH and temperature studies

The effect of pH on the lipase activity was determined spectrophotometrically using *p*NPP as substrate. The substrate was prepared in 50 mM buffer volumes of various pH values. At pH 7-8 sodium phosphate buffer was used; for pH values of 8.5 and 9, a glycine/NaOH buffer was used. Carbonate buffer was used for pH values 9.5 – 10.5, and Na_2HPO_3/NaOH buffer was used for pH values 11-12. The effect of pH on lipase stability was determined by incubating the lipase active samples for 2 hours at 30 °C in 50 mM Na_2HPO_4/KH_2PO_4 solution, pH 8.5. Residual activity was assayed spectrophotometrically. The optimum temperature

for lipase activity was determined over a range of 30-70 °C using the spectrophotometric assay. The assay mixture was equilibrated at the required temperature before the addition of the enzyme. The effect of temperature on lipase stability was determined by incubating lipase containing samples for 30 min in 50 mM Na_2HPO_4/KH_2PO_4, pH 8.5. Residual activity was measured using the spectrophotometric assay.

2.3. Results

2.3.1. Lipase production on agar plates

The two bacterial isolates were identified as *B. pumilus* and *B. licheniformis*. The isolates showed extracellular lipase production on Rhodamine B/olive oil, Tween 80/$CaCl_2$ and tributyrin agar plates. Rhodamine B/olive oil agar plates resulted in pink zones around the bacterial colonies, an indication of extracellular lipase being produced. Under UV light, orange fluorescent halos surrounded the bacterial colonies (Figure 2.1.a). Tween 80 / $CaCl_2$ plates resulted in white opaque halos around lipase positive colonies. The halos were the result of precipitation of Ca^{2+} salts of fatty acid (Figure 2.1b). The microbial isolates exhibited zones of clearance around the colonies on tributyrin agar plates (Figure 2.1c(i). The natural opacity of tributyrin agar plates is due to the presence of micro-droplets of tributyrin. The produced lipase converts the tributyrin micro-droplets into water-soluble butyric acid and thereby removing the opacity.

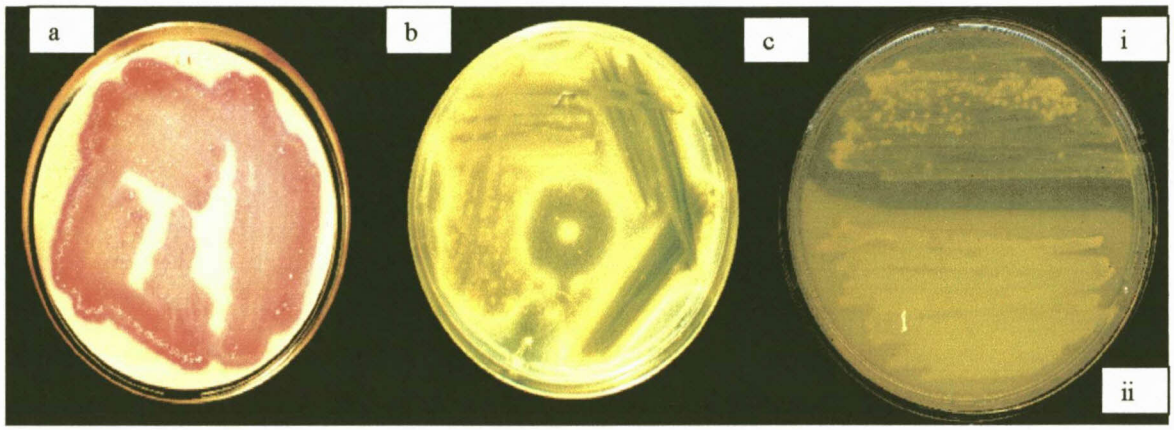

Figure 2.1. Photographs showing lipase production by *B. licheniformis* growing on [a] Rhodamine B/olive oil [b] Tween 80/$CaCl_2$ and [c(i)] tributyrin agar plates. c(ii) shows a lipase deficient *Escherichia coli* JM 109 growing on tributyrin agar plate.

2.3.2. The effect of different carbon sources on growth and lipase production

A range of different carbon sources in mineral medium were screened for their capacity to support growth of *B. licheniformis* and lipase production. The microorganism could not grow in minimal medium supplemented with Tween 20, Tween 80, caprylic acid and caproic acid. The mineral medium supplemented with triolein, olive oil, tricaprylin, tributyrin, glycerol and glucose supported growth of the microorganism, but very low lipase activity was produced (less than 20 U/l). Nutrient broth supported growth and production of lipase activity with activity levels approaching 500 U/l during the stationary phase of *B. licheniformis* growth (Figure 2.2).

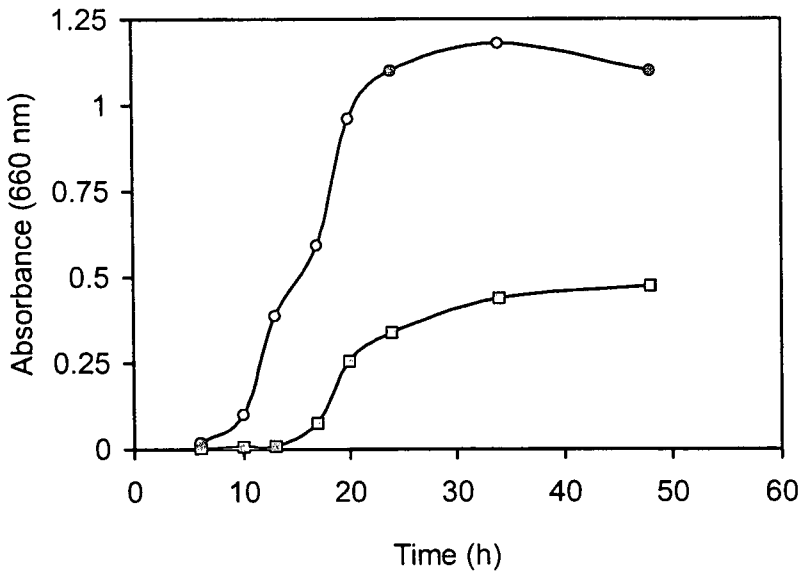

Figure 2.2. Growth curve ( ) and corresponding extracellular lipase production ( ) of *B. licheniformis* culture growing on nutrient broth.

2.3.3. The influence of carbon sources on the level of lipase production

The repressive or inductive effects of carbon sources were investigated by growing *B. licheniformis* on nutrient broth until an extracellular lipase activity of about 0.3 U/ml was achieved. The carbon source was then added and the level of lipase production monitored over a 15 hour period (Figure 2.3).

Glucose, glycerol tributyrin, tricaprylin, triolein, olive oil and fatty acids reduced the level of lipase production when added into a culture of *B. licheniformis* that was actively engaged in lipase biosynthesis. The incubation of the enzyme in lipase hydrolytic products [glycerol, and free fatty acids (caproic and caprylic)] prior to assay, and enzymes assays in the presence of lipase hydrolytic products in concentrations of up to 2 mg/ml, did not have inhibitory effects on the activity of the lipase enzyme (data not shown). This suggested that the reduction in lipase activity observed in cultures was a repressive effect of the carbon source on the biosynthesis of the lipase enzyme, and not the inhibitory effect on the enzyme itself. Free fatty acids, glucose and glycerol exhibited slow repressive effects while triacylglycerols showed a pronounced and rapid repressive effect, with more than 70% reduction in lipase production level being observed one hour following addition of the carbon source. The repressive effects of triolein was however, slower than that of the other triacylglycerols.

Addition of Tween 20 and 80 to the culture growing on nutrient broth increased the level of lipase production. To elucidate the inductive effect of Tween 20 and 80 on lipase production, *B. licheniformis* was grown on glucose or glycerol mineral media, supplemented with either Tween 20 or 80. Although good growth was observed in both media, no lipase production was observed. This demonstrated that Tween 20 and 80 do not have the inductive effects for lipase production on their own; they probably enhance the expression of the lipase gene, probably by interacting at the level of lipase protein translocation.

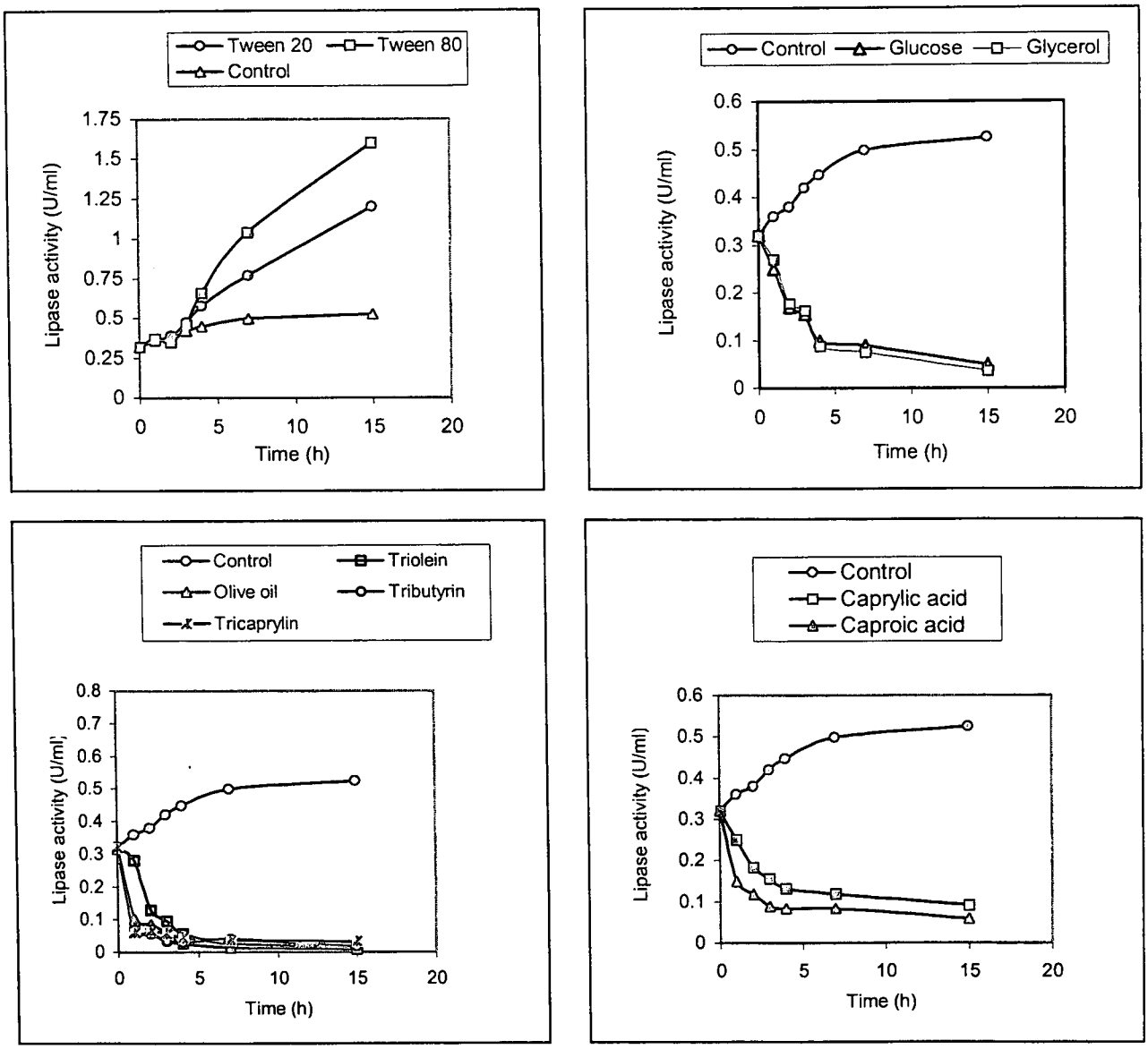

Figure 2.3. The effects of (A) Tween 20 and 80, (B) glucose and glycerol, (C) triacylglycerols and (D) fatty acids on the level of lipase production by *B. licheniformis* growing on nutrient broth.The data represent the average of two independent experiments.

To test the hypothesis that Tween facilitates the translocation of the protein to the extracellular medium, an experiment was set where Tween 20 and 80 were separately added into the culture of lipase-producing *B. licheniformis*. The lipase-producing culture was divided among several flasks and grown under similar conditions except that the cultures were harvested by centrifugation following various times of growth. The intracellular and extracellular lipase activities were determined and compared with that of the control culture grown under similar conditions, but in the absence of detergent. The control cultures were also harvested by centrifugation at various times of growth (Table 2.1).

There was a lag period of about 6 hours before the effect of Tween 80 resulted in increased level of extracellular lipase. This point coincided with a 50% reduction in the level of intracellular lipase activity compared to the control, and is immediately followed by an increase in intracellular lipase activity to the almost constant value of about 90 units/l. Induction of lipase production with Tween 20 gave a similar trend of lipase activity with the lag period of about 8 hours required. The initial point of high extracellular lipase activity coincided with the reduced level of intracellular lipase activity, which was also followed by an increase in intracellular lipase activity to an almost constant activity value of about 80 units/l. Intracellular lipase activity of the control culture attained a near constant activity value of about 48 units/l.

Table 2.1.

Total activity of extracellular and intracellular lipase of *B. licheniformis* growing in nutrient broth in the absence of Tween (control) or in the presence of Tween 20 or Tween 80. The growth of cultures was stopped at different times by removal from the 30°C incubator followed by immediate centrifugation at 4 °C. The intracellular and extracellular lipase activities were assayed with the *p*NPP assay. The values indicate the average values of two independent experiments.

Time	Extracellular lipase activity (units/l)			Intracellular lipase activity (units/l)		
(h)	Control	Tween 20	Tween 80	Control	Tween 20	Tween 80
2	180	190	240	15	17	22
4	300	208	280	27	11	19
6	500	475	882	42	40	21.2
8	480	840	45.3	52	27	90
12	500	1130	1520	46	76	94
15	600	1600	1760	50	88	88
18	640	1920	2160	48	86	90

2.3.4. Purification of B. licheniformis lipase

Purification of *B. licheniformis* lipase was attempted using traditional methods of chromatography with the aim of sequencing the N-terminal of the purified extracellular lipase protein and thereafter designing a DNA probe that could be used in gene cloning. Ion-exchange chromatography of the lipase enzyme on DEAE-Toyopearl resulted in the separation of most contaminating proteins from the enzyme (Figure 2.4). There was a strong interaction between the lipase enzyme and the chromatographic resin as evidenced by the requirement for the high salt concentration before the enzyme could elute from the column. The active fractions where loaded onto hydrophobic interaction chromatography

columns, but the enzyme failed to elute even at high concentrations of ethylene glycol. Although the enzyme could bind to the hydroxylapatite chromatography column, there was an irreversible interaction between the protein and the resin. The chromatography of the pooled lipase active fraction on size exclusion column did not result in the purification of the protein as evidenced by a low specific activity and SDS-PAGE (data not shown).

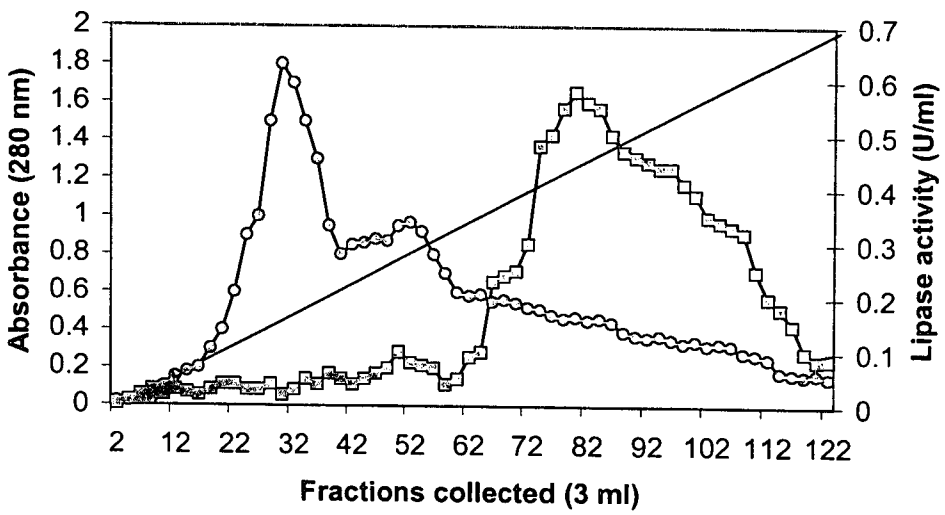

Figure 2.4. DEAE–Toyopearl chromatography of *B. licheniformis* lipase. [$A_{280\ nm}$ ( ) and lipase activity ( )]. The line across the graph represents the salt (KCl) gradient (0-1.5 M) of the elution buffer.

2.3.5. Characterization of Bacillus lipases

The bacterial isolate identified as *B. pumilus* also produced extracellular lipase activity when grown on nutrient broth supplemented with Tween 80 (details not discussed). The crude *B. pumilus* lipase and the partially purified lipase from *B. licheniformis* were characterized with respect to thermostability, pH optima and stability, and substrate preference. The lipase enzymes exhibited maximum activities at temperatures between 40 and 60 °C with *B. licheniformis* lipase showing maximum activity at 50-60 °C (Figure 2.5). The lipase enzymes were not thermostable. Incubation of lipases at the temperature of 50 °C for 30 min resulted in a sharp decrease in activity, with less than 20 % activity remaining for both lipases.

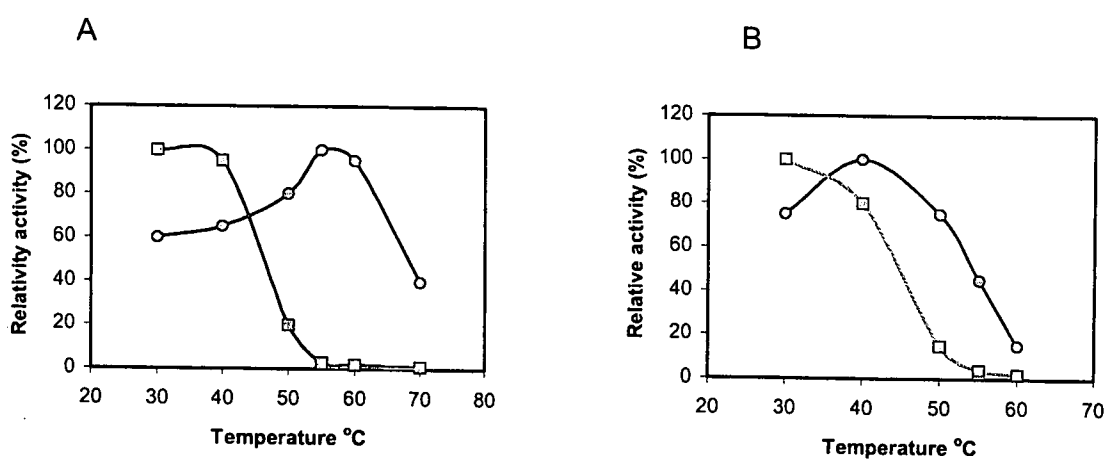

Figure 2.5. Effect of temperature on (A) *B. licheniformis* lipase and (B) *B. pumilus* lipase. The results are expressed as a percentage of the maximal activity in temperature optimum curve ( ) or as a percentage of the initial activity in temperature stability curve ( ). The data represent the arithmetic mean of at least three determinations with maximum deviation of less than 6%.

The enzymes displayed optimal catalytic activities towards *p*NPP in the alkaline region of pH 8-11 (Figure 2.6). The lipase from *B. licheniformis* showed a broad range of pH optimum with the highest activity being obtained at pH 10. *B. pumilus* showed maximum activity at pH 9.5. Both lipases were stable at alkaline pH values of 7-10.

A

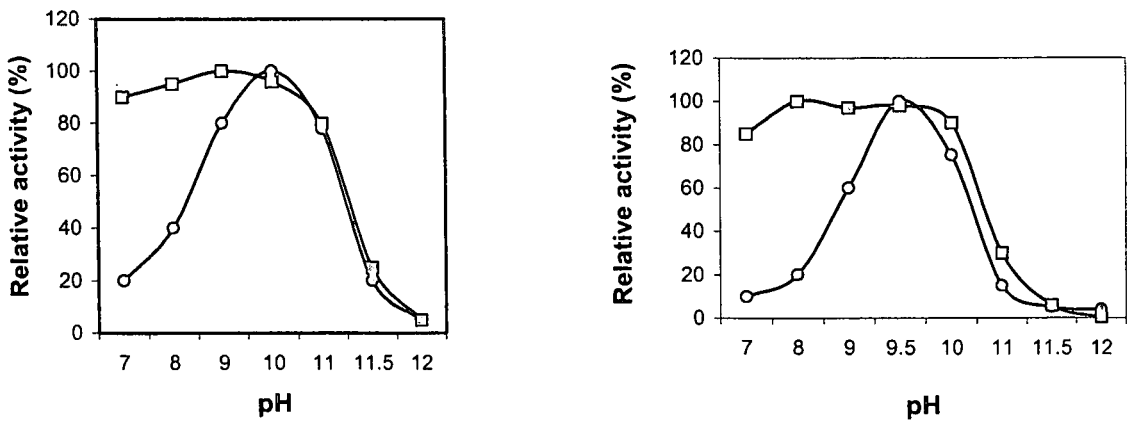

Figure 2.6. Effect of pH on (A) *B. licheniformis* and (B) *B. pumilus* lipase activities. The results are expressed as a percentage of the maximal activity in pH optimum curve ( ) or as a percentage of the initial activity in pH stability curve ( ). The data represent the arithmetic mean of at least three determinations with maximum deviation of less than 6%.

2.3.6. Substrate specificities

The relative activities of lipases toward selected triacyglycerols were investigated (Table 2.2). Activity on each substrate was expressed as the percentage of tricaprylin. Although lipase activities were detected on all triacylglycerols investigated, the highest activity for both lipases was obtained with tricaprylin (C8) indicating the preference of the lipases for mid-chain carboxylic esters. The

preference for the mid-chain fatty acyl esters has also been reported for *B. subtilis* lipase (Lesuisse *et al.*, 1993).

Table 2.2

Relative activities of *Bacillus* lipases on different substrates. Lipase activities were assayed at fixed 10 mM concentrations of the triacylglycerol using the pH-stat assay. Maximal deviations of not more than 8% were recorded.

Triacylglycerol	*B. licheniformis* lipase relative activity (%)	*B. pumilus* lipase relative activity (%)
Tributyrin	68	76
Tricaprylin	100	100
Triolein	20	32
Olive oil	40	46

2.3.7. Cloning and sequence analysis of B. pumilus *lipase gene*

The PCR primers to amplify the *B. pumilus* DNA fragment encoding lipase activity were designed based on the published nucleotide sequence of the lipase gene from *B. pumilus* DSM strain (GenBank accession No. A34992). These primers amplified a DNA fragment of about 800 bp (Figure 2.7), which was subsequently cloned into pGem-T vector to construct the plasmid denoted pGem-pumilip. The cloned DNA fragment was sequenced (Figure 2.8).

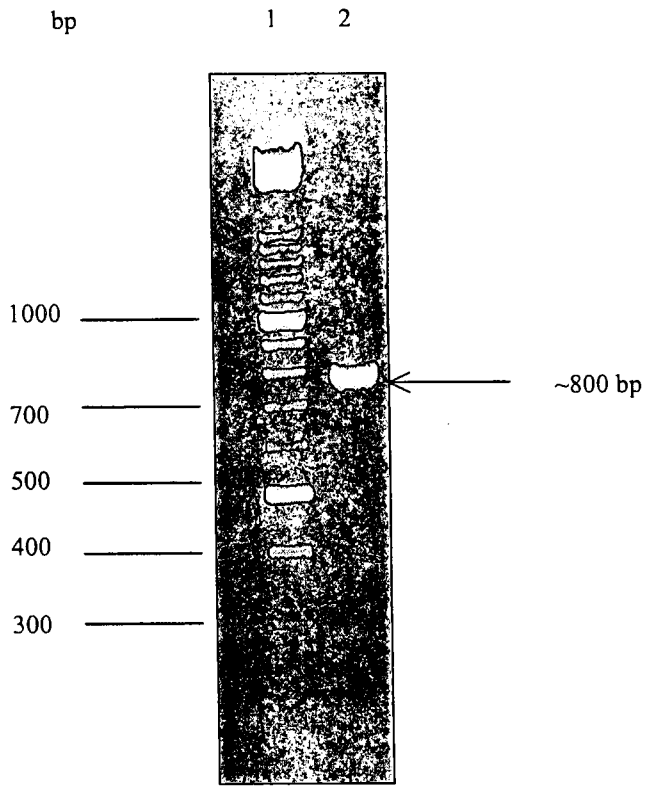

Figure 2.7. Agarose gel electrophoresis of a PCR product (Lane 2) obtained with *B. pumilus* genomic DNA as the template. Lane 1, DNA molecular weight marker with selected fragment size indicated.

The cloned DNA fragment displayed a single open reading frame of 639 bp preceded by the promoter sequence. The –35 and –10 sequences of the promoter were identified as TTCACT and TATAAA, respectively, by comparison with promoter elements that are recognized by *B. subtilis* sigma factors. These elements resemble the consensus sequences for the promoter region recognized by σ^A RNA polymerase of *B. subtilis*, which have been reported to be TTGACA and TATAAT (Haldenwang, 1995). The two promoter elements are separated by

a sequence of 19 bp and are found at positions commonly occupied by promoter regulator elements. Although there is an ATG codon immediately upstream of the ribosome binding sequence (AAGGGGG), initiation of translation is likely to start at the GTG codon located 6bp upstream of the rbs site as a result of the requirement for a 6-9 nucleotide spacer between the rbs and the initiation codon for efficient translation (Mountain, 1989). The open reading frame is terminated by a single TAA stop codon. The plasmid containing this DNA fragment exhibited lipase activity when transformed into lipase deficient *E. coli* cells and streaked on tributyrin agar plates (data not shown). This indicated that the lipolytic gene was successfully cloned.

The major open reading frame encodes a protein consisting of 213 amino acids, with a predicted molecular weight of 22 983 Da. Extracellular proteins produced by *Bacillus* species are synthesized as pre-proteins with the amino-terminal signal peptide sequence. The signal peptide is needed for proper targeting of the protein, and is removed before the mature protein is released into the extracellular medium (Simonen and Palva, 1993). A possible signal peptide could be predicted by the SignalP computer program (Nielsen *et al.*, 1997) and the putative cleavage site was predicted to lie between the two Ala residues at positions 32 and 33 (Figure 2.8). Cleavage of the signal peptide at this point releases a mature lipase protein of 181 amino acid residues with the predicted M_r of 19.3 kDa and pI of 9.43 (Bjellqvist *et al.*, 1993, 1994; Wilkins *et al.*, 1998)

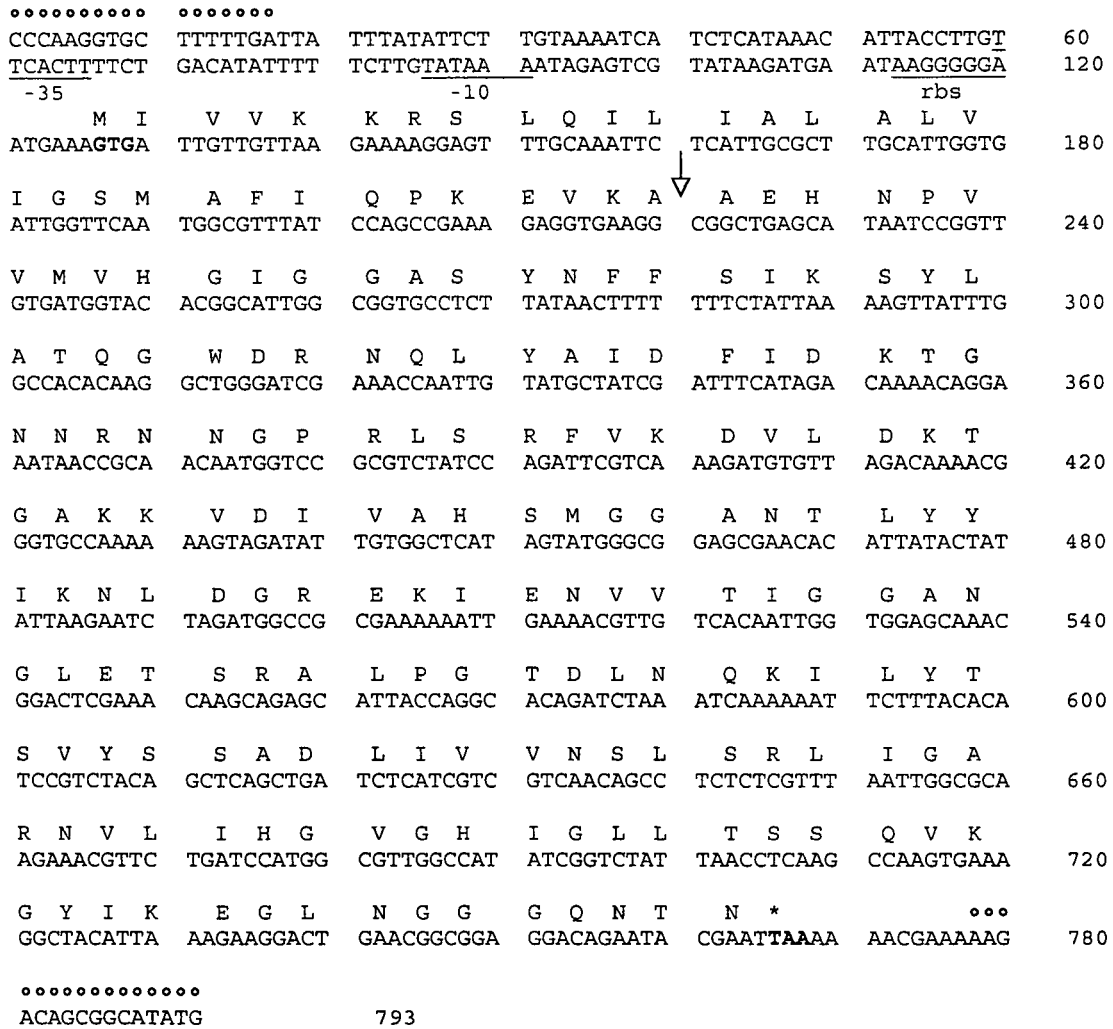

Figure 2.8. DNA sequence encoding *B. pumilus* lipase and the deduced amino acid sequence. The putative transcription and translation signals are underlined. The vertical arrow indicates the predicted cleavage site of the signal peptide. The stop codon is represented by (*). The annealing positions of the primers are indicated by the dotted lines.

The amino acid sequence of *B. pumilus* (UOFS) strain was compared with the amino acid sequences of lipases produced by *B. pumilus* DSM strain and *B. subtilis* (Figure 2.9). The first 32 amino acids occupying the N-terminal regions of the lipases from the two *Bacillus* species do not significant show homology. These segments were predicted as signal peptides, with the cleavage site located between the first two consecutive Ala residues. The mature segments of the lipases exhibit sequence homology of more than 80 %. The lipases from the two *B. pumilus* strains show an amino acid identity of 96%, indicating that the primers used in this study succeeded in amplifying the targeted lipase gene. The putative "conserved" pentapeptide Ala-X_1-Ser-X_2-Gly containing the nucleophilic serine residue essential for catalysis was identified at position 106-111.

```
                                                  ▽
B.pumilus UOFS      MIVVKKRSLQILIALALVIGSMAFIQPKEVKA AEHNPVVMVHGIGGASYNFFSIKSYLAT 60
B.pumilus DSM       MIFVKKRSLQILIALALVIGSMAFIQPKEAKA AEHNPVVMVHGIGGASYNFFSIKSYLAT 60
B.subtilis          MKFVKRRIIALVTILMLSVTSLFALQP-SAKA AEHNPVVMVHGIGGASFNFAGIKSYLVS 59
                    * .**:* : ::  * * : *:  :** ..** ****************:** .*****.:

B.pumilus UOFS      QGWDRNQLYAIDFIDKTGNNRNNGPRLSRFVKDVLDKTGAKKVDIVAHSMGGANTLYYIK 120
B.pumilus DSM       QGWDRNQLYAIDFIDKTGNNRNNGPRLSRFVKDVLDKTGAKKVDIVAHSMGGANTLYYIK 120
B.subtilis          QGWSRDKLYAVDFWDKTGTNYNNGPVLSRFVQKVLDETGAKKVDIVAHSMGGANTLYYIK 119
                    ***.*::***:** ****.* **** *****:.***:**********************

B.pumilus UOFS      NLDGREKIENVVTIGGANGLETSRALPGTDLNQKILYTSVYSSADLIVVNSLSRLIGARN 180
B.pumilus DSM       NLDGGDKIENVVTIGGANGLVSSRALPGTDPNQKILYTSVYSSADLIVVNSLSRLIGARN 180
B.subtilis          NLDGGNKVANVVTVGGANRLTTGKALPGTDPNQKILYTSIYSSADMIVMNYLSRLDGARN 179
                    ****:*: ****:**** * :.:****** ********:*****:**:* **** ****

B.pumilus UOFS      VLIHGVGHIGLLTSSQVKGYIKEGLNGGGQNTN 213
B.pumilus DSM       ILIHGVGHIGLLTSSQVKGYIKEGLNGGGQNTN 213
B.subtilis          VQIHGVGHIGLLYSSQVNSLIKEGLNGGGQNTN 212
                    : ********** ****:. **************
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Figure 2.9. Alignment of amino acid sequences of lipases from various *Bacillus* species. The alignment was performed with CLUSTAL W (Thompson *et al.*, 1994). The asterisks (*) indicate identical or conserved amino acid residues in all sequences in the alignment. The point (.) and colon (:) respectively denote semi-conserved and conserved amino acid substitutions in the alignment. The vertical arrow indicates the predicted cleavage site of the signal peptide. The putative "conserved" pentapeptide is shown in bold, underlined letters. Lipase gene sequence accession numbers. *B. pumilus* (A34992), *B. subtilis* (M74010).

2.4. Discussion

Previous works on the physiology of lipase production showed that the mechanisms regulating lipase biosynthesis vary within the *Bacillus* genera. Lipase production by thermophillic *Bacilli* is strongly induced by long chain triacylglycerols, and repressed by glucose (Lee *et al.*, 1999; Sugihara *et al*, 1991, Schmidt-Dannert *et al.*, 1994). Results obtained with *B. subtilis* showed that lipase production is constitutive and independent of the addition of lipidic substrates to the culture medium (Lesuisse *et al.*, 1993). Divergent results on the effects of carbon sources on lipase production by *B. licheniformis* strains have been reported. Khyami-Horani (1996) reported increased lipase production in the medium containing glucose, while olive oil, detergents (Tween 20 and 80) had no significant effect on the level of lipase production by *B. licheniformis*. On the other hand, Fakhreddine *et al.*, (1998) showed that lipase production by *B. licheniformis* was sensitive to glucose repression with Tween 80 having stimulatory effects.

In our laboratory, we isolated *B. licheniformis* (UOFS) strain that produces extracellular lipase activity. This study describes how, in *B. licheniformis* (UOFS) selected carbohydrates and lipidic substrates participate in the expression of the extracellular lipase activity. The microorganism could not grow in minimal medium containing Tween 20, Tween 80, caprylic acid and caproic acid as sole carbon sources. The mineral medium supplemented with tributyrin, triolein, olive

oil, tricaprylin, glycerol and glucose supported growth of the microorganism, but lipase activities at almost undetectable levels were achieved.

Bacillus species are known to release extracellular enzymes during late exponential and stationary phase growth (Sharp *et al.*, 1989). During these phases of growth, preferred energy sources are limiting or depleted, and the organisms by releasing extracellular enzymes to degrade other carbon sources that might be available in the culture media. Growing the *B. licheniformis* on triacylglycerols, glucose and glycerol to stationery phase was, however, not accompanied by the release of extracellular lipase activity. This indicated the requirement for the lipase-inducing factor. Nutrient broth supported both growth and production of extracellular lipase activity, indicating the presence of a lipase-inducing factor in the rich medium. Production of lipase was predominantly achieved during the stationary phase of growth implying a growth phase-dependent regulation of gene expression.

Although the inducing factor could not be established, it was observed that addition of triacylglycerols, free fatty acids, glucose and glycerol inhibited lipase production. Triacylglycerols were found to have more pronounced and rapid repressive effects as compared to the other carbon compounds. One can speculate that the increased repressive effects of triacylglycerols resulted from compounded effects of the hydrolytic products of the lipase. Glycerol and free fatty acids on their own exhibited slow repressive effects. Addition of

triacylglycerols in lipase-containing medium should result in the hydrolysis of the triacyglycerols (Figure 2.1), yielding free fatty acids and glycerol, and the compounded effects of each of these, could have resulted in increased repression. This is further supported by the observation that triolein, which was the least preferred substrate for the *B. licheniformis* lipase, showed relatively slow repressive effect as compared to the other triacylglycerols.

This speculation suggests the occurrence of two pathways by which the investigated triacylglycerols repress the production of the lipase enzyme. One pathway responds to the presence of fatty acids whereas the other responds to the presence of glycerol. The two pathways function independently of each other as evidenced by the rapid and pronounced repression in the presence of triacylglycerols. This speculation is on the basis that the two carbon compounds are not acting synergistically on one repressive pathway.

Data obtained with media supplemented with Tween 20 and 80 showed enhanced levels of extracellular lipase activities. Although Tween has been shown to enhance lipase production by other strains of *Bacillus* species (Gowland *et al.*, 1987, Fakhreddine *et al.*, 1998, Emanuilova *et al.*, 1993), the mechanism of induction has not been explained. This study has demonstrated that Tween on its own does not have the lipase inducing effect. However, it enhances the production of the lipase by *B. licheniformis*. The inducing effect of Tween could be hypothesized to be at the level of lipase protein translocation

from the intracellular to the extracellular medium. This is supported by the coincidence of the initial observable increase of extracellular lipase activity with reduced intracellular lipase activity. There is probably the existence of a rate-limiting step in the transport of newly synthesized protein. In the presence of Tween, the rate of lipase protein translocation to the extracellular medium is increased. The cells respond to the enhanced protein secretion by increasing the rate of lipase biosynthesis. It is known that surfactants can increase yeast cells permeability, facilitating the export of several compounds across the cell through the membrane (Espinosa *et al.*, 1990, Christova *et al.*, 1996). Therefore, it is likely that Tween 20 and 80 increased the permeability of *B. licheniformis* cell envelope, and consequently the rate of protein secretion and expression.

Although the lipase enzyme from *B. licheniformis* could not be purified to homogeneity, some properties could however be investigated. The lipase is an extremely basic tolerant protein, resembling the lipases produced by *B. pumilus* and *B. subtilis* (Möller *et al.*, 1991; Lessuisse *et al.*, 1993). The three *Bacillus* lipases show high activities toward substrates with mid-chain fatty acyl esters, and are not thermostable. The cloning and sequencing of the lipase gene from *B. pumilus* and comparison of the sequences with that of *B. subtilis* lipase gene revealed that the biochemical properties exhibited by the two lipases have some structural basis. The amino acid sequences of the mature lipases showed striking identities, suggesting that the mature lipase of *B. licheniformis* might also share

significant amino acid similarity with the mature lipases from *B. pumilus* and *B. subtilis*.

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CHAPTER 3

Over-expression and properties of a purified recombinant *Bacillus licheniformis* lipase: A comparative report on *Bacillus* lipases

3.1. Introduction

Lipases (E.C. 3.1.1.3) constitute a group of enzymes having the ability to hydrolyse triacylglycerols at a lipid-water interface (Sarda and Desnuelle, 1958). They remain active in a variety of organic solvents, where they can catalyse various transformations other than the hydrolytic reaction by which they are defined (Margolin and Klibanov, 1987). Microbial lipases have attracted considerable attention owing to their biotechnological potential, ranging from their use as additives in laundry detergents to stereospecific biocatalysis (Jaeger *et al.*, 1994). Lipases are secreted into culture medium by many fungi and bacteria and have been purified and extensively characterized in pursuit of lipases with novel properties for industrial applications.

Alkaliphilic and thermophilic microorganisms have been the focus of a number of investigations into sources of lipases that are stable and function optimally at extreme alkaline pH values and high temperatures. Isolates of *Bacillus* species have been found to produce lipolytic enzymes under alkaline conditions (Lindsay *et al.*, 2000). Lipases from *B. subtilis* (Lesuisse *et al.,* 1993) and *B. pumilus* (Moller *et al.*, 1991) have been of particular interest as they exhibit optimal activity and stability at extreme alkaline pH values (pH values above 9.5). These enzymes, however, are thermolabile. This is in contrast to lipases from *B. stearothermophilus* (Kim *et al.*, 1998), *B. thermocatenulatus* (Rua *et al.*, 1997) and *B. thermoleovorans* (Lee *et al.*, 1999), which are thermotolerant, and display

maximal activity and stability at moderate alkaline pH values (pH 7-9.5). The genes encoding these *Bacillus* lipases have been sequenced and the predicted amino acid sequences found to lack the characteristic Gly-X-Ser-X-Gly motif centered on the lipase active serine residue.

Purification of *Bacillus* lipases for biochemical characterization and crystallographic studies is difficult, mainly due to stringent regulatory mechanisms of their synthesis, which often result in low levels of production. For example, purification of about 100 mg of *B. subtilis* lipase required initial construction of a lipase overproducing *Bacillus* strain followed by the construction of a 30-litre fermentor (Misset *et al.*, 1994). Purification of most *Bacillus* lipases is further complicated by the use of hydrophobic substrates as inducers for lipase production (Gowland *et al.*, 1987). In our laboratory, we attempted to purify the lipase enzyme from *B. licheniformis* using traditional methods of protein purification. The presence of Tween 80 as lipase inducer in the culture media, protein aggregation, lack of interaction and irreversible interaction with chromatographic resins resulted in only partially pure lipase preparations.

To fully understand the catalytic properties of *Bacillus* lipases, the three-dimensional structures of these proteins are required. At the time of planning and execution of this study, there was no known three-dimensional structure of lipase proteins from the *Bacillus* genus. The problem of low production and difficulties associated with traditional protein purification procedures could be alleviated by

over-expressing *Bacillus* lipase genes in suitable expression systems as recombinant proteins, followed by purification by affinity chromatography.

In this study, the author describes the cloning, sequencing, over-expression and characterization of a purified recombinant *B. licheniformis* lipase. The analysis of the predicted amino acid sequences of cloned *Bacillus* lipase genes and the biochemical characterization of isolated proteins, lead us to propose two distinct groups of *Bacillus* lipases.

3.2. Materials and methods

3.2.1. Chemicals

All chemicals were commercially available and of analytical or molecular biology grade. Tryptone, yeast extract, nutrient broth and agar were purchased from Biolab Diagnostics (Johannesburg, South Africa). Lysozyme, ampicillin, isopropyl β-D-thiogalactoside (IPTG), agarose, oligonucleotide primers, deoxynucleotides and *Taq* polymerase were purchased from Roche (Germany). T7 and Sp6 promoter sequences, endonuclease restriction and ligase enzymes were purchased from Promega (Madison, USA). Molecular mass marker proteins were purchased from Bio-rad (California, USA). Unless mentioned otherwise, all reagents were purchased from Sigma (St. Louis, USA).

3.2.2. *Bacteria, plasmids and media*

B. licheniformis DSM 12369 and *B. licheniformis* UOFS were used. Initial screening for lipolysis was performed as described by Labuschagne *et al.*, 1997. The two *B. licheniformis* strains served as sources for genomic DNA. *Escherichia coli* cells JM109 and JM109 (DE3) (Promega, Madison, USA) were used as cloning and expression hosts, respectively. Plasmids pGem-T (Promega, Madison, USA) and pET 20b(+) (Novagen, Madison, USA) were used for cloning and expression, respectively. The *B. licheniformis* bacterium was grown at 42 °C in nutrient broth for the genomic DNA preparation. The *E. coli* hosts carrying plasmids were grown in Luria-Bertani (LB) medium containing 100 μg ampicillin per ml.

3.2.3. *DNA preparation and transformation*

Genomic DNA was prepared as essentially as described by Errington (1984).. Plasmid DNAs were isolated using a nucleospin plasmid isolation kit (Macherey-Nagel, Germany). Transformation of *E. coli* with recombinant plasmids was performed using standard $CaCl_2$ procedures (Sambrook *et al.*, 1989).

3.2.4. Cloning and sequencing of the lipase gene

A pair of degenerate primers was designed according to the sequences available of the *B. pumilus* lipase gene (GenBank accession no. A34992) and the *B. subtilis* lipase gene (GenBank accession no. M74010). The restriction sites for *Nde* I and *Xho* I were incorporated into the forward and reverse primer sequence, respectively. The primers used were forward 5'-GGG CAT ATG GCT GAR CAY AAY CCN GTN GTN ATG GTN-3' and reverse 5' GGG CTC GAG ATT CGT ATT CTG TCC YTG KCC YCC GCC–3'. *Taq* polymerase was used to perform PCR with *B. licheniformis* genomic DNA as the template. The PCR conditions were as follows: 1 initial denaturation step at 94 °C for 2 min, 35 cycles at 94 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 1 min, except for the final cycle where extension proceeded for 10 min.

The PCR product was electrophoretically resolved on a 1% (w/v) agarose gel, and the appropriate DNA fragment recovered with an Agarose Gel DNA Extraction kit (Roche, Germany). The purified DNA fragment was ligated into a pGEM-T vector system, according to the manufacturers' recommendations, transformed into *E. coli* JM109 competent cells, and plated on LB agar plates containing 100 μg ampicillin/ml. A clone containing the correct insert was identified by restriction enzyme analysis, and denoted pGem-Lichlip. The nucleotide sequence of the *B. licheniformis* (UOFS strain) lipase gene in pGEM-

Lichlip plasmid was determined (T7-Sequencing kit, Pharmacia), and deposited into the Genbank sequence databases under the accession number AJ297356.

3.2.5. Data search and analysis

Sequences retrieved from databases were analysed with SignalP (Nielsen *et al.*, 1997), Compute pI/MW (Bjellqvist *et al.*, 1993, 1994; Wilkins *et al.*, 1998) and CLUSTAL W (Thompson *et al.*, 1994).

3.2.6. Over-expression of the lipase gene

A 549 bp fragment was released from pGem-Lichlip by digesting with *Nde* I and *Xho* I restriction enzymes and ligated into similarly cleaved pET-20b(+) plasmid. The construct, referred to as pET-Lichlip, was transformed into *E. coli* JM109 (DE3) cells and plated on LB agar plates containing 100 μg/ml ampicillin. A single colony containing pET-Lichlip was grown in 50 ml LB containing ampicillin to an absorbance of approximately 0.8 at 600nm. The culture was then adjusted to 0.5mM IPTG, and incubation continued at 30 °C for 10h. Cells were harvested by centrifugation at 5 000 X *g* for 10 min, resuspended and incubated in 10 mM phosphate buffer, pH 8.0 containing 1mg/ml lysozyme for 30 min, followed by sonication on ice with a Branson Sonifier Cell Disrupter B-30 (settings: 100% duty cycle at 30 W for 6 X 10-sec pulses) to release intracellular proteins. The cell-free extract was centrifuged at 10 000 X *g* for 20 min to remove cell debris,

and assayed for lipase activity. The culture broth obtained from the first centrifugation step was collected and assayed for secreted or extracellular lipase activity. Fractions containing intracellular and extracellular lipase activity were combined, lyophilized, and stored at -20°C.

3.2.7. Purification of the recombinant lipase enzyme

The purification procedure was performed at room temperature. Lyophilized crude extract (0.5g) was dissolved in 5 ml loading buffer (50 mM sodium phosphate buffer pH, 8.0; 300 mM NaCl; 20 mM imidazole) and then mixed with 5ml of the 50% Ni^{2+} -chelated nitriloacetic acid (Ni-NTA) resin slurry (Qiagen, CA, USA). The crude extract-NTA mixture was loaded into a chromatographic column and washed with 50 ml loading buffer. Elution of bound protein was effected with the loading buffer containing 250 mM imidazole. The resultant enzyme solution was dialyzed overnight against 10 l of 5 mM carbonate buffer, pH 10.0.

3.2.8. Enzyme assays

Enzyme activity was measured spectrophotometrically at 410nm with *p*-nitrophenyl palmitate (*p*NPP) as substrate at 37 °C in 50 mM phosphate buffer pH 8.5, 0.1% (w/v) gum arabic and 0.2% (w/v) sodium deoxycholate according to the method of Winkler and Stuckmann (1979). One unit of enzyme activity was

defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol from *p*NPP per minute.

3.2.9. Protein determination

Protein concentration was determined with the bicinchoninic acid (BCA) protein assay kit (Pierce, IL, USA) using the enhanced method according to the manufacturer's instructions.

3.2.10. Electrophoresis

SDS-PAGE was performed on 12% running gels as described by Laemmli (1970) and resolved proteins visualised by Coomassie staining following standard procedures. A broad range protein standard (Bio-Rad, U.S.A.), was used as molecular mass markers.

3.2.11. pH optimum and stability

The effect of pH on the lipase activity was determined spectrophotometrically using *p*NPP as substrate as described above. The substrate was prepared in 50 mM buffer volumes of various pH values. At pH 7-8 sodium phosphate buffer was used; for pH values of 8.5 and 9, a glycine/NaOH buffer was used. Carbonate buffer was used for pH values 9.5–10.5, and Na_2HPO_3 /NaOH buffer was used for pH values 11-12. The effect of pH on lipase stability was determined by

incubating aliquots of pure lipase in buffers of different pH values for 4 hours at 30 °C. Residual activity was assayed spectrophotometrically with *p*NPP as the assay substrate.

3.2.12. Temperature optimum and stability

The optimum temperature for lipase activity was determined over a range of 30–75 °C using the spectrophotometric assay. The assay mixture was equilibrated at the required temperature before the addition of enzyme. The temperature stability of the lipase was determined by incubating aliquots of pure lipase for 30 min in 50 mM carbonate buffer, pH 10.0 at temperatures various temperatures. Residual activity was measured using the spectrophotometric assay.

3.2.13. Substrate preference

Substrate preference of the enzyme was determined spectrophotometrically using the following *p*NP fatty acid esters: propionic acid (C_3), butyric acid (C_4), caproic acid (C_6), caprylic acid (C_8), lauric acid (C_{12}), myristic acid (C_{14}), palmitic acid (C_{16}) and stearic acid (C_{18}). The molar substrate concentrations of each fatty acid ester were adjusted to the same value based on absorbance at 280 nm. The results were expressed as a percentage of the substrate that gave maximal activity.

3.2.14. Effect of various agents on lipase activity

The activity of the purified lipase were determined spectrophotometrically following incubation with 1mM concentration of metal chlorides, ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl (PMSF), dithiothreitol (DTT) and 2-mercaptoethanol at 30 °C for 30 min. The remaining activity was determined spectrophotometrically in the presence of an appropriate concentration of a given agent and expressed as a percentage of the activity without the agent.

3.3. Results

3.3.1. Production of B. licheniformis lipase

The wild type *B. licheniformis* organism exhibits lipase activity when grown on plates containing Rhodamine B/olive oil and Tween 80/$CaCl_2$. We cultured *B. licheniformis* in nutrient broth containing various carbon sources as inducers of lipase activity (Chapter 2). Maximum lipase production of approximately 600 uints/l was achieved during the stationary phase of growth with nutrient broth as the sole carbon source. Addition of Tween 20 or 80 to the *B. licheniformis* culture growing on nutrient broth increased lipase production to activity levels of about 2000 units/l.

3.3.2. Cloning and sequencing of the lipase gene

Degenerate oligonucleotide primers, based on the known lipase sequences from *B. pumilus* and *B. subtilis*, were used to amplify a 560 bp fragment from genomic DNA isolated from *B. licheniformis* strains (Figure 3. 1). The PCR product was sub-cloned into the pGem-T vector, and the insert sequenced. The major open reading frame identified in the sequence is predicted to code for a 19kD protein with a pI of approximately 9.5. This hypothetical protein shows significant sequence identity with *B. pumilis* and *B. subtilis* lipases (Figure 3. 2A). The "conserved" pentapetide Ala-X-Ser-X-Gly is similar to that of other *Bacillus* lipases.

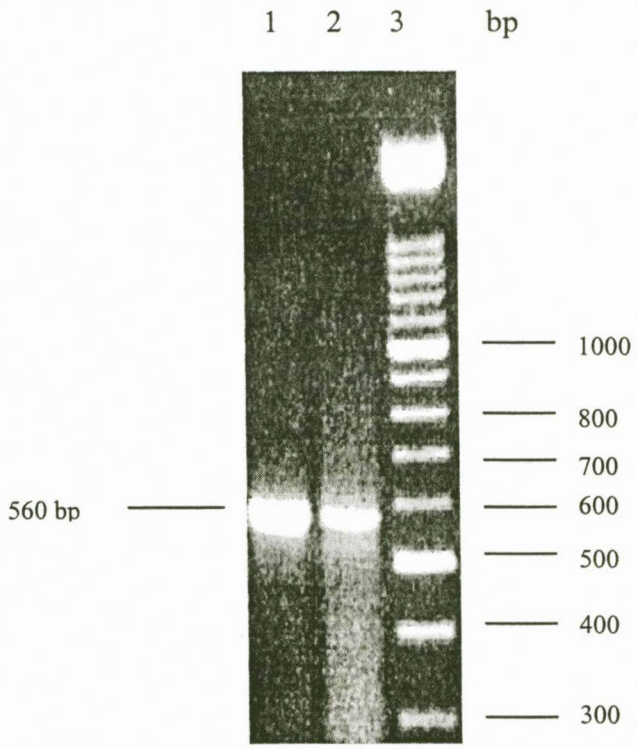

Figure 3.1. Agarose gel electrophoresis of the PCR product obtained with *B. licheniformis* UOFS (lane 1) and *B. licheniformis* DSM 12369 (lane 2) genomic DNAs as templates. Lane 3 shows DNA molecular weight markers.

A. Subfamily I.4

```
B. pumilus              AEHNPVVMVHGIGGASYNFFSIKSYLATQGWDRNQLYAIDFIDKTGNNRNNGPRLSRFVK 60
B. licheniformis        AEHNPVVMVHGIGGASYNFASIKSYLVGQGWDRNQLFAIDFIDKTGNNRNNGPRLSRFVK 60
B. subtilis             AEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNYNNGPVLSRFVQ 60
                        ****************:** .*****. ***.*::*:*:** ****.* **** *****:

B. pumilus              DVLDKTGAKKVDIVAHSMGGANTLYYIKNLDGGDKIENVVTIGGANGLVSSRALPGTDPN 120
B. licheniformis        DVLDKTGAKKVDIVAHSMGGANTLYYIKNLDGGDKIENVIPIGGANGLVSSRALPGTDPN 120
B. subtilis             KVLDETGAKKVDIVAHSMGGANTLYYIKNLDGGNKVANVVTVGGANRLTTGKALPGTDPN 120
                        .***:****************************:*: **:.:**** *.:.:******** 

B. pumilus              QKILYTSVYSSADLIVVNSLSRLIGARNILIHGVGHIGLLTSSQVKGYIKEGLNGGGQNTN 181
B. licheniformis        QKILYTSVYSSADLIVVNSLSRLIGARNVLIHGVGHIGLLTSSQVKGYIKEGLNGGGQNTN 181
B. subtilis             QKILYTSIYSSADMIVMNYLSRLDGARNVQIHGVGHIGLLYSSQVNSLIKEGLNGGGQNTN 181
                        *******:*****:**:* **** ****: ********** ****:. *************
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B. Subfamily I.5

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B. thermocatenulatus    ASPRANDAPIVLLHGFTGWGREEMLGFKYWGGVRGDIEQWLNDNGYRTYTLAVGPLSSNW 60
B. thermoleovorans      AASRANDAPIVLLHGFTGWGREEMFGFKYWGGVRGDIEQWLNDNGYRTYTLAVGPLSSNW 60
B. stearothermophilus   ASPRANDAPIVLLHGFTGWGREEMLGFKYWGGVRGDIEQWLNDNGYRTYTLAVGPLSSNW 60
                        *:.********************:*********************************

B. thermocatenulatus    DRACEAYAQLVGGTVDYGAAHAAKHGHARFGRTYPGLLPELKRGGRVHIIAHSQGGQTAR 120
B. thermoleovorans      DRACEAYAQLVGGTVDYGAAHAAKHGHARFGRTYPGLLPELKRGGRIHIIAHSQGGQTAR 120
B. stearothermophilus   DRACEAYAQLVGGTVDYGAAHAANDGHARFGRTYPGLLPELKRGGRVHIIAHSQGGQTAR 120
                        **********************:.*********************:*************

B. thermocatenulatus    MLVSLLENGSQEEREYAKAHNVSLSPLFEGGHHFVLSVTTIATPHDGTTLVNMVDFTDRF 180
B. thermoleovorans      MLVSLLENGSQEEREYAKAHNVSLSPLFEGGHHFVLSVTTIATPHDGTTLVNMVDFTDRF 180
B. stearothermophilus   MLVSLLENGSQEEREYAKEHNVSLSPLFEGGHRFVLSVTTIATPHDGTTLVNMVDFTDRF 180
                        ****************** ************:**************************

B. thermocatenulatus    FDLQKAVLKAAAVASNVPYTSQVYDFKLDQWGLRRQPGESFDHYFERLKRSPVWTSTDTA 240
B. thermoleovorans      FDLQKAVLEAAAVASNVPYTSQVYDFKLDQWGLRRQPGESFDHYFERLKRSPVWTSTDTA 240
B. stearothermophilus   FDLQKAVLEAAAVASNAPYTSEIYDFKLDQWGLRREPGESFDHYFERLKRSPVWTSTDTA 240
                        ********:*******.****:::************:*********************

B. thermocatenulatus    RYDLSIPGAEKLNQWVQASPNTYYLSFSTERTHRGALTGNYYPELGMNAFSAVVCAPFLG 300
B. thermoleovorans      RYDLSVSGAEKLNQWVQASPNTYYLSFATERTYRGALTGNYYPELGMNAFSAVVCAPFLG 300
B. stearothermophilus   RYDLSVPGAETLNRWVKASPNTYYLSFSTERTYRGALTGNYYPELGMNAFSAIVCAPFLG 300
                        *****:.***.**:**:**********:****:******************:*******

B. thermocatenulatus    SYRNEALGIDDRWLENDGIVNTVSMNGPKRGSSDRIVPYDGTLKKGVWNDMGTCNVDHLE 360
B. thermoleovorans      SYRNPTLGIDDRWLENDGIVNTVSMNGPKRGSSDRIVPYDGALKKGVWNDMGTYNVDHLE 360
B. stearothermophilus   SYRNAALGIDSHWLGNDGIVNTISMNGPKRGSNDRIVPYDGTLKKGVWNDMGTYKVDHLE 360
                        **** :****.:** *******:*********.********:********** :*****

B. thermocatenulatus    VIGVDPNPSFDIRAFYLRLAEQLASLRP 388
B. thermoleovorans      IIGVDPNPSFDIRAFYLRLAEQLASLRP 388
B. stearothermophilus   VIGVDPNPSFNIRAFYLRLAEQLASLRP 388
                        :**********:******************
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Figure 3. 2. Alignment of amino acid sequences of mature lipases from various *Bacillus* species. The alignment was performed with CLUSTAL W as described under Materials and Methods. The asterisks (*) indicate amino acid identity in all shown sequences. The point (.) and colon (:) respectively denote semi-conserved and conserved amino acid substitutions in the alignment. The putative "conserved" pentapeptide is shown in bold, underlined letters. Lipase gene sequence accession numbers. *B. pumilus* (A34992), *B. subtilis* (M74010), *B. licheniformis* (AJ297356), *B. thermocatenulatus* (X95309) *B. thermoleovorans* (AF134840), *B. stearothermophilus* (U78785).

3.3.3. Expression and purification of the recombinant lipase from Escherichia coli

The lipase expression vector, pET-Lichlip was constructed by ligating the 549 bp *Nde* I/*Xho* I fragment from pGem-Lichlip into *Nde* I/*Xho* I digested pET20b(+). This construct, which places the lipase gene under control of a T7 promoter and in-frame with a C-terminal region coding for 6 histidine residues, was transformed into *E. coli* JM 109 (DE3). The transformed host was grown to early stationary phase and the T7 promoter induced with IPTG. Lipase activity was detected in both the extracellular and intracellular extracts. No inhibition of growth was observed for the host strain expressing the lipase gene. The total lipase activity of about 800 units was obtained from a 50 ml culture, with a 5:3 distribution ratio for the extracellular:intracellular lipase activities. An average lipase activity of 20 Units/ml was obtained with a spectrophotometric assay using *p*NPP as substrate. Compared with the average lipase activity of about 0.6 Units/ml obtained when *B. licheniformis* was cultured in nutrient broth, the lipase expression level increased by more than 30-fold, from about 4.5 mg/l to more than 150 mg/l using the *E. coli* (DE3) host harbouring the pET-lichlip plasmid.

Purification of the recombinant *B. licheniformis* lipase enzyme was facilitated by the presence of 6 histidine residues at the C-terminus of the protein. This allowed a one-step purification of the lipase protein using Ni-NTA affinity chromatography. The histidine-tagged lipase was purified 80-fold with a yield of 72% and a specific activity of 130 units/mg (Table 3.1). The purified recombinant

B. licheniformis lipase appeared to be homogeneous on SDS-PAGE, and had a relative molecular mass of approximately 25 kDa (Figure 3. 3).

Table 3.1.

Purification of a recombinant *B. licheniformis* lipase.

Step	Total Activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	216	135	1.6	1	100
Ni-NTA affinity	156	1.2	130	80	72

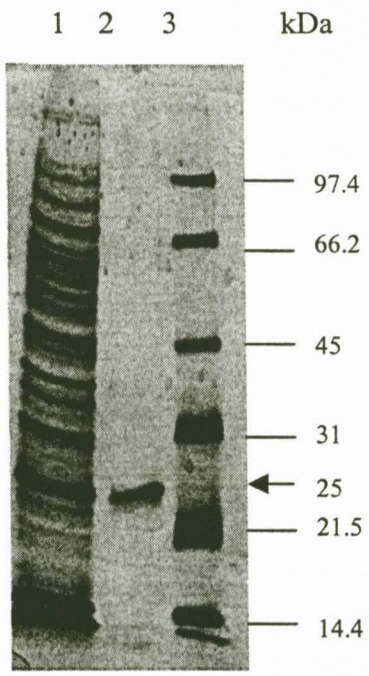

Figure 3.3. Purified recombinant lipase from *B. licheniformis* was resolved on a 12% SDS-PAGE gel, and the protein visualised by staining with Coomassie brilliant blue. Lanes: 1, crude extract; 2, purified lipase; 3, standard proteins: lysozyme (M_r 14.4 kDa), soybean trypsin inhibitor (M_r 21.5 kDa), carbonic anhydrase (M_r 31 kDa), ovalbumin (M_r 45 kDa), bovine serum albumin (M_r 66.2 kDa), phosphorylase b (M_r 97.4 kDa).

3.3.4. Characterization of the cloned lipase

The purified, recombinant lipase displayed optimal catalytic activity towards *p*NPP in the extreme alkaline region of pH 10.5-12.0 (Figure 3. 4). The pH optimum of the native, crude lipase from *B. licheniformis* was determined to be between pH 9.0 and 11, with less than 30% of the optimal activity observed at a pH above 11 (data not shown). The recombinant lipase retained more than 80 % of its activity following incubation for 2h at 30 ^{0}C in various buffers over a pH ranging from 8.0 to 12.0, indicating that the recombinant enzyme is extremely alkali tolerant protein (Figure 3.4). Incubation of the native crude lipase at pH values above 10 decreased the activity of the enzyme to less than 60% activity, with almost no activity remaining after incubation at pH 12 (Chapter 2, Figure 2.6).

The enzyme exhibited maximum activity at temperatures between 50 and 60 °C and retained 90% activity after incubation for 30 min at 45 °C (Figure 3.5). Above this temperature, the stability of the enzyme decreased sharply with less than 25% activity remaining after incubation for 30 min at 50 °C. Similar results were obtained with the non-recombinant crude lipase (Chapter 2, Figure 2.5).

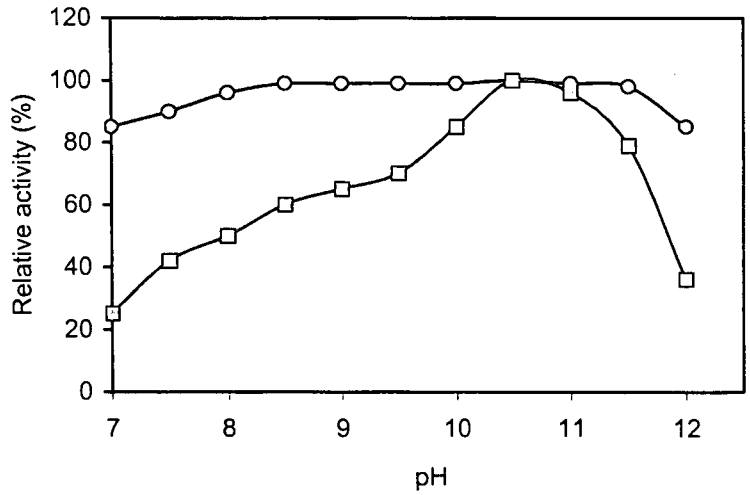

Figure 3.4. Effect of pH on recombinant *B. licheniformis* lipase activity (□) and stability (○). The results are expressed as a percentage of the maximal activity (in pH activity curve) or as a percentage of the initial activity (in pH stability curve). The data represent the arithmetic mean of at least three determinations. The maximum deviation in the data was no more than 6%.

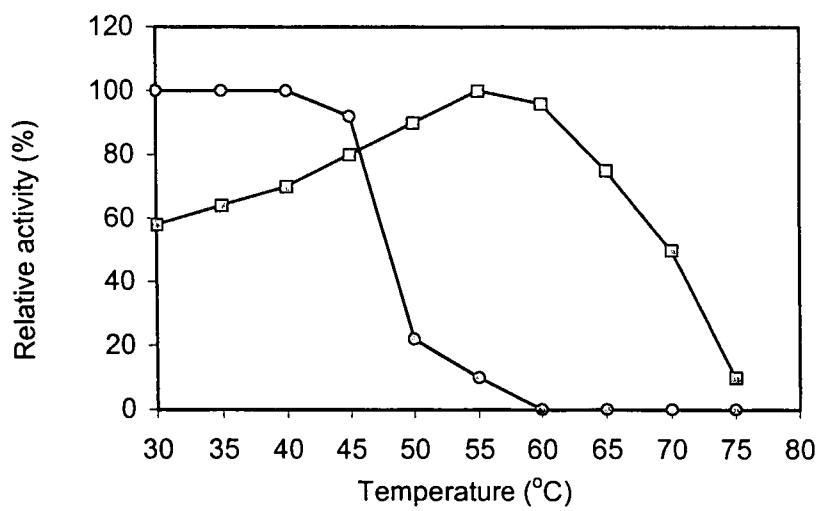

Figure 3.5. Effect of temperature on recombinant *B. licheniformis* lipase activity and stability. The activity of recombinant, purified lipase is shown as a percentage of the maximal activity (■). The temperature stability curve (○) is shown as a percentage of the initial activity. The data represent the arithmetic mean of at least three determinations with a maximum deviation of less than 8%.

The substrate specificity for the lipase enzyme was studied with *p*-nitrophenyl fatty acid esters of various chain lengths (Table 3.2). The highest hydrolysis rates were obtained with *p*NP-caproate (C6) and *p*NP-caprylate (C8), indicating the enzyme's preference for medium-size acyl chain lengths. *p*NP esters of palmitic (C16) and stearic acid (C18) were also good substrates, whereas *p*NP-myristic acid (C14) was hydrolyzed at a relatively low rate. The presence of good lipase activity towards long-chain fatty acid esters indicates that it is a true lipase in contrast to esterases that hydrolyze exclusively short chain fatty acid esters.

Table 3.2.
Activity of the recombinant *B. licheniformis* lipase towards various *p*-nitrophenyl esters

Chain length of acyl group	Relative activity (% ± SD)
C_3	17 ± 10
C_4	39 ± 12
C_6	200 ± 3
C_8	182 ± 4
C_{12}	102 ± 5
C_{14}	77 ± 6
C_{16}	100 ± 7
C_{18}	98 ± 8

Hydrolysis of *p*NP esters was measured at fixed molar concentration of substrates at pH 8.5. The activities are expressed relative to the activity obtained with *p*NPP, and represent the arithmetic mean and SD of at least three determinations.

The effect of some divalent cations on the enzyme activity was assessed by measuring the *p*NPP hydrolysis activity of the purified recombinant lipase in the presence of various ions (as chloride salts) at concentrations of 0.05 - 5mM. The

results obtained at 1mM concentrations are shown in Table 3.3. Ca^{2+}, Mg^{2+} and Sn^{2+} had a negligible effect on the activity of the enzyme, with 98, 95 and 92% activity being obtained, respectively. The metal ions Cu^{2+}, Fe^{2+}, Ba^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} inhibited the lipase moderately, whereas no activity was detectable in the presence of Hg^{2+}. The lipase was activated when incubated and assayed in the presence of 1mM EDTA. About 60% inhibition was observed when the enzyme was incubated and assayed in the presence of the classical serine-specific inhibitor PMSF. DTT activated the activity of the lipase enzyme to 142%. Mercaptoethanol did not affect the activity of the enzyme.

Table 3.3.
Effect of various agents on the activity of purified *B. licheniformis* recombinant lipase

Agent (1 mM)	Relative activity (% ± SD)
Ca^{2+}	98 ± 2
Mg^{2+}	95 ± 2
Zn^{2+}	16 ± 3
Co^{2+}	32 ± 4
Fe^{2+}	60 ± 6
Sn^{2+}	92 ± 3
Hg^{2+}	Undetectable
Ba^{2+}	64 ± 3
Mn^{2+}	65 ± 2
Cu^{2+}	78 ± 6
Ni^{2+}	60 ± 5
EDTA	125 ± 5
PMSF	41 ± 3
DTT	134 ± 3
2-mercaptoethanol	100 ± 4

The data represent the arithmetic mean and SD of at least three determinations.

3.4. Discussion

Production of an extracellular lipase by a *B. licheniformis* strain has been reported previously (Khyami-Horani, 1996), but no study describing the purification of this enzyme has been published to date. Biochemical characterisation of a *B. licheniformis* lipase partially purified in our laboratory showed kinetic properties very similar to those of lipases from *B. pumilus* (Moller *et al.*, 1991) and *B. subtilis* (Lesuisse *et al.*, 1993). The genes encoding the latter lipases have been cloned and sequenced. These lipases are synthesized as pre-proteins with an amino-terminal signal peptide sequence. This signal peptide is needed for proper targeting of the protein, and is removed before the mature protein is released into the external medium (Simonen and Palva, 1993). The aligned lipase gene sequences from *B. pumilus* and *B. subtilis* exhibit significant homology within the sequences encoding the mature lipase proteins. Degenerate oligonucleotide primers were therefore designed, based on these gene sequences, and were used in the amplification and cloning of the *B. licheniformis* lipase gene. The cloned DNA fragment encoded a functional lipase protein.

Arpigney and Jaeger (1999) classified bacterial lipolytic enzymes into eight families based on conserved sequence motifs and biological properties. The first family has been divided into six subfamilies with lipases from *B. subtilis* and *B. pumilus* comprising a group of their own, subfamily I.4. The results of this study suggest that the lipase from *B. licheniformis* must be included in subfamily I.4 on

the basis of amino acid sequence identity and biochemical properties. A comparison of the mature lipases produced by this subfamily reveals proteins of 181 amino acids in length, which share an amino acid sequence identity of 80%. The lipases from *B. licheniformis* and *B. pumilus* has an amino acid sequence identity of 96% (Figure 3.2A). The computer analysis of the three lipases suggests that they are extremely basic proteins with pI values exceeding 9.2, and relative molecular masses of approximately 19 kDa (Table 3.4). It is notable that all three lipases lack cysteine amino acid residues, suggesting the absence of stabilizing disulphide bridges. The retention of full lipase activity in the presence of disulphide bond reducing agents DTT and 2-mercaptoethanol supports this idea. This suggests that these proteins assume a flexible tertiary structure that may facilitate conformational changes. This may be required for enzymatic activity when a water-soluble enzyme reacts with a hydrophobic lipid, as suggested by Verger (1980). These lipases are not thermostable; their activities decreased significantly at temperatures above 45 °C. They exhibit extreme alkaline tolerance, with maximum lipase activities at pH values between 9.5 and 12. The recombinant *B. licheniformis* lipase described in this study has the highest pH optimum reported to date, exhibiting maximum activity at pH 11.0-11.5. The six C-terminal histidine residues may contribute to the very high pH optimum.

Lipases from *B. thermocatenulatus*, *B. stearothermophilus*, *Staphylococcus hyicus, S. aureas and S. epidermidis* constitute an additional group, subfamily I.5

(Arpigny and Jaeger, 1999). We propose that lipases identified in genus *Staphylococcus* from subfamily I.5 be assigned to their own group, subfamily I.6. This proposed reassignment is supported by the high level of conservation of the mature lipase domains and the biochemical properties of these lipases. *Staphylococcus* lipases are common in that they are synthesized as precursors containing large pro-peptides (207-267 amino acid residues in length), which are subsequently cleaved to yield mature lipases of approximately 400 residues. The amino acid identity between the mature lipases from *S. epidermidis* (GenBank accession no. AF090142) (Farrel *et al.*, 1993), *S. haemolyticus* (GenBank accession no. AF096928) (Oh *et al.*, 1999), *S. hycus* (accession no. X02844) (Gotz *et al*, 1985) and *S. aureus* (GenBank accession no. M12715) (Rollof and Normark, 1992) is 50-70%; 29% of the non-identical residues are conservative changes (data not shown). Although *Staphylococcus* pro-lipases are cleaved to yield mature proteins with predicted molecular masses similar to that of lipases identified in *B. thermocatenulatus*, *B. stearothermophilus* and *B. thermoleovorans*, the amino acid sequence identity is less than 30%, confirming the lack of close evolutionary relatedness.

We further propose that lipases identified in *B. thermocatenulatus*, *B. stearothermophilus* and *B. thermoleovorans* be classified as subfamily I.5. *Bacillus* lipase genes from this subfamily code for proteins with signal peptides that are cleaved to yield mature lipases of 388 amino acid residues in length (Figure 3. 2B). The amino acid sequence identity amongst these three *Bacillus*

lipases is more than 91%, with deduced molecular masses of approximately 43kD and pI values of 6.2 (Table 4). In addition, these three lipases posses two cysteine residues at positions 64 and 295. Whether these cysteine residues participate in the formation of a disulphide bond that may stabilize the protein against thermal inactivation is currently unknown. Treatment of the lipases isolated from *B. thermoleovorans* and *B. stearothermophilus* with β-mercaptoethanol or DTT reduced lipase activities by approximately 15% (Kim *et al.*, 1998; Lee *et al.*, 1999). This group of *Bacillus* lipases is thermostable as it retains activity at temperatures above 50 °C. Despite an amino acid sequence identity of more than 91%, these lipases show different pH optima. The lipase from *B. thermocatenulatus* exhibited maximum activity at pH 8.5 (Rua *et al.*, 1997), *B. stearothermophilus* lipase at pH 9.5 (Kim *et al.*, 1998), while *B. thermoleovorans* lipase has maximum activity at pH 7.5 (Lee *et al.*, 1999).

There is little amino acid homology between the two subfamilies of *Bacillus* lipases, suggesting that they are evolutionally only distantly related. Although lipases from other genera contain the conserved pentapetide **Gly**-X-Ser-X-Gly around the active serine, this is not the case with the *Bacillus* lipases. The conserved pentapeptide **Ala**-X_1-Ser-X_2-Gly is found in *Bacillus* lipases. X_1 is a His residue and is conserved in subfamilies 4 and 5 of *Bacillus* lipases, but X_2 is Met and Gln in subfamily I.4 and subfamily I.5, respectively. The conserved pentapeptide amongst *Bacillus* lipases at a region surrounding the putative

nucleophilic Ser is therefore, Ala-His-Ser-X-Gly, with X representing either Met or Gln.

Table 3.4
Biochemical properties of *Bacillus* lipases

Bacillus isolate	M_r[a](kDa)	pI[a]	Optimum pH	Temperature stability [c]	Reference
Subfamily I.4					
B. licheniformis[b]	19.2	9.46	11	< 25%	This study
B. subtilis	19.3	9.25	10	<25%	(Lesuisse *et al*, 1993)
B. pumilus	19.3	9.43	9.5-10	<25%	(Moller *et al*, 1991)
Subfamily I.5					
B. thermocatenulatus	43.2	6.37	8.5	>90%	(Rua *et al*, 1997)
B. thermoleovorans	43.1	6.19	7.5	>90%	(Lee *et al.*, 1999)
B. stearothermophilus	43.2	6.15	9.5	>90%	(Kim *et al.*, 1998)

[a]The experimental values have been overlooked in favour of the theoretical M_r and pI deduced by the Compute pI/MW software program (Bjellqvist *et al.*, 1993, 1994; Wilkins *et al.*, 1998).
[b]Reflects properties of the recombinant lipase.
[c]Represents residual activity obtained after incubation of the lipase protein at 50 °C for 30 min.

3.5. Conclusion

Analysis of the current literature reveals the emergence of two distinct groups of *Bacillus* lipases, based on amino acid sequences and biochemical properties. The two groups of lipases should be classified according to the proposal of Arpigney and Jaeger, 1999, as subfamilies I.4 and I.5. *Staphylococcus* lipases should constitute their own group, subfamily I.6, with *Propionibacterium acnes*

and *Streptomyces cinnamoneus* constituting subfamily I.7. Despite the high amino acid similarity of lipases from Gram-positive bacteria within a given group, they exhibit different properties, particularly with respect to pH and possibly substrate specificities. A detailed comparison of the kinetic properties of lipases in the given groups could lead to a more thorough understanding of the structural determinants of the specificity associated with each lipase. The availability of the three-dimensional structures of bacterial lipases in each group would greatly facilitate the understanding of the structural basis of thermostability and alkali tolerance exhibited by the respective *Bacillus* lipase groups. This will enable the construction of a recombinant protein that can function at extremes of pH and temperature, and in the presence of desired chemical additives. The expression and purification systems described here could be scaled up to achieve a purification of more than 100 mg of pure lipase protein per liter of growth culture, and could be applied to the over-expression and purification of other closely related lipases from *Bacillus* species thus circumventing problems associated with the traditional production and protein purification methods. The expression system described in this paper could aid to alleviating problems of restrictive expression of lipase enzymes associated with *Bacillus* species. The availability of lipase proteins in quantities and qualities sufficient for X-ray crystallographic studies could be achieved by using this purification system.

3.6. References

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CHAPTER 4

The kinetic properties of *Bacillus licheniformis* lipase modified by site-directed mutagenesis

4.1. Introduction

Lipases are a class of enzymes that catalyse the hydrolysis of acylglycerols to free fatty acids and glycerols in lipid-water interfaces (Jaeger *et al.*, 1994). The fact that lipases are water-soluble but act on non-soluble substrates could raise the argument that this might impose unique restrictions on the molecular architecture so that the enzymes can be stable in both polar and non-polar environments while undergoing specific structural changes in moving from one to the other. All the lipases whose three-dimensional structures have been elucidated by X-ray crystallography [*Rhizomucor miehei* (Brady *et al.*, 1990), *Geotrichum candidum* (Schrag *et al.*, 1991), *Candida rugosa* (Grochulski *et al.*, 1993), *Candida antartica* (Uppenberg *et al.*, 1994) *Burkholderia glumae* (Noble *et al.*, 1993), *Burkholderia cepacia* (Kim *et al.*, 1997; Schrag *et al.*, 1997) and *Bacillus subtilis* (van Pouderoyen *et al.* 2001) (the structure of *Bacillus subtilis* lipase was published during the writing of this report) are α/β-type proteins with a mixed central β-pleated sheet containing the catalytic residues. The α/β-fold is recognised as a general folding pattern for different hydrolases (Ollis *et al.*, 1992) such as acetylcholine esterase (Sussman *et al.*, 1991), serine carboxypeptidase (Liao and Remington, 1990), haloalkane dehalogenase (Franken *et al.*, 1991), and dienelactone hydrolase (Pathak *et al.*, 1988).

The catalytic centre of the lipase contains a serine-protease-like triad consisting of Ser-His-Asp residues, and the active site serine residue is

located in a β-εSer-α motif (Figure 4.1) (Derewenda and Derewenda, 1991; Derewenda and Sharp, 1993).

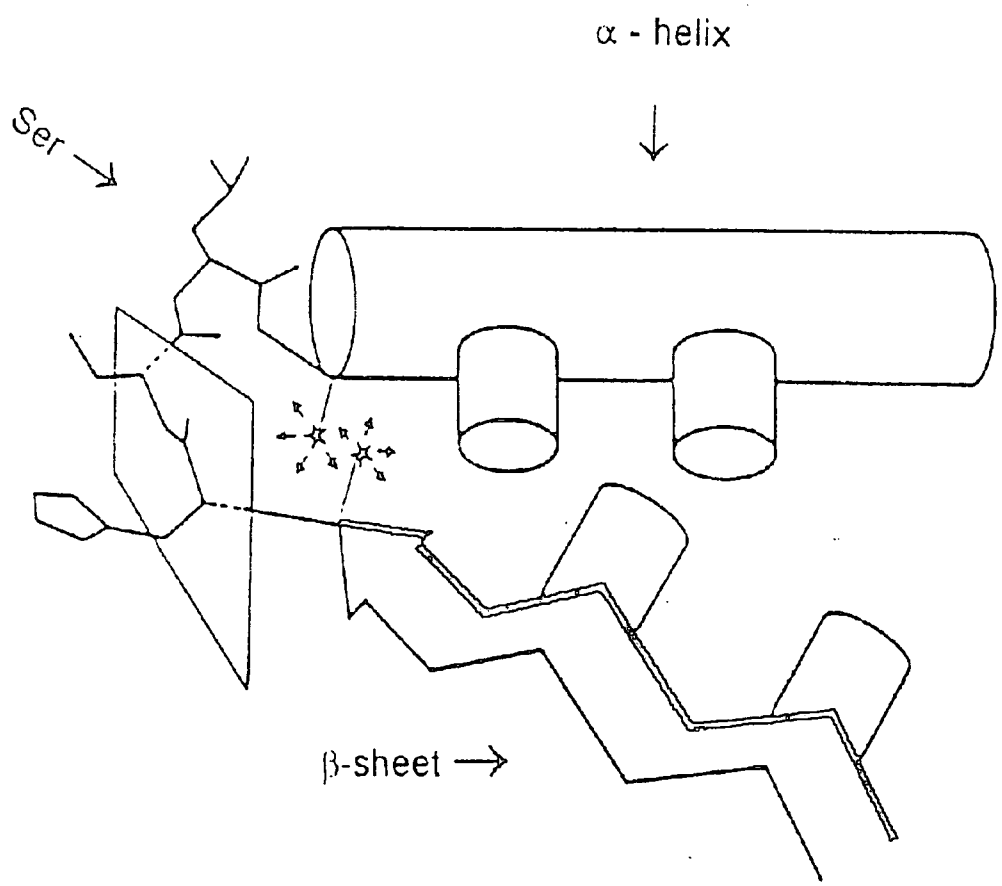


Figure 4.1. A schematic drawing showing the packing within the β-εSer-α motif of enzymes exhibiting this feature. The helix and strand pack against each other with four amino acids (tinted) forming the interface. The residues nearer the turn are in closer contact and therefore their sidechains are restricted to those of smaller hydrophobic amino acids. The plane of the central peptide of the turn is perpendicular to the axis of the motif, which forces the catalytic Ser to adopt a strained ε conformation. The two stars show the positions that β-carbons of amino acids other than Gly would occupy if the two invariant Gly residues of the GXSXG pentapeptide were mutated. (Taken from Derewenda and Sharp, 1993).

This motif consists of a six-residue β-strand a four-residue type II' turn with serine in the ε conformation, and a buried α-helix packed parallel against 4 and 5 of the central β-sheet. The invariant first and last glycine residues in the consensus sequence Gly-X-Ser-X-Gly (where X represents any amino acid) of this motif are in extended and helical conformations, respectively, which are conserved because of the steric requirements imposed by the packing stereochemistry of the β-εSer-α motif (Derewenda and Sharp, 1993; Derewenda and Derewenda, 1991). The consensus sequence found in lipases around the active serine, was however not found in *Candida antarctica* lipase (Uppenberg *et al.*, 1994). The sequence around the Ser 105 had the highest similarity to the consensus sequence, but the first conserved glycine had been replaced by Thr to give **Thr**-Trp-Ser-Gln-Gly. The lipases from *Bacillus* strains also appeared to lack the conserved pentapeptide purported to play an essential role in catalysis (Chapter 3, Figure 3.2). Alignments of several microbial lipase sequences showed significant homology between this conserved peptide and the sequence **Ala**-His-Ser-Met-Gly present in the lipases of *Bacillus* species, which could conceivably carry out identical enzymatic functions. The first Gly of the conserved sequence has been replaced by Ala. It is generally assumed that the central serine is the nucleophilic residue involved in the hydrolytic mechanism (Faustinella *et al.*, 1991) and is part of the catalytic triad Ser-Asp-His equivalent to the active residues seen in the serine proteinases (Brady *et al.*, 1990). The role of the flanking glycines could be less essential in catalysis. These residues may function rather to increase flexibility of this protein region and to reduce steric hinderance in order to optimise accessibility of the catalytic Ser. This

hypothesis could explain the relative tolerance of the consensus sequence towards a mutation of the first Gly into Ala. Furthermore, in subtilisin from various microorganisms, the sequence of the conserved pentapeptide was reported to be Gly-X-Ser-X-Ala (Brenner, 1988). Recently, the three-dimensional structure of an esterase enzyme from an *Alcaligenes* species has been resolved (Bourne *et al.*, 2000). The enzyme lacked the lipase/esterase consensus; the two Gly positions are occupied by Ser residues.

Determination of the three-dimensional structure of different lipases has confirmed their classification as "serine hydrolases". The active site is composed of three residues; a serine residue hydrogen-bonded to a histidine residue, and a carboxylate residue. In most lipases, the carboxylate residue is an aspartate, but in *Candida cylindracea* (Longhi *et al.*, 1992) and *Geotrichum candidum* (Schrag *et al.*, 1991) glutamate replaces aspartate.

The architecture of the catalytic triad of lipases is very similar to the one found in serine proteases (Winkler *et al.*, 1990; Brady *et al.*, 1990). During the reaction a tetrahedral intermediate is formed which decomposes into an acyl-enzyme complex. The free lipase is regenerated by a hydrolytic reaction mediated by a water molecule. Figure 4.2 describes the reaction mechanism of lipases hydrolysing an ester bond. First a nucleophilic attack of the oxygen of the serine side chain on the carbonyl carbon atom of the ester bond leads to the formation of the tetrahedral intermediate (Figure 4.2, reaction 1). The histidine assists in increasing the nucleophilicity of the serine hydroxyl group. The histidine imidazole ring becomes protonated and positively charged. The

positive charge is stabilised by the negative charge of the acid residue (Figure 4.2, reaction 2). The tetrahedral intermediate is stabilised by two hydrogen bonds formed with amide bonds of residues which belong to the oxyanion hole. Finally the alcohol is liberated leaving behind the acyl-enzyme complex. (Figure 4.2, reaction 3). By nucleophilic attack of a hydroxyl ion, the fatty acid is liberated and the enzyme regenerated (Figure 4.2, reaction 4).

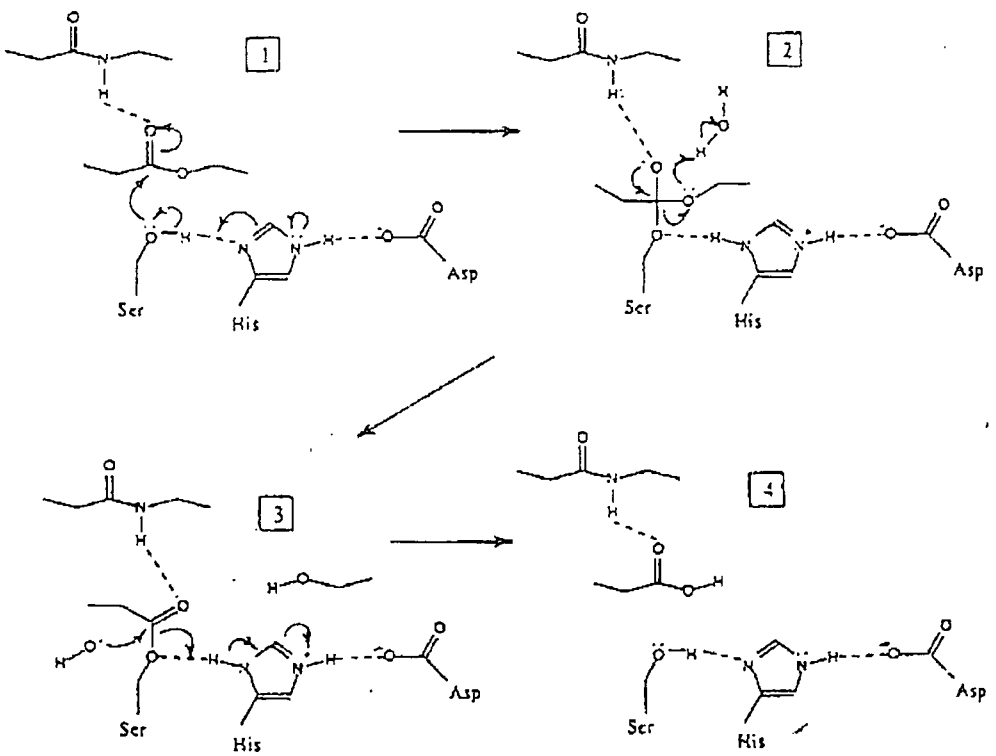


Figure 4.2. Mechanism of hydrolysis of an ester bond by a lipase. (Taken from Jaeger *et al.*, 1994).

Homology–derived three-dimensional (3-D) structure prediction has been used to predict protein folding of a number of lipolytic enzymes (Longhi *et al.*, 1992; Jaeger *et al.*, 1993, Misset *et al.*, 1994; Beer *et al.*, 1996; Manco *et al.*, 2000). Homology modeling is, however, limited by a very low level of

sequence identity between groups of lipase esterase family whose 3-D structures are known. The sequence similarity between lipolytic enzymes is usually limited to short spans located around the active site residues. However, the 3-D structures of lipases, in their cores, share a common fold motif, known as the α/β hydrolase fold (Ollis *et al.*, 1992). Comparison of the model structure of *Pseudomonas aeruginosa* built on the basis of sequences and structures of various hydrolases which were found to possess a common folding pattern (α/β hydrolase fold), with the X-ray structure of the *Burkholderia glumae* lipase revealed that it is possible to correctly build the structure of the core of a protein even in the absence of obvious sequence homology with protein of known 3-D structure (Misset *et al.*, 1994). Manco *et al.*, (2000) hypothesized for *Archaeoglobus fulgidus* esterase the α/β fold on the basis of secondary structure predictions and a secondary structure-driven multiple sequence alignment with very remote homologous proteins of known three-dimensional structure. The availability of the 3-D structure of the brefeldin A esterase from *Bacillus subtilis* (Wei *et al.*, 2000) confirmed their predictions, and this allowed them to built a refined 3-D structure of the esterase (Manco *et al.*, 2000).

Bacterial lipases are classified into eight families (Arpigny and Jaeger, 1999). *Bacillus* lipases have been placed in Subfamilies I.4 and I.5 (Arpigny and Jaeger, 1999; Chapter 3, Figure 3.2). The two subfamilies have in common that Gly_1 and X_1 are Ala and His, respectively in the "conserved" Gly-X-Ser-X-Gly pentapetide around the active site Ser residue. Subfamily I.4 consists of four members LipA and LipB from *Bacillus subtilis* (Dartois *et al.*, 1992; Eggert

et al., 2000), the lipases from *Bacillus pumilus* (Möller *et al.*, 1991) and *Bacillus licheniformis* (Chapter 3). Subfamily 1.4 lipases share among them about 75% amino acid sequence identity. Although Misset *et al.*, (1994) reported on the successful preparation of X-ray crystals for *Bacillus subtilis* LipA, no further data about the 3-D structure of the enzyme has been reported during the planning and execution of this study. [The 3-D structure of *Bacillus subtilis* has just been published (van Pouderoyen et al., 2001)].

Earlier work done with the aim of identifying the active site residues of *Bacillus subtilis* lipase (LipA) implicated Ser77 and Asp133 as members of the catalytic triad, but the His residue remained unidentified (Misset *et al.*, 1994). We constructed the lipase proteins containing mutations in the mentioned amino acid residues, together with His156 and His 76. The Ala75 residue was mutated by site directed mutagenesis into Gly to restore the common lipase consensus sequence. This study aims to report on the kinetic properties of the lipase protein mutants. In Chapter 3, the construction and purification of a C-terminally 6X His-tagged lipase by affinity chromatography was described. In this study, the author also describes the removal of the histidine tag, and the properties of the purified lipase without the histidine tag are compared with that of the C-terminal histidine-tagged lipase.

4.2. Materials and methods

4.2.1. Materials

Tryptone, yeast extract, nutrient broth and agar were purchased from Biolab Diagnostics (Johannesburg, South Africa). Lysozyme, ampicillin, isopropyl β-D-thiogalactoside (IPTG), agarose, deoxynucleotides and *Taq* polymerase were purchased from Roche, Germany. T7 and Sp6 promoter sequences, endonuclease restriction and ligase enzymes were purchased from Promega (Madison, USA). Molecular mass marker proteins were purchased from Bio-rad (CA, USA). The recombinant TEV (rTEV) protease was obtained from Life Technologies Inc (Madison, USA). Oligonucleotide primers were purchased from IDT Inc, USA. *Escherichia coli* cells JM109 and JM109 (DE3) (Promega, Madison, USA) were used as cloning and expression hosts, respectively. Plasmids pGem-T (Promega, Madison, USA) and pET 20b(+) (Novagen, Madison, USA) were used for cloning and expression, respectively. Plasmid pET-lichlip was constructed as described in Chapter 3. Plasmid DNAs were isolated using a nucleospin plasmid isolation kit (Macherey-Nagel, Germany). All other chemicals were commercially available and of analytical or molecular biology grade.

4.2.2. Homology modeling

Homology modelling of the *Bacillus lichemiformis* lipase was done with the Swiss-Model server (http://www.expasy.ch/swissmod/SWISS-MODEL.html) using the published structure of *Bacillus subtilis* as template.

4.2.3. Site-directed mutagenesis

Mutations on the mature *Bacillus licheniformis* lipase were introduced by PCR, firstly by identifying sequences that could generate silent mutations in the vicinity of the site where mutation was desired (Figure 4.3). Primers containing the silent restrictions sites and appropriate base alterations were constructed as shown in Table 4.1. *Taq* polymerase was used to perform PCR with the following primer pairs: (A) Primers 1 and 2, (B) primers 3 and 8, (C) primers 4 and 8, (D) primers 1 and 5, (E) primers 6 and 8 and (F) primers 7 and 8. pET-Lichlip plasmid was used as the template. The PCR conditions were as follows: 1 initial denaturation step at 94 °C for 2 min, 25 cycles at 94 °C for 30 sec, annealing at 55 °C for 20 sec extension at 72 °C for 1min, except for the final cycle where extension proceeded for 10 min.

The PCR fragment A was digested with *Nde* 1 and *Sal* 1 followed by ligation into pET vector digested with *Nde* I and *Xho* 1. This vector was circularized by the ligation of PCR fragments B and C digested with *Sal* 1 and *Xho* 1 to create pET-A75G and pET-H76N plasmids, respectively. The pET-D133N was constructed by digestion of PCR products D and E with a combination of *Nde* I / *EcoR* 1 / *Xho* 1 restriction enzymes, followed by ligation into pET-vector digested with *Nde* 1 and *Xho* 1. To construct pET-H156N, the *Nco* 1 / *Xho* 1 flanked fragment within the pET-lichlip was released by digestion with the mentioned enzymes, and replaced with fragment F digested with similar enzymes.

Figure 4.3. The DNA sequence encoding *Bacillus licheniformis* lipase activity subcloned into pET-Lichlip and the deduced amino acid sequence. Restriction enzymes introducing silent mutations in the vicinity of the sites of mutations together with sequences used as primers are shown. The silent mutations introduced by restriction sites and the mutated codons were designed taking into consideration the codon usage of *Bacillus* species (Mountain, 1989)

Table 4.1. Mutagenesis, upstream and donwstream primers. Mutated codons are in bold underlined letters. The silent restriction sites introduced by mutagenesis primers are shown in Italic letters.

Primer	Primer sequence (5'-3')	Function(s)
1. *Nde1* B.lips For	GGG *CAT ATG* GCT GAG CAC AAT CCG GTC GTC ATG	Upstream primer. Introduces the ATG initiation codon and *Nde* 1 site.
2. *Sall* 225 Rev	CAC AAT *GTC GAC* TTT TTT GGC ACC CG	Introduces *Sal* 1 site near the A75G an **H76N** mutations
3. *Sal*l A75G For	GGG *GTC GAC* ATT GTG **GGC** CAT AGT ATG	Contains *Sal* 1 site and introduces th **A75G** mutation
4. *Sal*l H76N For	GGG *GTC GAC* ATT GTG GCG **AAC** AGT ATG	Contains *Sal* 1 site and introduces th **H76N** mutation.
5. *Eco*RI D133N Rev	AAG *AGA ATT* CAC GAT GAG **GTT** TGC CGA G	Contains *EcoR* 1 site and introduce the **D133N** mutation.
6. *Eco*RI-140-For	GCA **AAC** CTC ATC GTC GT*G AAT TCT* CTT TCT CGG	Contains *EcoR* 1 site and the **D133** mutation.
7. *Nco*I H156N For	CTG ATC *CAT GGC* GTT GGC **AAC** ATC GGT	Introduces **H156N** mutation.
8. *Xho1* B.lips Rev	GGG *GAG CTC* ATT CGT ATT CTG TCC TCC ACC	Downstream primer that also introduce Xho 1 site and removes the stop codon

The constructed pET20-derived expression vectors were named to reflect the point and type of mutation carried by each. For example, pET-H76N carries the lipase gene mutated to give a lipase protein that would contain Asn at position 76 instead of His. The constructed vectors were transformed into *Escherichia coli* JM 109 (DE3) cells and plated on agar plates containing 100 µg ampicillin/ml. Transformation of *Escherichia coli* with recombinant plasmids was performed using standard $CaCl_2$ procedures (Sambrook *et al.*, 1989). Single colonies were picked and used to inoculate 50 ml LB containing ampicillin, and grown to an absorbance of approximately 0.8 at 600 nm. The cultures were adjusted to 0.5 mM IPTG, and incubation continued at 30 °C for 8 hours. Cells were harvested by centrifugation at 5, 000 X g for 10 min. The cultures were assayed for intracellular and extracellular lipase activity and subjected to SDS-PAGE analysis.

4.2.4. Purification of the recombinant lipase enzymes

The purification procedure was performed at room temperature. The pH of the lipase active supernatants was adjusted to 8.5. NaCl and imidazole were added to 10 ml of the crude enzyme to a final concentration of 0.3M and 20 mM, respectively. The crude enzyme was then mixed with 5ml of the 50% Ni^{2+}-chelated nitriloacetic acid (Ni-NTA) resin slurry (Qiagen, CA, USA). The crude enzyme-NTA mixture was loaded into a chromatographic column and washed with 5 volumes of 50 mM phosphate buffer pH 8.5, containing 0.3 M NaCl and 20 mM imidazole. Elution of bound protein was effected with the phosphate buffer containing 250 mM imidazole. The resultant enzyme solution was dialyzed overnight against 10l of 5 mM phosphate buffer, pH 8.5.

4.2.5. Removal of the 6X Histidine tag

Oligonucleotides shown in Figure 4.4 were used as primers with the 560 bp PCR product obtained in Chapter 3 (Figure 3.1) as the template. The resultant PCR fragment was digested with *Nde* I and *Xho* 1, and ligated into similarly cleaved pET-20b(+). The construct, referred to as pETLipNHis-tag, was transformed into *Escherichia coli* JM 109 (DE3) cells and plated on LB/ampicillin agar plates. Single colonies were used to transform 50 ml LB medium. Over-expression and purification of the N-terminal His-tagged protein were done as already described. The purified protein was subjected to protease cleavage using rTEV protease at room temperature according to manufacturer's instructions. The digested lipase protein was loaded into the

Ni-NTA chromatographic column and the non-binding protein solution was collected and subjected to SDS-PAGE and N-terminal sequencing. The lipase activity of this protein solution is referred to as the non-tagged lipase in this study.

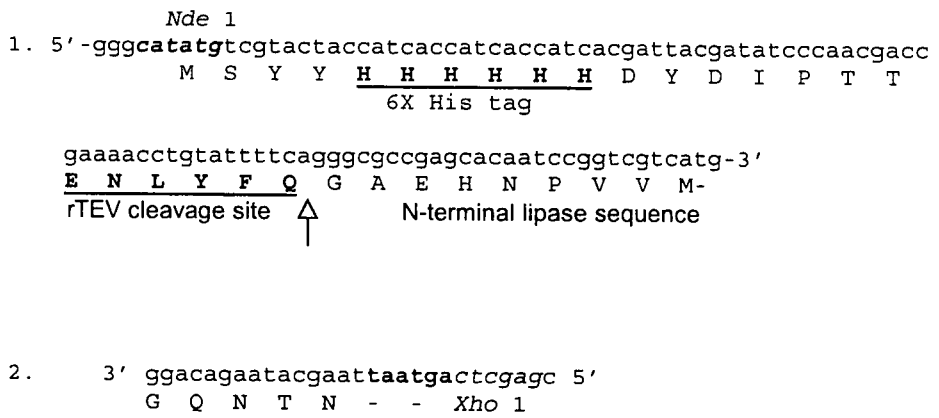


Figure 4.4. Oligonucleotide primers: 1. The upstream primer showing the sequences encoding the 6X His tag, the rTEV protease cleavage site and the first 7 amino acids of the N-terminal region of the mature lipase protein. 2. The downstream primer showed in the 3'-5' orientation. The primer encodes the last 5 C-terminal amino acid residues and introduces 2 stop codons and the *Xho* 1 site immediately after the lipase open reading frame. The vertical arrow indicates the rTEV protease cleavage site.

4.2.6. Enzyme assays

4.2.6.1. Spectrophotometric assays

Enzyme activity was measured spectrophotometrically at 410 nm with *p*-nitrophenyl fatty acid esters [propionic acid (C_3), butyric acid (C_4), caproic acid (C_6), caprylic acid (C_8), lauric acid (C_{12}), myristic acid (C_{14}), palmitic acid (C_{16}) and stearic acid (C_{18})] as substrate at 37 °C in 50 mM phosphate buffer pH 8.5, 0.1% (w/v) gum arabic and 0.2% (w/v) sodium deoxycholate according to

the method of Winkler and Stuckmann (1979). One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol of *p*-nitrophenol from *p*NPP per minute.

4.2.6.2. pH-stat assay

The substrate preference for the purified lipase enzymes was investigated using 10 mM of triacetin, triproprionin, tributyrin, tricaprylin, and triolein as substrates. A Metrohm 641 Impulsomat pH stat was used with a thermostatically controlled vessel at 30 °C. Substrates were mixed with the assay buffer (1 mM Tris-HCl buffer, 0,1 M NaCl, pH 8.5), in the titration vessel and the emulsion formed by vigorous stirring using a propeller agitator. The reaction was initiated by adding 100-200 µl enzyme sample. The hydrolysis rates of the emulsified triacyglycerols were measured potentiomerically by titrating the liberated fatty acids with standardized NaOH. One unit was defined as the amount of enzyme which released 1 µmol of fatty acid per min.

4.2.7. Protein determination

Protein concentration was determined with the bicinchoninic acid (BCA) protein assay kit (Pierce, IL, USA) using the enhanced method according to the manufacturer's instructions.

4.2.8. Electrophoresis

SDS-PAGE was performed on 15% running gels as described by Laemmli (1970), and resolved proteins visualized by Coomassie staining following standard procedures. A broad range protein standard (Bio-Rad, U.S.A.), was used as molecular mass markers.

4.2.9. pH optimum and stability

The effect of pH on the lipase activity was determined spectrophotometrically using *p*NPP as substrate. The substrate was prepared in 50 mM buffer volumes of various pH values. At pH 7-8 sodium phosphate buffer was used; for pH values of 8.5 and 9, a glycine/NaOH buffer was used. Carbonate buffer was used for pH values 9.5 – 10.5, and Na_2HPO_3 / NaOH buffer was used for pH values 11-12. The effect of different pH values on lipase stability was determined by diluting pure lipases 3X in mentioned buffers and incubation was done for 20 hours at 22 °C. Citric acid / Na_2HPO_4 buffer (50 mM) was used for pH 4-7 values. Residual lipase activity was assayed spectrophotometrically with *p*NPP as the assay substrate.

4.2.10. Temperature optimum and stability

The effect of temperature on lipase stability was determined by incubating aliquots of pure lipase for 2 hours in 50 mM phosphate buffer, pH 8.5 at 45 °C. Enzyme samples were withdrawn at different time intervals and assayed for

residual lipase activity using the spectrophotometric assay with *p*NPP as the assay substrate.

4.2.11. Sequencing of DNA

The nucleotide sequences of the mutant lipase genes were performed with the BigDye Terminator Cycle sequencing kit (Perkin Elmer), in a 377 Perkin Elmer DNA sequencer using the T7- promoter and T7-terminator primers.

4.2.12. N-terminal protein sequencing

The N-terminal sequencing of the lipase protein was kindly performed by Chauhan Ramola of the Department of Biochemistry, University of Natal, Pietermaritzburg, South Africa, using Edman degradation chemistry on a Procise 491 automated protein sequencer (Applied Biosystems, California, USA).

4.3. Results

4.3.1. The catalytic triad

All the lipases whose three-dimensional structures are known share the α/β-hydrolase folding pattern. The active site of the enzymes consists of the catalytic triad, which is composed of the nucleophilic Ser located in the relatively conserved Gly_1-X-Ser-X-Gly_2 pentapetide motif, an Asp or Glu residue and a histidine residue. In *Bacillus* species, the consensus pentapetide was however, found to be Ala-His-Ser-X-Gly. Previous work on *Bacillus subtilis* lipase (LipA) had implicated Asp133 as the carboxylic member of the catalytic triad while exonerating His152. His 76 and His156 were left as the only other potential candidates as His3 and His10 were close to the N-terminal and would probably be on the surface of the molecule and not be involved in the catalytic triad. The His76Asn, Asp133Asn and His156Asn lipase protein variants were therefore constructed. To investigate the significance of the conserved Gly-X-Ser-X-Gly commonly found in lipases, Ala75Gly variant of the *Bacillus licheniformis* lipase protein was constructed by PCR-based site-directed mutagenesis.

The mutagenesis primers were constructed taking into account the codon usage of *Bacillus* species (Mountain, 1989). The designed primers resulted in the amplification of PCR products of expected sizes: A, 230 bp; B 330 bp; C, 330 bp; D, 400 bp; E, 160 bp and F, 90 bp (Figure 4.5). The PCR products were digested with appropriate restriction enzymes and the 549 bp ligation

products were inserted into the pET-20b(+) expression vector, in-frame with the C-terminal region of the vector encoding six histidine residues. The presence of the desired mutations in the lipase genes inserted in the expression vectors was confirmed by DNA sequencing. The mutant lipase proteins were over-expressed in *Escherichia coli* JM 109 (DE3) cells to comparable protein levels (Figure 4.6).

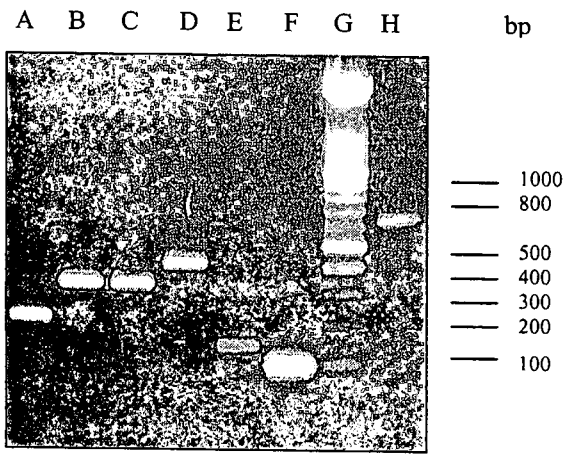


Figure 4.5. Agarose gel (1% w/v) electrophoresis of PCR products generated by primer pairs A (1 and 2), B (3 and 8), C (4 and 8), D (1 and 5), E (6 and 8), F (7 and 8). Lane H is the PCR product obtained using oligonucleotide primers shown in Figure 4.4. Lane G shows DNA molecular weight markers.

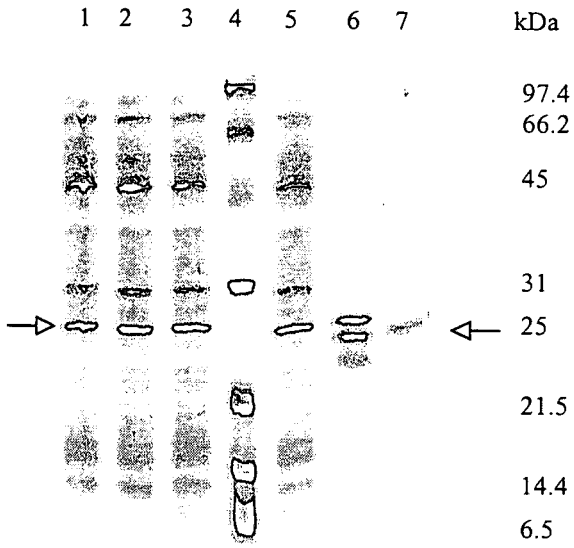


Figure 4.6. SDS-PAGE analysis of the over-expression of (1) A75G, (2) H76N, (3) D133N and (4) H156N lipase proteins in *Escherichia coli* JM109 cells containing the respective pET-derived expression vectors. Lane 4 is the protein molecular mass marker. Lane 6 contains the Ni-NTA purified N-terminal His-tagged lipase protein before rTEV protease cleavage. Lane 7 is the purified non-tagged lipase protein obtained after protease cleavage. The arrows point to the lipase protein band of about 25 KDa. The proteins were visualized by staining with the standard Coomassie Brilliant Blue dye.

Although Asp133Asn and His156Asn variants of the lipase protein were expressed in *E. coli* cells, no activity could be detected with the *p*NPP assay. The Ala75Gly cell cultures exhibited lipase activity of about 20 U/ml while the His76Asn variant yielded average lipase activities of about 1.0 U/ml with the *p*NPP assay. These data indicated that although His76 of the *Bacillus licheniformis* lipase is essential for optimal catalytic activity of the enzyme, it is not a member of the catalytic triad. Asp133 and His 156 together with Ser77 are likely to be the members of the catalytic triad as evidenced by the total lack of lipase activity despite the lipase proteins being expressed.

4.3.2. Removal of the C-terminal His tag

The histidine tag was removed from the C-terminal of the lipase protein by constructing the downstream primer that introduced two stop codons immediately after the lipase open reading frame. The upstream oligonucleotide primer was a 102 bp fragment that contained sequences encoding 6X His tag and the rTEV protease recognition site. The primers specified the amplification of a 650 bp DNA fragment (Figure 4.5, lane H). The 650 bp PCR fragment was digested with *Nde* 1 and *Xho* 1 and ligated into similarly digested pET-20b(+). The gene was successfully over-expressed although the resultant protein had a low specific activity (68 U/mg) towards *p*NPP as a result of the 24 amino acid long peptide introduced at the N-terminal sequence of the protein. The peptide contained the 6X His tag and the protease cleavage site to facilitate the purification of the protein with Ni-NTA chromatography and subsequent removal of the peptide by rTEV protease cleavage. The cleaved lipase protein was purified from the rTEV protease and the cleaved peptide containing the His tag by loading the protein solution into the Ni-NTA column. The Ni-NTA resin bound the His-tagged rTEV protease and peptide fragment, while the non-tagged lipase passed through the column without binding. Although the purified N-terminal His-tagged lipase active fraction showed multiple bands on SDS-PAGE, a single protein band was obtained after protease cleavage and re-loading the protein solution on Ni-NTA column (Figure 4.6; Lanes 6 and 7). The failure of the protein to bind to the Ni-NTA resin suggested the successful removal of the histidine tag from the lipase protein. This was supported by the increase in

specific activity (~130 U/mg with *p*NPP as substrate) for the cleaved lipase protein. The N-terminal sequencing of the protein confirmed the successful removal of the 23-long N-terminal peptide, where the first six amino acid residues were identified as Gly-Ala-Glu-His-Asn-Phe. This protein is therefore similar to the wild-type extracellular lipase except that it has an additional Gly residue at the N-terminal.

4.3.3. The catalytic properties of the lipase variants

The lipase protein variants Ala75Gly, His76Asn, the C-terminal His-tagged (Chapter 3), and the non-tagged lipase protein were purified by Ni-NTA chromatography to SDS-PAGE homogeneity and characterized with respect to thermal and pH stabilities, pH optimum, and substrate preferences.

4.3.4. Thermostability

The temperature stability profiles of the non-tagged, C-terminal tagged, Ala75Gly, and His76Asn at 45 °C are shown in Figure 4.7. The non-tagged and C-terminal tagged lipase proteins were relatively stable, retaining activities of about 80% upon incubation for 1 hour at 45 °C. The Ala75Gly and His76Asn lipase protein variants displayed remarkable temperature sensitivity, with almost no lipase activity remaining after 30 min of incubation at 45 °C. Freezing the His76Asn lipase variant resulted in a complete loss of lipase activity, indicating that freezing denatures the His76Asn mutant lipase protein.

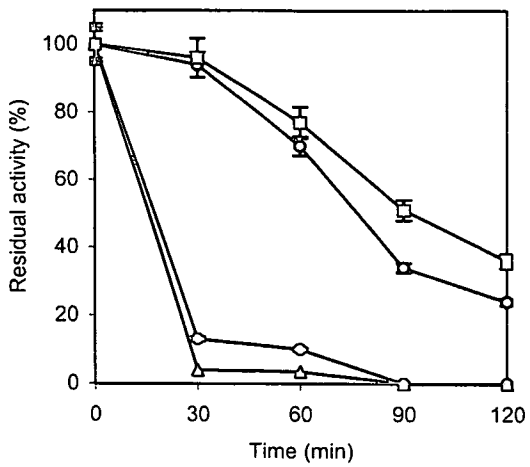


Figure 4.7. Thermal stability of ( ) C-terminal His-tagged, ( ) non-tagged, ( ) Ala75Gly and ( ) His76Asn lipase proteins. The lipase enzymes were incubated at 45 °C for various times and the residual lipase activities were determined with the spectrophotometric assay using *p*NPP as the substrate. Standard deviations are shown as error bars.

4.3.5. pH stability

The lipase proteins showed remarkable stabilities when incubated for 20 hours at pH values between 4-12 where they retained residual lipase activities of more than 60% (Figure 4.8). It was also observed that the incubation of lipase enzymes in alkaline pH buffers enhanced lipase activities in comparison to the initial activity obtained after dialysis of the enzymes in phosphate buffer, pH 8.5.

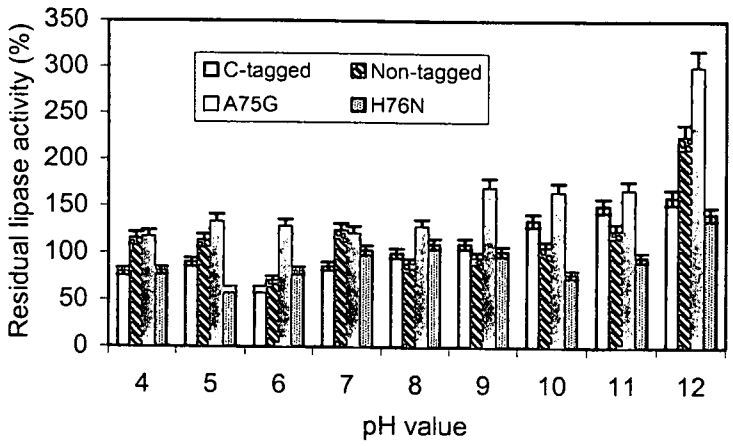


Figure 4.8. The effect of pH on the stability of C-terminal His-tagged, non-tagged, Ala75Gly, and His76Asn lipase proteins. The purified enzymes were incubated at 22 °C in buffers of various pH values for 20 hours. The residual activity was determined using the *p*NPP assay. The results are expressed as a percentage of the initial activity at pH 8.5 (the pH of the dialysis buffer). The standard deviations are shown as error bars.

4.3.6. pH optima for C-terminal tagged and non-tagged lipases

The presence of the six histidine tag at the C-terminal was suggested to influence the optimum pH of the lipase (Chapter 3). The effect of the histidine tag was investigated by determining the specific activities of both the purified C-terminal His-tagged and the non-tagged lipases at various alkaline pH values (Figure 4.9). The data obtained indicated the enhancement of the specific activity of the C-terminal tagged lipase at pH values 10-11.5.

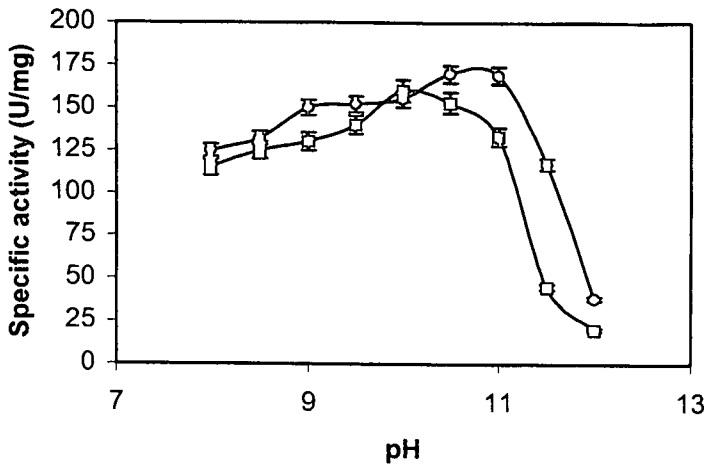


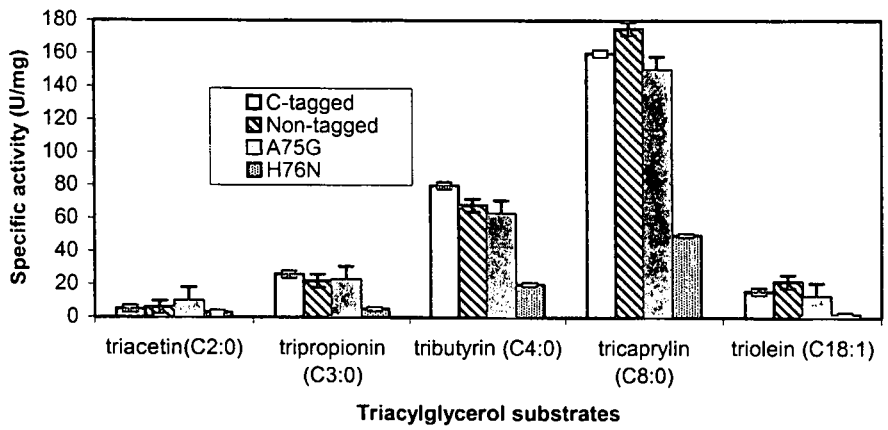
Figure 4.9. The influence of C-terminal 6X His tag on the specific activities of the lipase enzyme at alkaline pH values. (∘) Histidine tagged lipase and ( ) non tagged lipase).

4.3.7. Substrate specificity of the lipase enzymes

The substrate preference for the lipase enzymes was investigated using *p*NP-fatty acid esters and triacylglycerols (Figure 4.10). Triacylglycerols and *p*NP fatty acid esters of mid-length fatty acid chains were the preferred substrates for the lipase enzymes, with maximal activities achieved with substrates containing C6 and C8 fatty acid substrates. The specific activities of the C-terminal His-tagged, non-tagged and Ala75Gly lipase enzymes were comparable, being about 150 U/mg and 240 U/mg with tricaprylin and *p*NP-caprylate as substrates, respectively. Short-chain fatty acyl esters and triolein were poor substrates for the lipase enzymes. The His76Asn lipase variant

hydrolysed the two types of substrates with specific activities of less than 20% of the other lipase enzymes.

A



B

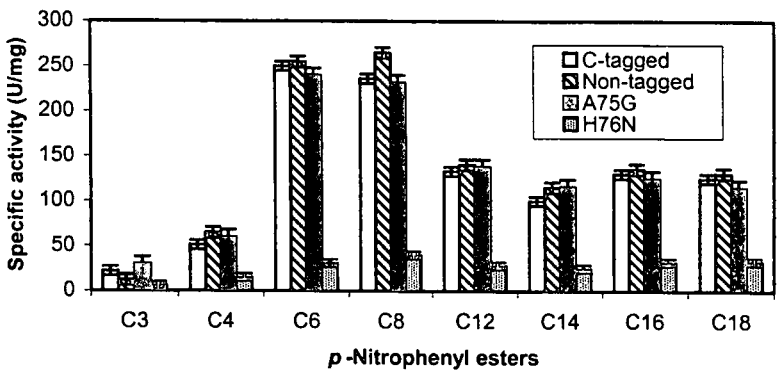


Figure 4.10. Substrate specificities of purified lipase variants towards (A) triacylglycerols and (B) *p*-Nitrophenyl esters. Activities were determined by the pH-stat assay for the triacylglycerols and with the spectrophotometric assay for the *p*-Nitrophenyl esters. Standard deviations are shown as error bars.

4.4. Discussion and Conclusion

The crystal structures of several lipases have been resolved. They have been found to share a common folding pattern called the α/β–hydrolase fold with the Ser, Asp or Glu and His residues forming the catalytic site. The His76Asn mutant resulted in a significant reduction of lipase specific activity. Due to the fact that lipase activity was obtained with the His76Asn mutant, it can be concluded that the His76 residue is not a member of the catalytic triad.

Asp133 and His156 were also investigated as potential members of the catalytic triad. The Asp133Asn and His156Asn mutants resulted in the complete loss of lipase activity. These data suggested that the Asp133 and His156 residues together with the Ser77 serve as the catalytic triad for *Bacillus licheniformis* lipase. Although Misset *et al.*, (1994) identified Ser77 and Asp133, and exonerated His152 as members of the active site for *Bacillus subtilis* LipA, they did not implicate His156 in the catalytic role. This study indicated that His156 is the other member of the catalytic site. The conclusion is in good agreement with the recent work done by Eggert *et al.*, (2000) who identified Ser78, Asp134 and His157 residues as members of the active site for *Bacillus subtilis* LipB. These residues correspond to Ser76, Asp133 and His156 for extracellular lipases from *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus licheniformis*.

investigated. The His76 residue shares a cavity in close proximity to the nucleophilic Ser (Figure 4.11).

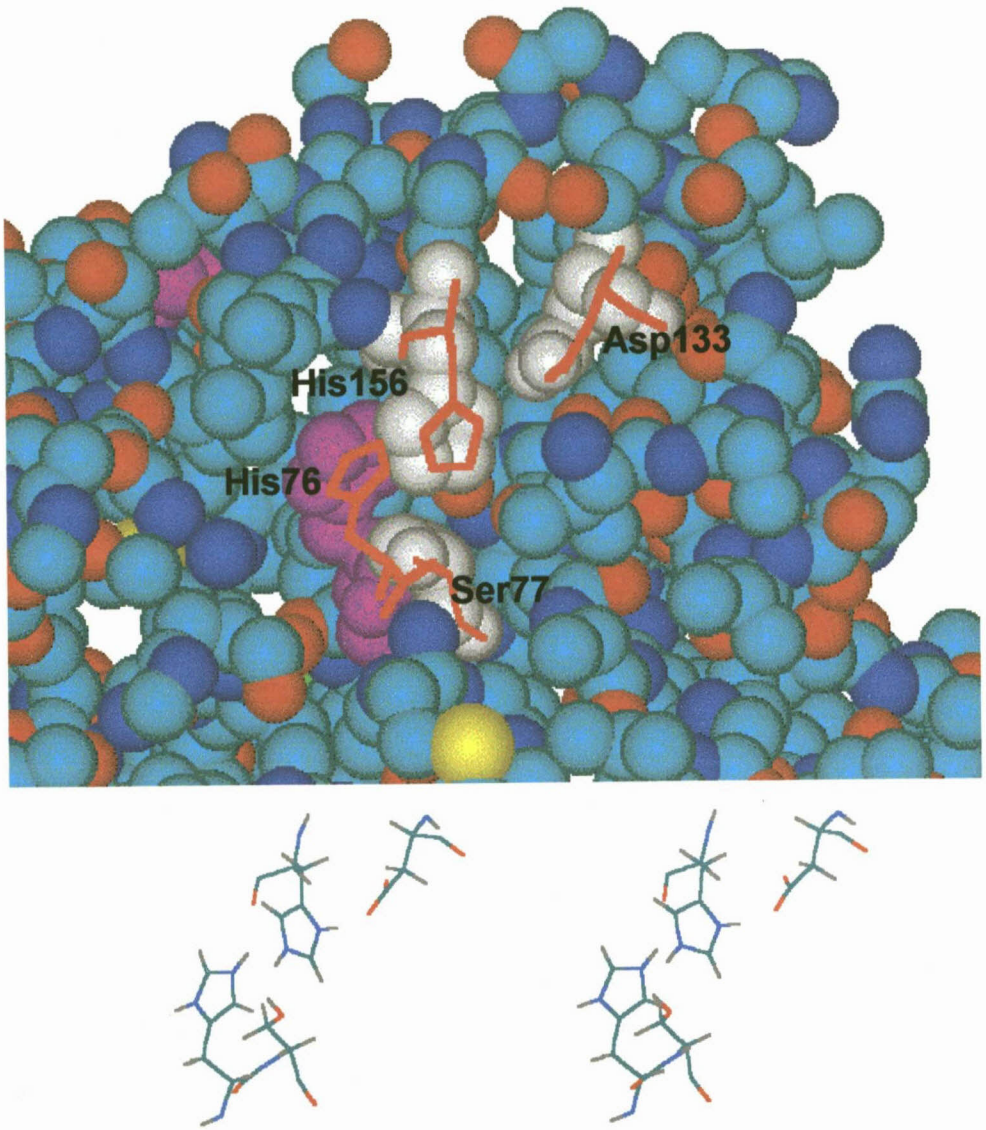


Figure 4.11. Catalytic triad of the *Bacillus subtilis* lipase showing the close proximity of the His76 to His156 and Ser77. The diagram below shows a stereo view of the residues shown in red.

His76 was mutated to Asn using Hyperchem and the co-ordinates of the *Bacillus subtilis* lipase (Figure 4.12). After geometry optimisation using a conjugate gradient method, the conformation of the catalytic triad was determined. The replacement of the His with Asn had almost no effect on the

position of any of the catalytic residues so it is unlikely that this could be the reason for the lower activity. The Asn could result in a slightly different H-bonding pattern affecting the position of water molecules in the catalytic site leading to reduced activity.

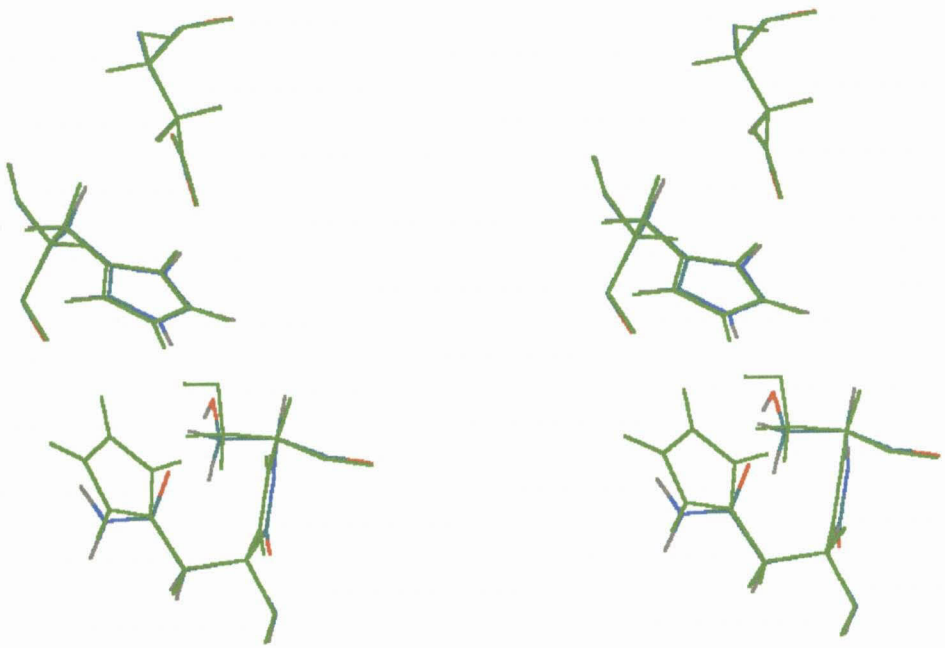


Figure 4.12. The catalytic triad of *Bacillus subtilis* with His to Asn mutation. The bonds shown in green are the unmutated residues while the bonds containing the coloured atoms are the mutant..

The Ala76Gly mutant lipase protein was constructed to restore the typical lipase consensus sequence surrounding the nucleophilic Ser. The specific activity of the Ala75Gly mutant was comparable with the non-mutated lipases. The two variants of the lipase protein exhibited dramatic instabilities at 45 °C, with almost no activity remaining upon incubation for 30 min. The Ala to Gly mutants at corresponding positions for LipA and LipB from *Bacillus subtilis*

mutants at corresponding positions for LipA and LipB from *Bacillus subtilis* lipases also resulted in marked reduction in lipase activity with LipB exhibiting half-life of 8 min at 45 °C (Misset *et al.*, 1994; Eggert *et al.*, 2000).

The lipase proteins and variants exhibited broad pH stability with more than 60% activity remaining after incubation in buffers of pH ranging from 4-12 for 20 hours. It was also observed that incubation of lipase proteins particularly in alkaline regions resulted in the enhancement of residual lipase activity. This is probably due to the fact that the *Bacillus licheniformis* lipase protein, being optimal in extreme alkaline conditions assumed a more catalytically favoured conformation at extreme alkaline pH values as compared to the reference pH 8.5. This effect was more pronounced with the Ala75Gly variant which showed a two-fold enhancement of lipase activity. The C-terminally extended enzyme (containing the His_6 tag) showed a slightly more alkaline pH-activity profile. The extension would somehow fold on the surface of the molecule, possibly forming ionic interactions with some surface negative charges. Both these phenomena can however not be explained at this stage. These experiments revealed that the evolutional acquisition of Ala and His by *Bacillus* species secreting Family 1.4 lipases at positions near the nucleophilic Ser has functional significance - they are required for optimal activity and thermal stability.

4.5. REFERENCES

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CHAPTER 5

Cloning, nucleotide sequencing and expression in *Escherichia coli* of a new carboxylesterase gene from *Bacillus licheniformis*.

5.1. Introduction

Hydrolases form a class of enzymes that shows wide substrate specificity, capable of hydrolyzing peptides, amides and halides in addition to esters and triacylglycerols. The fact that some esterases show hydrolytic activities on non-ester bonds, raises interesting questions on the terminology and classification of these enzymes (Junge and Kirsch, 1973; Heymann, 1980; Fojan *et al.*, 2000). Historically, esterases have been classified according to their known substrate specificity. They were defined as enzymes that catalyze the cleavage of ester bonds by the addition of water. Whitaker (1972) proposed a classification scheme for esterases based on the specificity for the acid moiety of the substrate, such as the carboxylic ester hydrolases for esterases that catalyze the hydrolysis of carboxylic ester. In addition to the mentioned carboxylic esterases, aryl esterases, acetyl esterases, choline esterases, cholesterol esterases and lipases belong to this group of hydrolytic enzymes. For the classification of these enzymes, either substrate specificity or place of enzymatic action has been used (Biely *et al.*, 1985; Krejci *et al.*, 1991; Hemilä *et al.*, 1994; Carrière *et al.*, 1998).

Carboxylesterases (3.1.1.1.) differ from lipases (3.1.1.3) in substrate specificity and type of enzyme kinetics. Whereas carboxylesterases preferentially hydrolyze ester bonds of substrates with shorter fatty acid chains, lipases display a much broader range than carboxylesterases. It appears that the physical state of the substrate is the most likely contributing factor towards substrate specificity. Long-

chain triacylglycerols, which are normal substrates of lipases, have hydrophobic properties. In aqueous environments, they form emulsions (lipid-water interfaces) at points of maximum concentration. By contrast, short-chain triacylglycerols posses a distinct solubility due to a higher hydrophilicity. They yield monomers at low concentrations and micelles in more concentrated solutions. Thus the lipase has to be capable of identifying an insoluble or heavily aggregated substrate. Since lipases are active towards aggregated substrates, lipase activity has been directly correlated with the total substrate and not with the substrate concentration (Verger, 1998). Esterase activity is found to be highest towards more water-soluble substrates.

Carboxylesterases are widely distributed throughout animals (Mentlein *et al.*, 1987; Long *et al.*, 1988; Ozols, 1989), plants (Gahan *et al.*, 1983; Melati *et al.*, 1996) and microorganisms (Goullet and Picard, 1990). Microbial esterases have been of particular interest due to the versatility of their properties, relatively easy procedures for production, cultivation conditions and recovery. The genes for several microbial esterases have been cloned and sequenced. Sequence analysis of esterases from diverse array of bacteria have shown that these enzymes contain the Ser-His-Asp(Glu) catalytic triad characteristic of serine hydrolases (Jaeger *et al.*, 1994). Comparison of the amino acid sequences of the enzymes reveals that most esterases display a conserved sequence motif, GESAG, around the central active site serine residue (Drabløs and Peterson,

1997). This motif has been used as an indicator for an esterase classification of an amino acid sequence.

The physiological functions of many esterases are not clear. Some of these enzymes are known to be involved in metabolic pathways that provide access to carbon sources (Dalrymple *et al.*, 1997; Degrassi *et al.*, 2000). In some pathogenic bacterial and fungal strains esterase activities are believed to be pathogenic factors (Mcqueen and Schottel, 1987). Some carboxylesterases have been shown to play a role in the detoxification of biocides. Insecticide resistance often results from the expression of genes for esterases that hydrolyze the insecticides (Blackman, *et al.*, 1995; Newcomb *et al.*, 1997). Some insecticides and neurotoxins inhibit acetylcholine esterase, which is essential in neurotransmittance. The fusidic acid resistance of *Streptomyces lividans* is due to a specific esterase, which inactivates the antibiotic (von den Haar *et al.*, 1997). A *B. subtilis* esterase that hydrolyzes the phytotoxin brefeldin A has been described (Wie, *et al.*, 1996).

Although applications of esterases are also found in the food industries (Crow *et al.*, 1994), their major impact is in the pharmaceutical and fine chemical industries where they are used in the resolution of racemic mixtures (Tombo *et al.*, 1987; Toone, *et al.*, 1990). An esterase from *Arthrobacter globiformis* was used in the resolution of ethyl chrysanthemate derivatives (Nishizawa *et al.*, 1993, 1995), which are key compounds during the synthesis of pyrethrin

insecticides. A novel carboxylesterase from *Rhodococcus* sp H1 was able to hydrolyze heroin into morphine followed by a further degradation to morphinone by a morphine dehydrogenase (Rathbone *et al.*, 1997). A *B. subtilis* carboxyl esterase has been used for stereospecific resolution of *R,S*-naproxen esters to *S*-naproxen, which is an antiflammatory drug (Quax and Broekhuizen, 1994). Although the *B. licheniformis* strain showing high stereospecifity (95%) in the resolution of *R, S*-naproxen esters has been noted (Quax and Broekhuizen, 1994), there has not been an esterase that has been implicated in this particular reaction. Zock *et al.*, (1994) reported a *B. subtilis* carboxylesterase that showed high catalytic activities towards β-lactam *p*-nitrobenzyl esters. This enzyme was genetically engineered for efficient synthesis of cephalosporin-derived antibiotics (Moore and Arnold, 1996).

In this study, the author describes the nucleotide sequence and expression in *E. coli* of the carboxylesterase from *B. licheniformis* sharing significant amino acid sequence identity with the *p*-nitrobenzyl esterase from *B. subtilis*. The original aim of the study was to clone a DNA fragment containing the full length of *B. licheniformis* lipase gene, thus including the promoter region and the sequence encoding the signal peptide. A DNA fragment encoding a carboxylesterase was however, fortuitously cloned. It must be mentioned that the production of esterase activity by *B. licheniformis* isolate has been reported previously (Fakhreddine *et al.*, 1998; Alvarez-MaCarie, *et al.*, 1999a), and the encoding gene has been cloned and sequenced (Alvarez-Macarie *et al.*, 1999b). The

carboxylesterase gene described in this study has never been reported before, indicating that we have cloned a new *B. licheniformis* carboxylesterase gene.

5.2. Materials

Tryptone, yeast extract, nutrient broth and agar were purchased from Biolab Diagnostics (Johannesburg, South Africa). RNase, proteinase K, lysozyme, ampicillin, agarose, deoxynucleotides, *Taq* polymerase, Digoxigenin -11-dUTP (DIG) labelling and High Pure PCR kits were purchased from Roche (Germany). *E coli* cells JM109 and JM109 (DE3), pGem-T, Sp6 and T7 promoter sequences, restriction and ligase enzymes were purchased from Promega (Madison, USA). Oligonucleotide primers were purchased from IDT Inc, USA. pET 20b(+) was obtained from Novagen (Madison, USA). *B. licheniformis* DSM 12369 was obtained from the German Collection of Microorganisms. The nucleospin plasmid isolation kit was supplied by Macherey-Nagel (Germany). The nylon membranes for Southern blotting were purchased from Osmonics (MA, USA). All other chemicals were commercially available and of analytical or molecular biology grade.

5.3. Methods

5.3.1. Growth media and conditions

The *B. licheniformis* bacterium was grown at 42 °C in nutrient broth (16 g/l) for the genomic DNA preparation. The *E. coli* hosts carrying plasmids were grown in Luria-Bertani (LB) medium [10g tryptone, 5g yeast extract, 10g NaCl, per liter; pH 7.0]. To prepare tributyrine agar medium, agar (12g) and the emulsion (prepared by sonication) of 1% (v/v) tributyrin and 1%(w/v) gum arabic were added to the LB medium. Ampicillin was added to the final concentration of 100 μg/ml.

5.3.2. DNA preparation and transformation

B. licheniformis cells were grown in a 250 ml culture until the late exponential phase of growth, harvested by centrifugation and re-suspended in TNE (10mMTris, 10mM NaCl, 10 mM EDTA)] buffer, pH 8.0. The cells were lysed by treatment with 10 mg/ml lysozyme at 37 °C for 30 min. Sodium dodecyl sulphate (10% w/v) solution was added to a final concentration of 3.5 mg/ml, heated for 10 min at 60 °C and cooled on ice for 10 min. Proteinase K was added to a final concentration of 25 μg/ml followed by incubation at 37 °C for 1 hour. Denatured proteins were removed by extraction with equal volumes of phenol followed by centrifugation at low speed to separate the organic and aqueous phases. The top aqueous phase was collected and the DNA was precipitated with 100% ethanol in the presence of 1/5 volume of 3M sodium acetate buffer, pH 5.2. The

precipitated DNA was re-suspended in 10 mM Tris-HCl buffer, pH 8.5 and RNAse was added to a final concentration of 0.2 µg/ml and incubated at 37 °C for 30 min. The treatment of DNA with SDS and proteinase K was repeated and a final extraction of proteins was done by adding equal volume of chloroform:isoamyl (24:1). Precipitation of DNA was done as previously mentioned, washed with 70% ethanol, vacuum-dried and re-dissolved in 10 mM Tris-HCl buffer, pH 8.5. Plasmid DNAs were isolated using the nucleospin plasmid isolation kit. Transformation of *E. coli* with recombinant plasmids was performed using standard $CaCl_2$ procedures (Sambrook *et al.*, 1989).

5.3.3. Southern blot analysis

The genomic DNA of *B. licheniformis* (5 µg) was digested to completion with *Aat* II, *EcoR* I, *Hind* III and *Pst* I restriction enzymes and electrophoresed on 1% agarose gel. Southern blotting onto nylon membranes was performed as described (Sambrook *et al.*, 1989). The membranes were cross-linked by ultraviolet light with the GS Gene Linker (Bio-rad, USA). DNA hybridization and stringent post-hybridization washes were done at 68 °C for 2 and 16 hours, respectively, according to the DIG System User's Guide for Filter Hybridization (Roche, Germany). The DNA probe was the 560 bp PCR product encoding the mature *B. licheniformis* lipase segment (Chapter 3, Figure 3.1). The PCR product was resolved on 1% agarose gel and purified from the gel using the High Pure PCR purification kit. Labelling of the DNA probe was done by random priming

using the DIG labelling kit and colorimetric detection of the labeled probe was performed with the DIG Nucleic Acid Detection kit, according to the manufacture's recommendations.

5.3.4. Screening for lipolytic activity

The *EcoR* I digested DNA fragments of appropriate size range were excised and purified from 1% agarose gel. The purified DNA fragments were ligated into pGemT-vector digested with *EcoR* I and dephosphorylated with calf intestinal alkaline phosphatase. The ligated mixture was used to transform *E. coli* JM109 cells and plated on LB agar medium containing 100 µg ampicillin /ml and grown overnight at 37 °C. Single colonies were picked and plated on tributyrin agar plates and then grown for 3 days at 37 °C. Production of lipolytic activity was detected by zones of clearance around *E. coli* colonies transformed with recombinant plasmids. One colony showed production of lipolytic activity, and the presence of the insert DNA fragment was confirmed by restriction analysis. The recombinant plasmid was denoted pGem-Bcest.

5.3.5. Sequencing of DNA

The nucleotide sequence of the gene encoding lipolytic activity was performed with the BigDye Terminator Cycle sequencing kit (Perkin Elmer), in a 377 Perkin Elmer DNA sequencer. The T7 and SP6 universal primers were used as starting

sequencing primers, and other primers were designed based on the DNA sequences that became known. The complete nucleotide sequence of the 3.5 kb DNA fragment has been submitted to the GenBank sequence database under the accession number AJ315954.

5.3.6. The PCR reaction

The primers used were forward (5'-GGG CAT ATG GCT GAR CAY AAY CCN GTN GTN ATG GTN-3') and reverse 5'-GGG CTC GAG ATT CGT ATT CTG TCC YTG KCC YCC GCC-3') specifying the amplification of the fragment encoding the mature *B. licheniformis* lipase (Chapter 3). *Taq* polymerase was used to perform PCR with pGem-Bcest plasmid as the template. The PCR conditions were as follows: 1 initial denaturation step at 94 °C for 2 min, 30 cycles at 94 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 1 min, except for the final cycle where extension proceeded for 10 min.

5.3.7. Data search and analysis

Sequences retrieved from databases were analyzed with SignalP (Nielsen *et al.*, 1997), Compute pI/MW (Bjellqvist *et al.*, 1993, 1994; Wilkins *et al.*, 1998) and CLUSTAL W (Thompson *et al.*, 1994). The PSORT program was used for the prediction of protein localization sites and transmembrane segments (Klein *et al.*, 1985).

5.3.8. Promoter analysis and expression of the gene in E. coli

The promoter for the cloned gene was identified firstly by nucleotide sequence comparison and confirmed by PCR-based deletion of the presumed promoter region. The effect of deletion of the putative promoter region on gene expression was investigated by incorporating the *EcoR* 1 restriction site into the upstream primers (Primers 330 and 460, see Figure 4) and *Xho* 1 restriction site into the downstream primer (Primer 2030). These primers were used to amplify the gene fragment that contained or lacked the putative promoter region. The conditions for the PCR were the same as described previously except that the elongation time between cycles was 2 min. The PCR-amplified fragments were digested with *EcoR* I and *Xho* 1 and ligated into pET-T7less plasmid digested with similar enzymes [pET-T7less vector was constructed by introducing an extra *EcoR* 1 site at the position immediately upstream of the *Bgl* II restriction site in the pET 20(b)(+) vector]. The ligated DNA fragments were transformed into *E. coli* JM 109 and streaked on LB tributyrin agar plates. Expression of the gene was observed by the presence of zones of clearance around the colonies.

5.4. Results

B licheniformis DNA was digested with restriction enzymes that predominantly generated fragments of less than 10 kb. Southern blot analysis showed hybridization of the probe with the digested genomic DNA, although yielding different hybridization intensities. The *EcoR* I digested genomic DNA revealed two regions of intense hybridization; regions corresponding to 3500 and 1200 bp (Figure 5.1). The intensities of the hybridization bands were different, with the 3500 bp region showing high probe intensity.

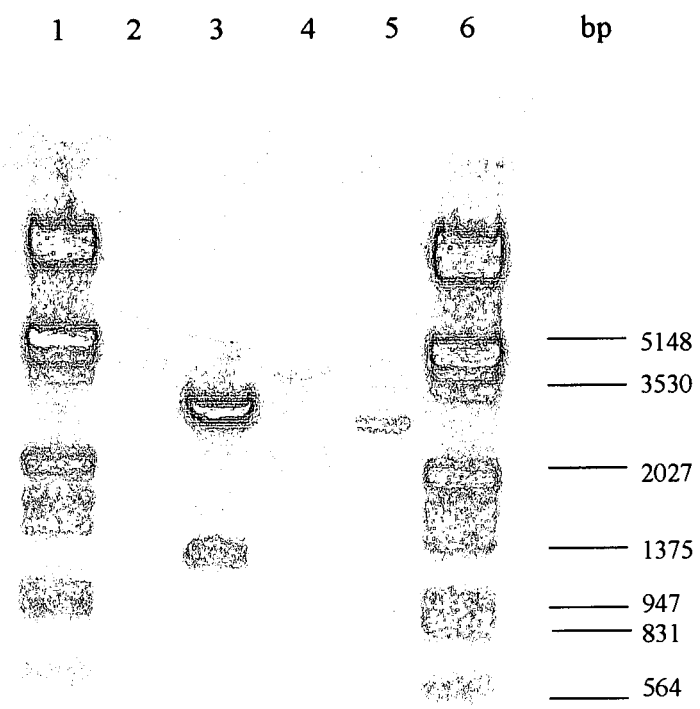

Figure 5.1. Southern hybridization analysis of *B. licheniformis* genomic DNA digested with *Aat* II (Lane 2), *EcoR* I (lane 3), *Hind* III (lane 4) and *Pst* I (lane 5). Lanes 1 and 6 are molecular weight markers. Hybridization was carried out with the DIG-labeled fragment encoding the mature gene for *B. licheniformis* lipase.

The DNA fragments located at the two regions of intense hybridization were excised and purified separately from the agarose gel and ligated into the pGemT-vector digested with *EcoR* I to create mini genomic libraries. The mini libraries were screened for lipolytic activity by transforming and plating *E. coli* cells on tributyrin plates. One clone of *E. coli* cells transformed with the mini genomic library of recombinant plasmids containing the large DNA fragments became surrounded by zones of clearance upon incubation on the otherwise opaque tributyrin agar plate (Figure 5.2).

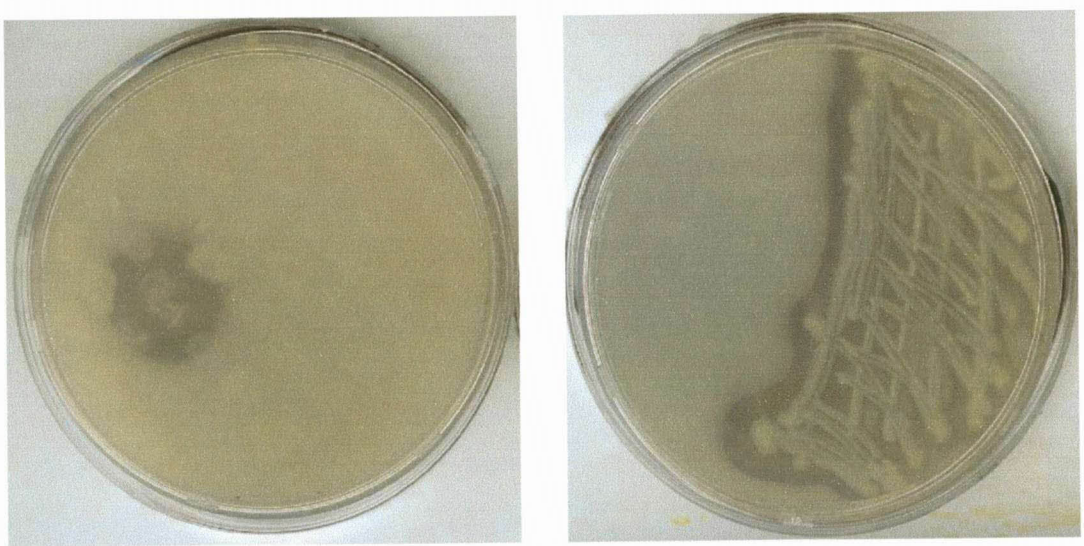

Figure 5.2. (a). Tributyrin agar plate showing zones of clearance surrounding the colony of *E. coli* cells transformed with pGem-Bcest. (b) The transformed *E. coli* isolate was streaked on a new tributyrin agar plate.

The plasmid harbored by the lipolytic positive clone was isolated and denoted pGem-Bcest. Restriction analysis of pGem-Cest revealed the presence of a DNA fragment that is about 3.5 kb in length (Figure 5.3). PCR amplification using pGem-Bcest as the template and primers specific for the 560 bp long fragment encoding the mature fragment of *B. licheniformis* lipase resulted in a fragment of about 560 bp (Figure 5.3).

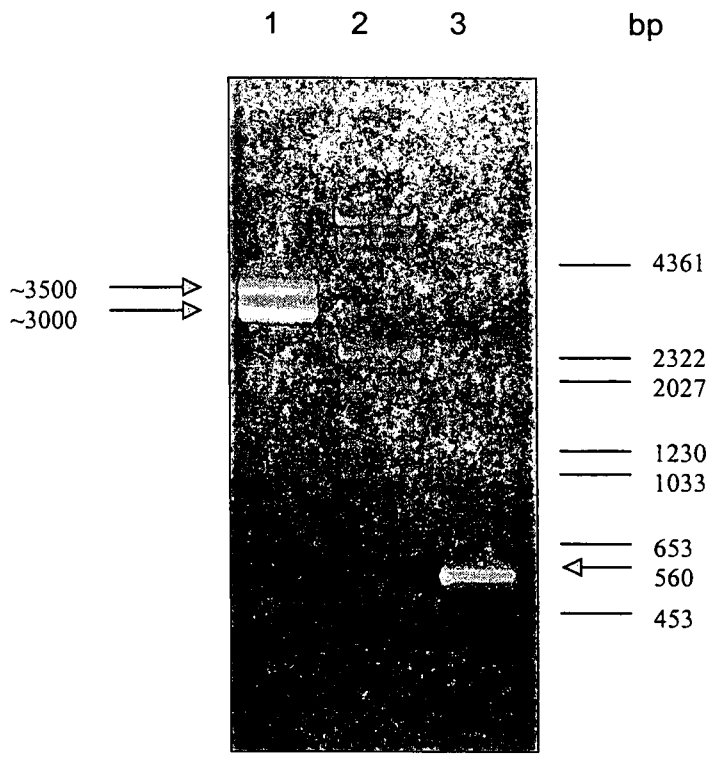

Figure 5.3. A 1% agarose gel showing the pGem-Bcest plasmid digested with *EcoR* I (Lane 1), molecular weight marker (Lane 2) and the PCR product obtained using primers specific for the amplification of the mature *B. licheniformis* lipase gene with pGem-Bcest as the template (Lane 3). The ~3500 and 3000 bp fragments on lane 1 represent the cloned DNA fragment and the pGem-T vector, respectively.

Nucleotide sequence analysis revealed an uninterrupted open reading frame of 1452 bp (nucleotides 447 to 1899) (Figure 5.4). The region of the DNA fragment serving as the promoter was found to lie between nucleotides 330-447. This was investigated by amplifying the DNA fragment with either Primer 330 or Primer 460 as upstream primers and common downstream Primer 2030. The amplified DNA fragments were ligated into pET-T7less plasmid digested with *EcoR* 1 and *Xho* 1. Digestion of pET-T7less with the mentioned restriction enzymes effectively removes the T7 promoter region and thereby eliminates the possibility that the introduced gene would be expressed from the T7 promoter. The DNA fragment amplified with Primer pairs 330 and 2030 showed zones of clearance when ligated into pET-T7less plasmid, transformed into *E. coli* cells and grown on tributyrin plates (data not shown). *E. coli* cells transformed with pET-T7less plasmid containing the DNA fragment amplified with primer pair 460 and 2030 did not show production of lipolytic activity, demonstrating that the promoter was effectively removed.

The ATG initiation codon of the cloned gene is separated from the AG-rich region (nucleotides 432-438) by an AT-rich spacer of 9 nucleotides. The AG-rich region presumably represents the ribosome-binding site. The –10 regulatory element was predicted to be TATCAT (nucleotides 417-422) due to its location and close resemblance to the TATAAT sequence of the promoters recognized by σ^A of *Bacillus* RNA polymerase (Haldenwang, 1995). The –35 element of the promoter could not be predicted with certainty although the sequence TTGAAA (361-366)

resembles the characteristic TTGACA of promoter element recognized by σ^A *Bacillus* RNA polymerase. This sequence is 50 bp upstream to the –10 promoter element whereas the spacer between the two elements is usually about 17 bp. The sequence located 17bp upstream to the –10 promoter element with some resemblance to the –35 element is TTAGAC (392-397), which is also likely to be the –35 regulatory element if the exact position of A need not be fixed within the consensus sequence. The open reading frame is terminated by two successive TGA stop codons followed by an inverted repeat of 8 nucleotides, which may function as a transcription termination signal.

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ATTCCTGCGT  TATTAACAAG  CACGTTTACT  TCCGGGTGGT  GCTCGGTTGT  CCATTCAAAC    60
AGCGCAAGCC  GTTCAGCTTC  ATCCGCTACA  TCGCACACAC  GCGTGATGAT  GCCCGGCAAT   120
GCTTGCTTTG  CTTCTTCAAG  CTTGTTTTTG  CGTCTGCCGC  ATATGATGAC  CGTATTTCCA   180
GCCTTCACGA  ATCGTTCGGC  AAAAGCGAAT  CCGATCCCTG  TTGCTCCTCC  TGTAATCAGT   240
ATGGTATTCC  CTGACAGCTC  CATGCAGATC  CCTCATTTCT  TGTTTTTTCG  AAGCGTTCCG   300
CTTATAGAAT  GGACAGCCTC  CATCATTCTA  TACCGGGACT  TGCTCCATAT  CCTATGATGA   360
                                Primer 330
TTGAAATATC  CTCAGTTTGC  AAGTGTCGCC  ATTAGACGGA  ATAGGAACGC  TTCCTTTATC   420
                                                                  -10
                            M   S  G  L  T   V  K  T   R  Y  G
ATGAAATGAT  GAAGGGAGTG  GTATAAAATG  TCTGGTCTCA  CAGTGAAAAC  CCGTTATGGA   480
             rbs                        Primer 460
A  L  K  G   T  M  Q   N  G  V   R  V  W  K   G  I  P   Y  A  K
GCGCTGAAAG  GAACGATGCA  GAACGGCGTC  CGCGTATGGA  AGGGCATCCC  TTATGCGAAG   540

P  P  V  G   K  W  R   F  K  A   P  Q  E  T   D  A  W   E  G  V
CCGCCTGTCG  GAAAGTGGAG  ATTTAAAGCT  CCGCAAGAAA  CCGATGCATG  GGAAGGAGTC   600

R  D  A  T   Q  F  G   S  I  C   P  Q  P  E   G  I  L   F  Q  L
AGGGATGCGA  CTCAATTCGG  CTCCATCTGC  CCCCAGCCTG  AAGGGATATT  GTTTCAATTA   660

E  R  V  E   K  S  E   D  C  L   C  L  N  V   F  A  P   Q  S  S
GAGAGGGTGG  AGAAGTCTGA  GGACTGCCTT  TGCTTAAATG  TGTTTGCTCC  ACAATCGTCC   720

G  E  N  R   P  V  M   V  W  I   H  G  G  A   F  Y  L   G  A  G
GGCGAAAACC  GGCCGGTGAT  GGTGTGGATT  CATGGCGGCG  CGTTTTACCT  CGGCGCAGGC   780

S  E  P  L   Y  D  G   S  H  L   A  A  D  G   D  V  I   V  A  T
AGCGAGCCTC  TATATGATGG  ATCTCATCTC  GCGGCTGATG  GAGATGTCAT  CGTAGCGACG   840

I  N  Y  R   L  G  P   F  G  F   L  H  L  S   S  V  N   Q  S  Y
ATCAATTATC  GCCTCGGTCC  GTTTGGATTT  TTGCACCTTT  CTTCTGTCAA  TCAATCCTAC   900

S  N  N  L   G  L  L   D  Q  I   A  A  L  K   W  V  K   E  N  I
AGCAACAATC  TCGGTCTGCT  TGACCAAATT  GCTGCGCTGA  AATGGGTGAA  GGAGAATATC   960
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S  S  F  G     G  D  P     D  N  I     T  V  F  G     E  S  A     G  S  M
TCATCCTTCG  GAGGAGATCC  TGACAACATC  ACGGTTTTTG  GAGAATCGGC  CGGTTCGATG     1020

S  I  A  S     L  L  A     M  P  D     A  K  G  L     F  Q  K     A  I  M
AGCATCGCCT  CGCTATTGGC  CATGCCAGAT  GCAAAAGGCT  TATTCCAAAA  AGCGATTATG     1080

Q  S  G  A     S  E  T     M  P  K     E  K  A  E     T  A  A     E  T  F
CAAAGCGGAG  CTTCCGAAAC  CATGCCGAAG  GAAAAAGCGG  AAACTGCGGC  AGAAACATTT     1140

L  H  I  L     N  I  D     P  D  H     S  E  Q  L     H  D  V     S  A  K
TTACACATTC  TCAATATCGA  TCCGGACCAT  TCGGAACAGC  TGCACGATGT  ATCTGCTAAA     1200

E  L  L  E     A  A  D     E  L  R     D  V  M  G     E  N  I     F  Q  L
GAGCTCCTTG  AAGCAGCGGA  TGAGCTTAGG  GATGTCATGG  GGGAAAATAT  TTTTCAATTG     1260

L  F  L  P     V  V  D     R  E  T     L  P  L  E     P  V  T     A  V  A
CTGTTTTTAC  CGGTCGTTGA  CAGGGAGACG  CTGCCGCTGG  AGCCTGTGAC  AGCCGTTGCA     1320

Q  G  A  A     D  D  I     K  L  L     I  G  T  N     R  D  E     G  V  L
CAAGGAGCGG  CGGATGACAT  CAAGCTGTTG  ATCGGGACAA  ACCGGGATGA  AGGCGTGCTA     1380

F  F  T  P     E  S  E     L  L  P     E  Q  K  K     A  E  I     L  R  E
TTTTTCACGC  CGGAATCGGA  GCTGTTGCCG  GAACAAAAAA  AGGCTGAGAT  TCTAAGAGAA     1440

H  V  G  G     E  L  A     K  T  A     A  E  L  Y     P  G  S     L  E  G
CATGTAGGGG  GCGAATTGGC  GAAAACAGCT  GCCGAATTGT  ATCCGGGATC  GCTGGAAGGC     1500

Q  I  N  M     M  T  D     I  L  F     W  R  P  A     V  A  F     A  A  G
CAAATCAATA  TGATGACAGA  CATCCTTTTT  TGGCGTCCGG  CCGTCGCGTT  TGCCGCAGGG     1560

Q  S  A  H     S  P  V     W  M  Y     R  F  D  W     H  S  E     H  P  P
CAGTCGGCGC  ATTCGCCCGT  ATGGATGTAC  CGGTTTGACT  GGCATTCCGA  ACATCCGCCG     1620

F  H  K  A     A  H  G     L  D  I     P  F  V  F     G  N  M     D  A  L
TTCCATAAAG  CGGCACACGG  TTTGGACATC  CCCTTTGTGT  TCGGCAACAT  GGATGCCCTT     1680

D  M  I  T     N  T  K     A  S  E     E  T  K  Q     L  S  Q     H  I  P
GACATGATCA  CGAATACGAA  AGCAAGCGAA  GAAACGAAAC  AGCTTTCGCA  ACATATTCCA     1740

G  L  P  G     F  H  L     H  I  R     E  V  R  P     L  K  P     S  A  G
GGGTTGCCTG  GCTTTCATTT  GCACATACGG  GAAGTCCGTC  CACTGAAGCC  TTCAGCTGGC     1800

R  T  M  I     R  T  H     E  K  R     S  F  S  N     T  T  I     L  I  E
CGAACTATGA  TAAGGACACA  CGAAAAACGC  TCATTTTCCA  ATACAACTAT  TTTAATAGAA     1860

E  D  P  D     A  E  K     R  K  K     L  K  I  *  *
GAAGATCCTG  ATGCTGAAAA  GAGAAAAAAA  CTGAAGATCTGATGATTGAC  CTCCCTCGTC     1920

ATGAGGGAGG  TTCTAAAGCC  TCGAATCATC  ATCAAATCAG  CCGCTTTTCT  GATACCGTTC     1980
ATTTTGAATG  TCTTCCAAAG  ATTTTCCCCT  CGTCTCCGGG  GCCATGAGAA  GGCCGATGAT     2040
                                                Primer 2030
CATATGAACG  GCGAGAAATG  TGATAATCAC  GCCTCCGGCC  GTTTTAAAGC  CAAGTATGGT     2100
AATCATGGCC  GGGACAACCA  AGGAAATCAA  GCCGACGCCG  ATCCGCACGA  CAAAGAACAT     2160
AAAGCCTTGG  GCCATT                                                     2176
```

Figure 5.4. The DNA sequence from *B. licheniformis* encoding lipolytic activity. The amino acid deduced from the nucleotide sequence is shown at the beginning of each codon. The putative transcription and translation signals are underlined. The stop codons are represented by (*). The italized nucleotides after the stop codons represent nucleotides forming the inverted repeat. Sequences used as primers in the promoter mapping experiments are underlined by dotted lines.

The open reading frame encodes a protein of 484 amino acids with the theoretical molecular weight of 53 353 Da and a pI of 5.38 (Bjellqvist *et al.*, 1993, 1994; Wilkins *et al.*, 1998). No signal peptide sequence was found (Nielsen *et al.*, 1997). The protein was predicted to be an intracellularly localized enzyme (Klein *et al.*, 1985). Database searches with the complete protein sequence deduced from the nucleotide sequence revealed similarities to esterases. The *p*-nitrobenzyl esterase from *B. subtilis* showed 59% amino acid identity, carboxylesterases from *Bacillus* sp BP-7, 60% identity and *Paenibacillus* sp-23, 43% identity (Figure 5.5). The esterase GE**S**AG characteristic motif was found at position 184-189.

```
Bacillus sp BP-7     MSESVVKTQYGTVKGISKNGVQTWKGIPYAKPPVGQLRFKAPDPPAAWEGVLDATAYGPV 60
B.subtilis           MTHQIVTTQYGKVKGTTENGVHKWKGIPYAKPPVGQWRFKAPEPPEVWEDVLDATAYGPI 60
B.licheniformis      MSGLTVKTRYGALKGTMQNGVRVWKGIPYAKPPVGKWRFKAPQETDAWEGVRDATQFGSI 60
Paenibacillus        MRELQVQTKYGKVQGELLQGASVWKGIPYAKPPVGEMRFQAPTQPESWDGIRQATEFGPE 60
                     *    * *:** ::*   :*.  ************: **:**  .  *:.: :** :*.

Bacillus sp BP-7     CPQPPDLLSYSYPELPRQSEDCLYVNVFAPDT-PGKNRPVMVWIHGGTFYLGAGSEPLYD 119
B.subtilis           CPQPSDLLSLSYTELPRQSEDCLYVNVFAPDT-PSQNLPVMVWIHGGAFYLGAGSEPLYD 119
B.licheniformis      CPQPEGILFQ--LERVEKSEDCLCLNVFAPQS-SGENRPVMVWIHGGAFYLGAGSEPLYD 117
Paenibacillus        NIQPRHDSEWMGGQKPPESEDSLYLNIWAPEKESSHPLPVMVWIHGASFVTGSGSLPVYD 120
                       **         :   :***.* :*::**:. ...  ********.::*  *:** *:**

Bacillus sp BP-7     GSNLAAQGDVIVVTLNYRLGPFGFLHLSSIDEAYSDNLGLLDQTAALKWVKDNISAFGGD 179
B.subtilis           GSKLAAQGEVIVVTLNYRLGPFGFLHLSSFDEAYSDNLGLLDQAAALKWVRENISAFGGD 179
B.licheniformis      GSHLAADGDVIVATINYRLGPFGFLHLSSVNQSYSNNLGLLDQIAALKWVKENISSFGGD 177
Paenibacillus        GTQLAVRGDVIVVTINYRLGPLGFLHMAPLGEGYVSNAGLLDQVAALQWVKDNITAFGGD 180
                     *::**. *:***.*:******:****::...:.* .* ***** ***:**::**::****

Bacillus sp BP-7     PENVTVFGESAGGMSIAALLAMPAAKGLFQKAILESGSSRTMTEEKAASTAHAFLRILGI 239
B.subtilis           PDNVTVFGESAGGMSIAALLAMPAAKGLFQKAIMESGASRTMTKEQAASTAAAFLQVLGI 239
B.licheniformis      PDNITVFGESAGSMSIASLLAMPDAKGLFQKAIMQSGASETMPKEKAETAAETFLHILNI 237
Paenibacillus        PNQVTVFGESAGSMSIAALMAMPAAKGLFQRAIMESGASQFMPAEQASALREGMLKVLGV 240
                     *:::********.****:*:*** ******:**::**:*. *. *:* :    :*::*.:
```

```
Bacillus sp BP-7     DGHHLDRLHTVSAEDLLKAADQLRKTENENIFQLFFQPALDPKTLPAEPEQAIAEGAADG 299
B.subtilis           NESQLDRLHTVAAEDLLKAADQLRIAEKENIFQLFFQPALDPKTLPEEPEKSIAEGAASG 299
B.licheniformis      DPDHSEQLHDVSAKELLEAADELRDVMGENIFQLLFLPVVDRETLPLEPVTAVAQGAADD 297
Paenibacillus        DRDNLEKLNSIPVEQIMAAAEVVKQQSGAG-MALLFQPVLDGETLPQVPLQAVSEGSAKD 299
                     :  : ::*: :..::::: **: ::     . : *:* *.:* :***  *  ::::*:*..

Bacillus sp BP-7     IPLLIGTNRDEGYLFFTPDSEVHSQETIDEALEYLLG-QPLAKKAADLYPRSLESQIHIM 358
B.subtilis           IPLLIGTTRDEGYLFFTPDSDVHSQETLDAALEYLLG-KPLAEKAADLYPRSLESQIHMM 358
B.licheniformis      IKLLIGTNRDEGVLFFTPESELLPEQKKAEILREHVG-GELAKTAAELYPGSLEGQINMM 356
Paenibacillus        VSILIGTTLHEGALFIQPHVPYSKDIDMVQGVNFMTPDLENRVAIADSYPKTADGQAQVM 359
                     : :****. .** **: *.     :       :.            *: ** : :.* ::*

Bacillus sp BP-7     TDLLFWRPAVACASAQSRYAPVWMYRFDWHP-DKPPYNKAFHALELPFVFGNLNGLKRMV 417
B.subtilis           TDLLFWRPAVAYASAQSHYAPVWMYRFDWHP-EKPPYNKAFHALELPFVFGNLDGLERMA 417
B.licheniformis      TDILFWRPAVAFAAGQSAHSPVWMYRFDWHS-EHPPFHKAAHGLDIPFVFGNMDALDMIT 415
Paenibacillus        TDMFFWRSALQYAAAQQQHAPVWMYRFDWVMPEHPLLKRAIHSIEMFFVFNTLDALK-FM 418
                     **::***.*:  *:.*. ::********   ::*  ::* *.::: ***.:::.*. :

Bacillus sp BP-7     QADITDEVKQLSHTIQ--SAWLAFAKTGNPSCEDVQWPAYTEDKRETLILNSELSIEHDP 475
B.subtilis           KAEITDEVKQLSHTIQ--SAWITFAKTGNPSTEAVNWPAYHEETRETVILDSEITIENDP 475
B.licheniformis      NTKASEETKQLSQHIPGLPGFHLHIREVRPLKPSAGRTMIRTHEK-RSFSNTTILIEEDP 474
Paenibacillus        KAEPDEAAKALALKVQ--DAWIAFAKDGKPSVAGIKWPEYSKD-RATLIFNHEIEVVHDP 475
                     ::.  : .* *:  :    .:  . :  .*       .    . :   : :  : : .**

Bacillus sp BP-7     DGEKRKKLLHS--- 486
B.subtilis           ESEKRQKLFPSKGE 489
B.licheniformis      DAEKRKKLKI---- 484
Paenibacillus        ESSKRELLGV---- 485
                     :..**: *
```

Figure 5.5. Amino acid alignment of carboxylesterases from *Bacillus* sp BP-7 (accession no AJ278066), *B. subtilis* (U06089), *B. licheniformis* (this study) and *Paenibacillus* (AJ238680). The alignment was performed with CLUSTAL W. The asterisks (*) indicate amino acid identity in all shown sequences. The point (.) and colon (:) respectively denote semi-conserved and conserved amino acid substitutions in the alignment. The characteristic esterase motif is shown in bold, underlined letters. The putative catalytic triad is shown in bold double underlined letters.

5.5. Discussion and Conclusion

While attempting to clone the promoter region of a gene encoding *B. licheniformis* extracellular lipase activity, a DNA fragment exhibiting lipolytic activity on tributyrin agar plate was obtained. Although the cloned DNA fragment consistently yielded a PCR product of 560 bp when used as the template with primers specifying the amplification of the 560 bp fragment encoding the mature segment of extracellular *B. licheniformis* lipase, complete sequencing of the cloned DNA did not reveal any sequence homologous to the *B. licheniformis* lipase gene. The protein did not show any considerable homology with known extracellular lipases from *Bacillus* species. A previously unknown lipolytic gene was, however cloned. Amino acid sequence analysis showed that the gene belongs to a family of esterases. The **GESAG** motif characteristic of esterases could be identified at position 184-189 of the protein. The serine residue contained within this motif serves as the nucleophile during catalysis with Glu^{308} and His^{397} as putative members of the catalytic triad. The amino acid sequence that could serve as the signal peptide could not be predicted which suggested that the enzyme is an intracellularly bound protein. *E. coli* cells transformed with the plasmid containing the cloned carboxylesterase gene, however exhibited extracellular lipolytic activity when grown on tributyrin agar plates without any indication of cell lysis.

The protein showed high amino acid identity with the carboxylesterases from *Bacillus* sp. Bp-7 and *Paenibacillus* sp. BP-23, which have been demonstrated to be membrane-bound enzymes (Prim *et al.*, 2000; 2001). Although the natural substrate of the cloned *B. licheniformis* carboxylesterase is not known, it showed high amino acid identity to the *p*-nitrobenzyl esterase from *B. subtilis*. This enzyme has been of particular interest, particularly in the synthesis of β-lactam antibiotics (Zock, *et al.*, 1994). The crystallographic structure of the *p*-nitrobenzyl esterase has been resolved at 1.5Å (Spiller *et al.*, 1999) and the amino acid residues stabilizing the protein against thermal deactivation have been identified (Giver *et al.*, 1998; Spiller *et al.*, 1999). Preliminary comparison of primary structures revealed that some of the amino acid residues occupying the active site cavity of the *B. subtilis* *p*-Nitrobenzyl esterase differ at corresponding positions of *B. licheniformis* carboxylesterase. It would therefore be of structural and functional importance to purify and study the biochemical properties of the *B. licheniformis* carboxylesterase.

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CHAPTER 6

SUMMARY (OPSOMMING)

SUMMARY

Lipases (EC 3.1.1.3) catalyze the hydrolysis of triacylglycerols and occur widely in nature. The lipase-catalysed reaction is reversible and a wide range of trans- and interesterification reactions can be catalyzed. These enzymes could be used to manufacture products which could not be obtained by conventional chemical processes, and as the advantages of the use of lipases relative to traditional chemical processes are more recognized, lipases may be expected to gain even more importance in the enzyme market. For these purposes, new lipases with a wide range of novel characteristics are required.

Bacterial isolates collected mainly from alkaline dairy washes were screened for lipase production on agar plates containing Rhodamine B/olive oil, Tween 80/$CaCl_2$ and tributyrin. Isolates that showed lipase production on agar plates were investigated for lipase production in liquid cultures in the presence of different carbon sources. *Bacillus licheniformis and Bacillus pumilus* were identified as best producers of lipase on the basis of relative activity, pH and temperature optimum using *p*-Nitrophenyl palmitate as the assay substrate.

The production of extracellular lipase by *Bacillus licheniformis* in the presence of selected carbohydrates and lipidic substrates was investigated. The microorganism could not grow in mineral medium containing Tween 20, Tween 80 and caproic and caprylic acids. Although the mineral medium supplemented

with tributyrin, triolein, olive oil, tricaprylin, glycerol and glucose supported growth of the microorganism, no lipase production was detected; probably as a result of the lack of the lipase inducing factor. When the microorganism was grown on nutrient broth, lipase activity of about 600 units/l was achieved. This indicated the presence of the lipase producing components in the rich nutrient broth medium.

Production of extracellular lipase was repressed by the addition of triacylglycerols, free fatty acids, glycerol and glucose. The repressive effects of triacylglycerols were found to be more rapid and pronounced as compared to the effects of free fatty acids and glycerol alone. The increased repressive effects of triacylglycerols could have occurred as a result of compounded effects of glycerol and free fatty acids, which are the hydrolytic products when the lipase hydrolyses the triacylglycerols. These observations suggested synergistic effect or the presence of two independent pathways by which free fatty acids and glycerol repress the production of lipase by *Bacillus licheniformis*.

Addition of detergents Tween 20 and Tween 80 enhanced the production of lipases by the microorganism. The extracellular lipase activity increased to levels of about 2000 units/l in the presence of the detergent.

The purification of the lipase from *Bacillus licheniformis* was attempted using a combination of ion-exchange chromatography, hydrophobic interaction chromatography, hydroxylapatite and size exclusion chromatography. However,

aggregation of lipase protein, lack of interaction, and irreversible interaction with chromatography resins resulted in only partially pure lipase preparations.

The partially purified lipases showed biochemical properties similar to lipases produced by *Bacillus pumilus* and *Bacillus subtilis*. The three lipases are thermolabile, alkali tolerant and function optimally in alkaline pH conditions. The cloning and sequencing of the lipase gene from *Bacillus pumilus* isolate followed by amino acid analysis revealed high sequence homology suggesting similar protein folds. This led to the hypothesis that the mature lipase secreted by *Bacillus licheniformis* could have significant homology with the mature lipase secreted by the other two *Bacillus* species.

Degenerate primers were consequently designed based on the sequences of mature lipases secreted by *Bacillus pumilus* and *Bacillus subtilis*. The primers amplified a DNA fragment of 560 bp encoding lipase activity with *Bacillus licheniformis* genomic DNA as the template. The DNA fragment encoding the mature lipase of *Bacillus licheniformis* was subcloned into the pET 20b(+) expression vector to construct a recombinant lipase protein containing 6 histidine residues at the C-terminal. High-level expression of the lipase by *Escherichia coli* cells harbouring the lipase gene-containing expression vector was observed upon induction with IPTG at 30 °C. A one step purification of the recombinant lipase was achieved with Ni-NTA resin. The histidine tag was removed by creating a 6X His-tag at the N-terminal of the protein followed by a rTEV protease

cleavage site. The lipase protein was purified by Ni-NTA affinity chromatography followed by cleavage with rTEV protease to remove the histidine tag. The C-terminal His-tagged and the non-tagged lipase proteins were characterized.

The specific activity of the purified enzyme was about 130 units/mg with *p*-nitrophenyl-palmitate as substrate. The enzyme showed maximum activity at pH 9.5-11.5 and was remarkably stable at alkaline pH values up to 20 hours. The C-terminal histidine tag was found to enhance the specific activity of the lipase in pH conditions between 10-11.5. The enzyme showed maximal activities toward *p*-nitrophenyl esters and triacylglycerols containing C_6 and C_8 fatty acyl groups. The metals that affected the lipase significantly were divalent Co, Zn and Hg which decreased the activity to less than 30% with Hg abolishing all the lipase activity. The enzyme was not inhibited by ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), or mercaptoethanol, while the classical serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) decreased the lipase activity to 40% at 1 mM concentration.

The amino acid sequence of the lipase shows striking similarities to lipases from *Bacillus subtilis* and *Bacillus pumilus*. Based on the amino acid identity and biochemical characteristics, we classified *Bacillus licheniformis* lipase as a member of Family 1.4 lipases, together with lipolytic enzymes produced by *Bacillus subtilis* and *Bacillus pumilus*. The consensus sequence of the lipases around the nucleophilic Ser deviated from the canonical Gly-X-Ser-X-Gly. In the

three *Bacillus* lipases, the consensus sequence was found to be Ala-His-Ser-X-Gly. This motif is shared with another group of larger lipases produced by thermophilic *Bacillus* species.

Site-directed mutagenesis of the Ala and His residues near the nucleophilic Ser suggested that the lipase enzymes acquired the amino acids during evolution for optimal activity and enhanced thermal stability. Asp133, His156 together with Ser77 were identified by site-directed mutagenesis as residues that form the catalytic triad of the lipase enzyme. This was confirmed by the three-dimensional structure model built using *Bacillus subtilis* Lipase A as the template.

While in pursuit of cloning the promoter region of *Bacillus licheniformis* lipase gene, a 3.5 kb DNA fragment that showed lipolytic activity on tributyrin agar plate was obtained. Sequence analysis of the cloned DNA fragment revealed that a new carboxylesterase gene with an open reading frame encoding a protein of 484 amino acids with estimated molecular mass of 53 kDa and a pI of 5.4 had been cloned. The cloned protein showed high amino acid identity with industrially significant enzymes belonging to the esterase family.

This study has advanced the biochemical knowledge on lipases secreted by *Bacillus* species. Although the biochemical properties of *Bacillus* lipases are becoming known, the molecular mechanisms regulating the biosynthesis of the enzymes remain unknown. Future studies should therefore also attempt to

elucidate the molecular mechanisms of *Bacillus* lipase biosynthesis. This would facilitate the bioengineering of *Bacillus* species to produce inducible lipase enzyme at quantities enough for application in for example, detergent and leather tanning industries. Current studies aimed at the cloning of the promoter region of the lipase from *Bacillus licheniformis* should be continued for the purpose of understanding the molecular regulation of the gene.

The role of *Bacillus* lipases in biocatalytic resolution of racemic mixtures has not been fully explored. The recent availability of the three-dimensional structure of Family 1.4 lipases will enable us to understand the structural determinants of lipase properties and to construct *Bacillus* lipases that suit desired functions. As an example, the mature lipase produced by *Bacillus pumilus (*UOFS*)* is more than 95% identical to *Bacillus lichenifomis* lipase at amino acid level, but the latter lipase shows a broader alkaline optimum pH profile. The determinant for the broad alkaline pH profile has not yet been identified.

The new carboxylesterase gene cloned in this study should be over-expressed and biochemically characterized. The biotechnological applicability for the enzyme should be assessed.

OPSOMMING

Lipases (E.C. 3.1.1.3), wat wyd in die natuur voorkom, kataliseer die hidrolise van triasielgliserole. Die lipase-gekataliseerde reaksie is omkeerbaar en 'n wye verskeidenheid trans- en interesterifiseringreaksies is moontlik. Die ensieme kan die sintese van produkte wat nie gewoonweg deur konvensionele chemiese metodes gemaak kan word, nie moontlik maak, en daar kan verwag word dat, soos die toepaslikheid van lipases meer bekend word, hulle steeds belangriker in die ensiem mark sal word. Daarom sal nuwe lipases met nuwe eienskappe benodig word.

Bakteriese isolate wat hoofsaaklik van alkaliese melkery afloopwater versamel is, is op Rhodamien B/olyfolie-, Tween 80/$CaCl_2$- en tributirien-bevattende agarplate getoets. Isolate wat lipase vervaardig het, is in skudkulture vir lipase-produksie in die teenwoordigheid van verskillende koolstofbronne getoets. *Bacillus licheniformis* en *Bacillus pumilus* is as die beste lipase-produseerders geïdentifiseer met *p*-nitrofeniel palmitaat as toetssubstraat en deur relatiewe aktiwiteit, optimum pH en temperatuur as maatstaf te gebruik.

Lipase produksie deur *Bacillus licheniformis* is ondersoek in die teenwoordigheid van verskillende koolhidrate en lipiedsubstrate. Die mikro-organisme kon nie groei in 'n mineraalmedium wat met Tween 20, Tween 80, kaproësuur of kapriliensuur gesupplementeer was nie. Dieselfde medium met tributirien,

trioleïen, olyfolie, gliserol of glukose gesupplementeer is, kon groei ondersteun maar geen lipase aktiwiteit kon gemeet word nie, waarskynlik weens die gebrek aan 'n induksiefaktor. Dit het daarop gedui dat daar in 'n ryk voedingsop medium waarskynlik 'n lipase induseerder teenwoordig was.

Die byvoeging van triasielgliserole, vrye vetsure, gliserol of glukose het die produksie van die ekstrasellulêre lipase onderdruk. Die onderdrukkende effek van triasielgliserole was vinniger en sterker as die van vetsure of gliserol afsonderlik, moontlik as gevolg van die gesamentlike effek van die twee verbindings wat beide die produkte van hidrolise is. Die waarnemings het gedui op 'n sinergistiese effek van moontlik twee afsonderlike bane waar deur vrye vetsure en gliserol die produksie van *Bacillus licheniformis* lipase onderdruk.

Byvoeging van Tween 20 of Tween 80 het die produksie van lipases deur die organisme versterk en 'n toename tot so veel as 2000 eenhede/ml veroorsaak.

Suiwering van die lipase is deur middel van 'n kombinasie van ioonuitruilings-, hidrofobiese interaksie-, hidroksielapatiet- en uitsluitingschromatografie was onsuksesvol. Aggregasie, 'n gebrek aan interaksie met die kolommedia of onomkeerbare binding van die lipase-proteïen aan die chromatografiemedium het die suiwering bemoeilik en slegs 'n gedeeltelik-gesuiwerde proteïen kon verkry word.

Die lipasepreparaat het dieselfde eienskappe as dié van *Bacillus pumilis* en *Bacillus subtilis* getoon. Al drie ensieme was termies labiel, kon alkali tolereer en het optimaal in alkaliese medium gefunksioneer. Die klonering en nukleotiedvolgordebepaling van die lipase geen van 'n *Bacillus pumilis* isolaat het daarop gedui dat hierdie lipase en die een van *Bacillus subtilis* dieselfde struktuur kon hê. Dit kon daarop dui dat die lipase van *Bacillus licheniformis* beduidende homologie met die ensiem van die ander twee bacilli kon toon.

Degenererende voorlopers is, met die nukleotiedopeenvolging van die *Bacillus pumilis* en *Bacillus subtilis* as basis, ontwerp. 'n DNA fragment van 560 basispare wat vir lipase aktiwiteit kodeer en met die genomiese DNA as templaat, is met die gebruik van die voorlopers geamplifiseer. Die DNA fragment wat vir die lipase kodeer is in 'n pET 20b(+) uitdrukkingsvektor gesubkloneer om 'n lipase konstruk te verkry met 6 histidienresidue aan die C-terminale van die proteïen toegevoeg. Hoë uitdrukkingsvlakke van die lipase is verkry met *Escherichia coli* selle wat die vektor bevat na induksie met IPTG by 30°C. 'n Enkel stap suiweringsprosedure deur chromatografie op Ni-NTA chromatografiemedium het gevolg. Die histidien merker kon verwyder word deur 'n 6X histidien volgorde aan die N-terminale punt van die proteïen te heg en dit na proteïenuitdrukking met rTEV protease te verwyder, Suiwering van die volledig geprosesseerde ensiem was daarna op die Ni-NTA kolom moontlik. Die ensiem met die C-terminale histidien merker sowel as die volledig geprosesseerde ensiem is daarna gekarakteriseer.

Die gesuiwerde ensiem het 'n spesifieke aktiwiteit van 130 eenhede/mg getoon met *p*-nitrofeniel palmitaat as substraat. Die ensiem was maksimaal aktief by pH van 9.5-11 en was besonder stabiele by alkaliese pH tot by pH 12. Die C-terminale histidien merker het die lipase aktiwiteit by pH 10-11.5 versterk. Die ensiem was maksimaal aktief met *p*-nitrofeniel esters of triasielgliserole met C_6 of C_8 asielgroepe. Divalente Co, Zn, en Hg het die grootste effek op die aktiwiteit gehad; eersgenoemde twee het die aktiwiteit tot minder as 30% laat afneem terwyl Hg die ensiem totaal gedeaktiveer het. Die ensiem was nie deur EDTA, ditiotreïtol of merkaptoetanol geïnhibeer nie maar die klassieke serien proteaseinhibeerder, feniel-metiel-sulfonielfluoried (PMSF) (1mM) het die lipase-aktiwiteit tot minder as 40% van die kontrole laat afneem.

Die aminosuuropeenvolging van die lipase het besondere ooreenkomste met lipases van *Bacillus subtilis* en *Bacillus pumilis* getoon. Met aminosuuropeenvolging en biologiese eienskappe as maatstaf is die *Bacillus licheniformis* lipase as 'n lid van die Familie 1.4 lipase geklassifiseer, saam met die lipases van *Bacillus subtilis* en *Bacillus pumilis*. Die konsensus-opeenvolging in die omgewing van die nukleofiliese serien het van die gewone Gly-X-Ser-X-Gly afgewyk. In die *Bacillus* lipases is die opeenvolging Ala-His-Ser-X-Gly, 'n motief wat ook in die groep groter lipases van die termofiele *Bacillus* spesies.

Setelgerigte mutagenese van die Ala en His residue na-aan die nukleofiliese Ser het aangedui dat die lipase-ensieme dié aminosure verkry het, waarskynlik

tydens evolusie vir optimale aktiwiteit en verhoogde termiese stabiliteit. Asp133, His156 en Ser77 is as die katalitiese drietal geïdentifiseer deur setelgerigte mutagenese. Dit is bevestig deur 'n driedimensionele model van die ensiem te bou met die *Bacillus subtilis* lipase A as templaat.

In 'n poging om die promotorgebied van die *Bacillus licheniformis* te kloneer, is 'n 3.5 kb DNA fragment verkry wat lipolitiese aktiwiteit op tributirien agarplate getoon het. Nukleotied opeenvolgingsanalise van die gekloneerde fragment het 'n nuwe karboksiel esterasegeen opgelewer met 'n oop leesraam wat kodeer vir 'n proteïen met 484 aminosure, 'n geskatte molekulêre massa van 53 kDa en 'n pI van 5.4.

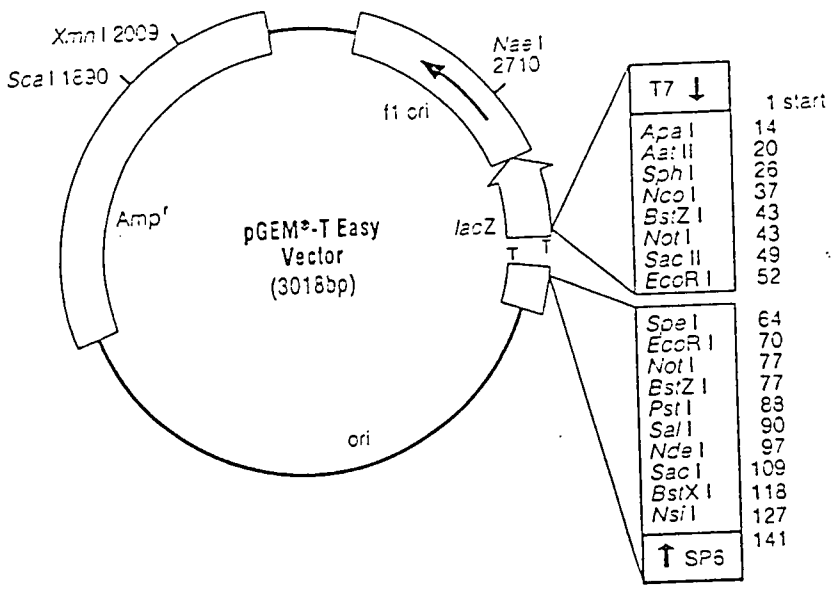
Hierdie studie het beduidend bygedra tot die beskikbare inligting aangaande die lipases van die *Bacillus* spesies. Alhoewel meer omtrent die molekulêre eienskappe van die *Bacillus* lipases bekend word, is daar min bekend oor die molekulêre meganismes wat die uitskeiding van die ensieme reguleer. Toekomstige navorsing behoort daarop gerig te wees om hierdie kwessie op te klaar. Dit sou die biogeniëring van *Bacillus* spesies fasiliteer en moontlik verhoogde uitskeidingsvlakke van die ensiem moontlik maak vir toepassing in die leer- of wasmiddelbedryf. Huidige studies wat gerig is om die promotorgedeelte van die lipase geen van *Bacillus licheniformis* te kloneer behoort daarom voortgesit te word.

Die toepassing van *Bacillus* lipases in die biokatalitiese skeiding van rasemiese mengsels is nog nie volledig ondersoek nie. Die onlangse publikasie van die driedimensionele struktuur van 'n Familie 1.4 lipase maak nou meer sinvolle konstruksie van pasgemaakte *Bacillus* lipases moontlik. As voorbeeld kan genoem word dat die aminosuuropeenvolging van die volledig geprosesseerde lipase van *Bacillus pumilis* (UOFS) meer as 95% ooreenkoms toon met die ensiem van *Bacillus licheniformis* maar laasgenoemde toon 'n wyer optimale pH profiel. Die rede vir die verskillende pH profiel is nog onbekend.

Die nuwe karboksiel esterasegeen wat gekloneer is behoort in groot maat uitgedruk te word en biochemies gekarakteriseer te word. Die biotegnologiese toepaslikheid van die ensiem kan dan ook bepaal word.

Appendix I

pGem-T Easy vector map

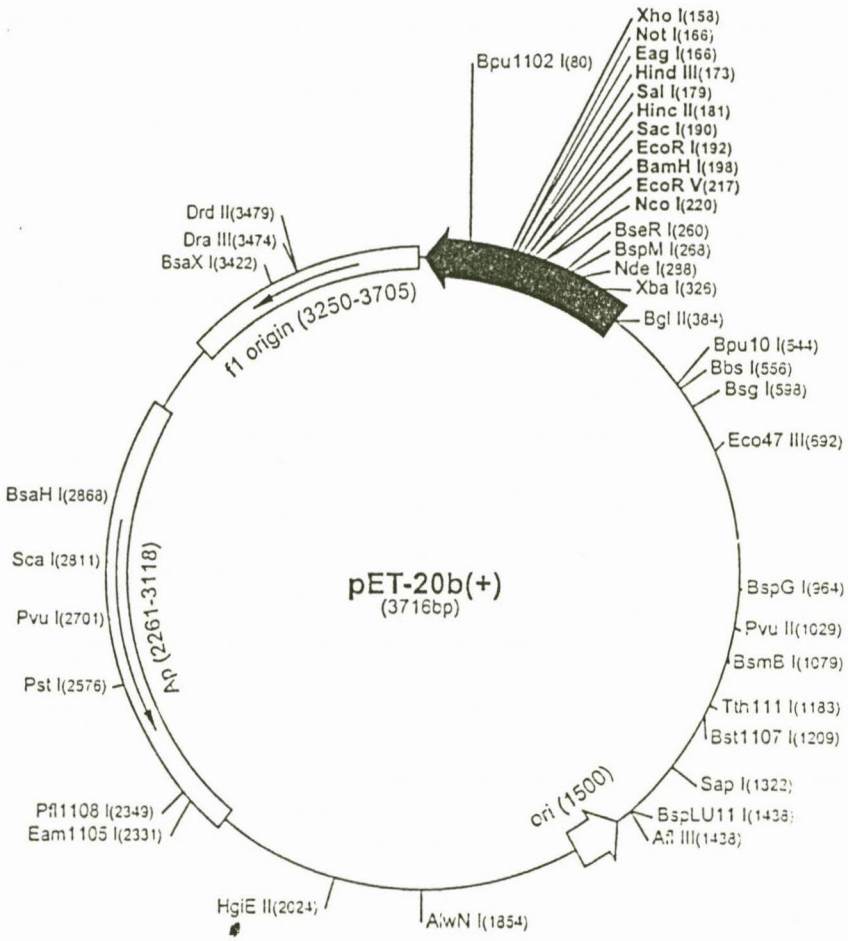


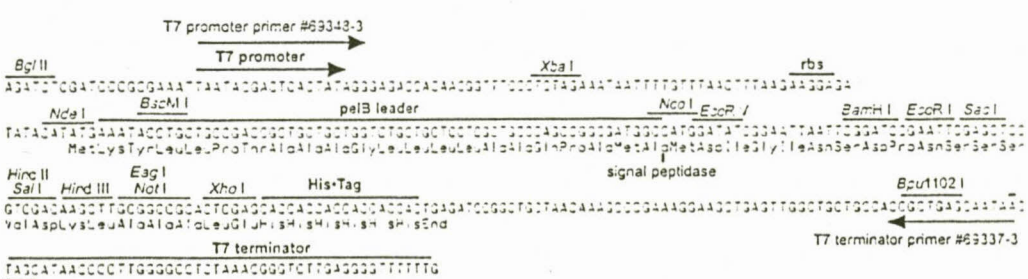
Appendix II

pET-20b(+) Vector map

The pET-20b(+) vector (Cat. No. 69739-3) carries an N-terminal *pelB* signal sequence for potential periplasmic localization, plus optional C-terminal His•Tag® sequence. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer (Cat. No. 69337-3).

T-20b(+) sequence landmarks	
promoter	353-369
transcription start	352
elB coding sequence	224-289
ultiple cloning sites	
Nco I - *Xho* I)	158-225
is•Tag coding sequence	140-157
terminator	26-72
BR322 origin	1500
a coding sequence	2261-3118
origin	3250-3705





pET-20b(+) cloning/expression region