PURIFICATION AND CHARACTERIZATION OF LIPASE FROM A THERMOACTINOMYCES SPECIES.

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Jana el Thoumy

In memory of Nelson P. Germanos

When we met, I was just about to start the thesis

And you left, when I was just about to finish it

Everything in this world has a start and has an end

In memory of our friendship,

In memory of our parental link,

In memory of these very rich but very short moments we had

together,

And even more than this,

I dedicate to you my work,

May your soul rest in peace my dear Nelsinho.

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ABSTRACT

PURIFICATION AND
CHARACTERIZATION OF
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by Jana Thoumy

Chairperson of the Supervisory Committee:Professor Fuad Hashwa
Department of Biology

An extracellular lipase with a molecular weight of 99 KDa from a *Thermoactinomycete* sp. isolated from an olive oil contaminated soil (Al Koura, Lebanon) was purified using ammonium sulphate , Sephadex G-25 and ion exchange chromatography (DEAE cellulose). The enzyme had more affinity to p- nitrophenyl laurate than to o- nitrophenyl laurate and it was stable after boiling for 1 hour while the purified fraction showed activity only at 60°C. Enzyme activity was measured in the presence of different substrates and a high affinity to p- nitrophenyl palmitate and p- nitrophenyl laurate, was detected. However, the enzyme didn't react at all with tripalmitin, trierucin, trielaidin, 1,3 diolein and triolein. The presence of metal ions like Ca^{2+} and Fe^{3+} activated the enzyme, while Co^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+} totally inhibited the lipase activity. Finally, the lipase had a short shelf life, even in the presence of Ca^{2+}.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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GLOSSARY

A: Absorbance

Ca^{2+}: Calcium ion

Co^{2+}: Cobalt ion

Cu^{2+}: Cupper ion

Fe^{2+}: Ferrous ion

Fe^{3+}: Ferric ion

IEC: Ion Exchange Chromatography

MA: MacConkey Agar

MCPM: Modified Czapeck Peptone Medium

Mg^{2+}: Magnesium ion

Mn^{2+}: Manganese ion

M.W.: Molecular Weight

o- NP: Ortho Nitrophenyl

OMW: Olive Mill Wastewater

p- NP: Para Nitrophenyl

PAGE: Polyacrylamide Gel Electrophoresis

SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SM: Sierra Medium

Zn^{2+}: Zinc ion

INTRODUCTION

Olive mill waste water (OMW) generated by the olive oil extraction process is the main liquid waste product of this industry. Approximately 1.8×10^6 tons of olive oil is produced annually worldwide with the majority (98%) of it being produced in the Mediterranean basin. It is reported that OMW resulting from the production process surpasses 30 million m^3 per year in the Mediterranean region (Sabbah et al., 2004).

OMW management has been a major issue of environmental concern for all olive oil producing countries. OMW can be a serious nuisance when disposed of untreated, due to its significantly high organic load, its phytotoxic properties, its relatively low biodegradability, its volatile solid, and its high concentration of phenolic compounds resistant to biological degradation (Komilis et al., 2005). On the other hand, Flouri et al., (1988) showed that OMW might have beneficial effects when applied to some plants at certain loading rates. OMW biologically derived compost was successfully used as a fertilizer during the cultivation of olive trees, grapes and potatoes (Balis et al., 1995). Bonari et al., (1993) performed irrigation experiments using OMW and concluded that the application of OMW to the soil 60 days after seeding, had no detrimental effects on the newly grown seeds as long as the OMW annual dosage was kept between 4 and 8 Tons per 1000 m^2.

The study conducted by Sabbah et al. (2004) showed that the seasonal production and high organic load of OMW makes the biological treatment a very reasonable treatment option for this type of aqueous waste. OMW has a substantial amount of olive oil divided into 4 parts: triolein (1600 mg/l),

diolein (400 mg/l), monoolein (200 mg/l) and oleic acid (50 mg/l) and other lipids (2100 mg/l). These "wasted" oils and lipids derivatives can be converted into diverse useful products for humans and animals alike. Industrially relevant enzymes, proteins and drugs are few examples of such products. The most significant enzyme group called "lipases" is the main target of this research. OMW can be utilized as a potential crude substrate for these needed lipolytically active proteins (lipases). These organically rich oils are known to be biodegraded by many thermophilic microorganisms possessing high and thermostable lipolytic activity (Markossian et al., 2000), which could be used as tools for the industry to recycle lipid-rich wastes. Enzymes such as lipases from thermophilic microorganisms have an advantage over enzymes derived from mesophiles as these are very stable at high temperatures. Additionally, enzyme resistance to denaturation in organic solvents was previously correlated with its thermostability in water and accordingly thermostable enzymes could be used both in aqueous and organic media (Kademi et al., 2000)

Bacillus thermoleovorans IHI-91 (Becker et al., 1997), *Bacillus thermoleovorans* ID-1 (Lee et al., 1999), *Bacillus thermocatenulatus* (Dannert et al., 1994), *Bacillus circulans* (Kademi et al., 2000) and *Thermoactinomyces vulgaris* (Elwan et al., 1978) are all lipase producing thermophilic bacteria; also, *Achromobacter* sp., *Alcaligenes* sp., *Arthrobacter* sp., *Pseudomonas* sp., *Staphylococcus* sp., and *Chromoacterium* sp. have been exploited for the production of lipases (Saxena et al., 2003).

Lipases are widely distributed in nature and have been found in many species of animals, plants, bacteria, yeast and fungi. Inspite of their wide distribution, the enzymes from microorganisms are most interesting because of their potential application in various industries ranging from the use in laundry

2

detergent to stereospecific biocatalysts (Maliszewska and Przemyslaw, 1992). Recently, there has been an interest in the basic properties and industrial applications of thermostable lipases. Most of these exhibit higher thermodynamic stability at elevated temperatures and in organic solvents, as a consequence of adaptation of the corresponding microorganisms to higher growth temperatures (Dannert et al., 1994; Dannert et al., 1996; Dannert et al., 1997; Iizumi et al., 1990; Omar et al., 1987; Sugihara et al., 1991; Sugihara et al., 1992).

Al Khudary et al., (2004) isolated a thermophilic Actinomycete (designated *Thermoactinomyces* sp. HRK-1) that demonstrated a highly thermostable lipase activity. This lipase was characterized based on studies done only on crude extracts. This Actinomycete was lost, and accordingly the aim of the aim of this work was to reisolate this Actinomyces same soil sample and characterize its lipase activity but using partially purified fractions.

Objectives:

1. Study the thermostability of the crude extract and partially purified fractions
2. Characterize the partially purified enzyme using different substrates
3. Determine the effect of metal ions on enzyme activity and stability
4. Assess the shelf life properties of the partially purified enzyme
5. Determine the molecular weight of this lipase and compare it with lipases from different organisms.

LITERATURE REVIEW

2.1 Characterization of the *Thermoactinomyces* sp.

The *Thermoactinomyces* sp. HRK-1 (Al Khudary et al., 2000) from Al Koura's soil-North Lebanon was a spore forming, gram positive, obligately thermophilic bacterium (a minimum growth temperature at 50°C) producing a highly thermostable lipase. Morphological studies indicated that the microorganism formed spores at the filamentous stage, while short rods were seen at the beginning of growth on a Sierra plate, which is considered as a characteristic feature of the *Thermoactinomyces* sp. (Brock et al., 1994).

2.2 Definition of the lipase

What exactly is a lipase? At present, there is no satisfying answer to this question.

Generally, bacterial lipases are glycoproteins but some extracellular bacterial lipases are lipoproteins. The production of extracellular lipases from bacteria is often dependant on nitrogen and carbon sources, inorganic salts, presence of lipids, temperature and availability of oxygen (Ghosh et al., 1996).

Lipases are widely distributed in animals, plants and microorganisms. These enzymes exhibit a great potential for commercial applications: they catalyze a variety of biotechnologically relevant reactions, e.g., production of free fatty acids, interesterification of oils and fats and synthesis of esters and peptides, and are available with a wide range of properties depending on their sources (Dannert et al., 1994). With respect to industrial applications, thermostable lipases are more promising, where thermostable proteins have several advantages. There is a considerable commercial pressure to develop

4

thermostable forms of biocatalysts in modern biotechnology (Dannert et al., 1994).

Fungal lipases were studied mainly due to their low cost of extraction, thermal and pH stability, substrate specificity and enzymatic activity in organic solvents (Saxena et al., 2003). The chief producers of commercial lipases are *Aspargillus niger, Candida cylindracea, Humicola lanuginose, Mucor miehei, Ryzopus arrizhus, R. delemar, R. japonicus, R, niveus and R oryzae* (Saxena et al., 2003).

2.3   Substrate specificity for lipases

Production of a thermostable lipase from thermophilic *Bacillus* sp. strain in the presence of tripalmitin at 70°C was described by Janssen et al. (1994). Media with tripalmitin, tristearin, and trimyristin as carbon sources were tested, and tripalmitin was found to be the best inducer of lipase activity. Dannert et al. (1994) working on a lipase from *Bacillus thermocatenulatus* showed that *p*-nitrophenyl palmitate and olive oil were the best substrates used as they induced maximum activity. However, Wang et al. (1995) showed that corn oil and olive oil were the best substrates and that the lipase produced by the *Bacillus* strain was active on triglycerides and on natural fats and oils. Similarly, Becker et al. (1997) found that olive oil was the best inducer to lipases from a thermophilic *Bacillus* strain. Imamura et al. (2000) working on a monoglycerol lipase from a moderately thermophilic *Bacillus* sp. H-257, showed that the enzyme hydrolyses monoacylglycerols with the highest activity occurring with monolauroylglycerol. The thermophilic *Bacillus* sp. J33 showed a high activity with *p*-nitrophenyl laurate as a substrate (Nawani et al., 2000). Lee et al. (2001) working on two lipases from (BTID-A & BTID-B) of the thermophilic bacterium *Bacillus thermolevorans* ID-1, indicated that the differences seen in substrate and positional specificities between the enzymes

suggested that they probably act synergistically to hydrolyze extracellular lipids. Sinchaikul et al. (2001) showed that the thermostable lipase from *Bacillus stearothermophilus* P1 showed that the lipase hydrolyzed synthetic substrates with optimal activity when using *p*-NP caprate as a substrate. On the other hand, Lin et al. (1996) studied the effect of adding Triton X-100 in medium containing olive oil. Results indicated that the production of the alkaline lipase by *Pseudomonas pseudoacaligenes* F-111 was increased by 50-fold compared to olive oil alone.

2.4   Thermostability of lipases from thermophilic microorganism

The lipase from *Bacillus thermocatenulatus* (Dannert et al., 1994) showed an optimum activity between 60 and 70°C, with olive oil and *p*-nitrophenyl palmitate as substrates. However, incubating the enzyme at above 40°C caused a decrease in stability after 30 minutes in phosphate buffer. Imamura et al. (2000) found that the lipase from *Bacillus* sp. H-257 was stable at 60°C when incubated for 10 min while the thermostable lipase from *Bacillus stearothermophilus* with an optimum activity at 68°C showed a quick decrease in activity at higher and lower temperatures (Kim et al., 2000). The thermostable lipase from *Bacillus stearothermophilus* P1 was most active between 45-65°C, with a maximal activity at 55°C. The enzyme was stable for 1 hour at 30-65°C. At 55°C, however, the enzyme was stable for more than 6 hours and had a half life of about 7.6 hours (Sinchaikul et al., 2001). The lipase from *Thermoactinomyces* sp. (HRK-1) had an optimal activity at 60°C and was stable at 100°C for 1 hour (Al Khudary 2002), while the thermostable lipase from *Aspergillus niger* had an optimal activity between 35-55°C. The enzyme was stable for 60 minutes with 50% of the original activity being detected at 60°C (Namboodiri and Chattopathyaya, 2000). Finally, the lipases I and II from *Pseudomonas aeruginosa* that were investigated for a period of 1

hour at temperature range from 40-70°C had the residual activity decreased as the exposure time and temperature increased (Saeed et al., 2005)

2.5   Effect of metal ions on lipase activity

The activity of the thermostable lipase produced by *Aspergillus niger* didn't change upon the addition of 1.0 M of Mg^{2+}, Ca^{2+}, Mn^{2+}, and Zn^{2+} while it was strongly inhibited when 50 mM Fe^{2+}, Fe^{3+} and Cu^{2+} were added (Namboodiri and Chattopathyaya, 2000). On the other hand, the study done by Al Khudary (2002) on lipase from crude extract of *Thermoactinomyces* sp. revealed an increase in the activity with 1mM of Fe^{2+} (6%), Fe^{3+} (25%) and Ca^{2+} (24%), and a decrease when Mg^{2+} was added (9.48%). Kim et al. (2000) working on thermostable lipase from *Bacillus stearothermophilus* reported that the enzyme was stable up to 65°C in the presence of calcium, while the thermally stable lipase from *Bacillus stearothermophilus* P1 was slightly inhibited upon the addition of $CaCl_2$ at different concentrations (Sinchaikul et al., 2001). Another study done by Saeed et al. (2005) showed that lipase I from *Pseudomonas aeruginosa* retained 47.74% of its original activity after 60 minutes of exposure to 70°C in the presence of Ca^{2+}, while lipase II retained about 36.7% of its original activity

2.6   Enzyme purification and molecular weight determination

Lipases from different microorganisms were purified using column chromatography and their molecular weight was determined using SDS-PAGE. Some studies used either a 2-step procedure for the enzyme's purification (Dannert et al., 1996; Rua et al., 1997; Kim et al., 2000), or a 1-step procedure (Nawani et al., 2000; Sinchaikul et al., 2001; Saeed et al., 2005) or a 5-step procedure (Imamura et al., 2000). The ion exchange chromatography combined to hydrophobic interaction chromatography was

used to purify the lipases from the following bacteria: *Bacillus thermocatenulatus, Bacillus stearothermophilus* (Dannert et al., 1996; Imamura et al., 2000; Kim et al., 2000), whereas ion exchange chromatography was only used for lipases from: *Bacillus thermocatenulatus* (Dannert et al., 1994), *Bacillus stearothermophilus* (Sinchaikul et al., 2001) and *Pseudomonas aeruginosa* (Saeed et al., 2005). On the other hand, hydrophobic interaction chromatography was the method of choice for the lipase from *Bacillus* sp. J 33 (Nawani et al., 2000), and gel chromatography for the lipase from *Lactobacillus plantarum* (Lopes et al., 2002). Rua et al. (1997) used gel filtration chromatography for the lipase from *Bacillus thermocatenulatus.*

The molecular weight of the lipases varied between 15.5 and 55 KDa (Dannert et al., 1996; Rua et al., 1997; Imamura et al., 2000; Kim et al., 2000; Nawani et al., 2000; Sinchaikul et al., 2001; Lopes et al., 2002; Saeed et al., 2005), whereas only *Thermoactinomyces* sp. studied by Al Khudary (2002) had a high molecular weight (80 KDa)

Chapter 3

MATERIALS AND METHODS

3.1   Screening, isolation and purification of lipase producers

The actinomycete that was lost in the lab was reisolated from soil. Soil sample was taken from Koura (North Lebanon), the same soil utilized by Al Khudary (2002). Lipase producing bacteria were isolated on agar plates supplemented with either 1% Tween [TM] 80, or 3% olive oil and a final pH of either 5.0 or 7.0. Enrichment for the soil samples was performed in 1 liter of MacConckey agar (MA) prepared according to Deutche Sammelong von Mikroorganismen (DSM catalog # 141). The cultures were incubated at 60°C, pH of 5.0 or 7.0 and a shaking speed of 200 rpm. After enrichment, 0.1 ml diluted innocula taken from the 1 L preparation were spread on MA plates and incubated at 60°C for 18 hours under humid atmosphere. The plates were sealed with plastic bags to minimize dryness of the medium (Becker et al., 1997). To study the morphological characteristics of the isolates, gram staining was performed to confirm the identity of the isolates (Elwan et al., 1978). Then, thermophilic Actinomycetes were screened for lipase on Sierra Medium Plates (SM) (Atlas, 1993) and Rhodamine B (Sigma Aldrich) agar plates (RBA) (Kouker et al., 1987). Positive colonies were further purified by using the 13-streak method on Rhodamine B plates. Pure isolates were stored on nutrient agar slants at 4°C.

3.2   Lipase production system

The Modified Czapeck peptone medium (MCPM) (Deutche Sammlung von Mikroorganismen und Zellkulturen: DSMZ list of Media) was used for

cultivation (Sigma Aldrich). The medium was modified by excluding sucrose and adding 0.2% of yeast extract and 0.5 % of peptone. Olive oil at a final concentration of 1% was used as the major growth substrate and agar (2% w/v) was added for solidification (Sigma Aldrich). The pH was adjusted to 7.3 and the medium was autoclaved for 15 min at 121°C. Isolates growing on Rhodamine B plates were streaked on Czapeck agar plates and incubated at 60°C

Batch cultures cultivation

Flasks containing 500 ml of MCPM and 1% olive oil were inoculated and incubated at 60°C in an orbital shaker at 250 rpm. Crude enzyme was obtained from the supernatant after centrifugation at 5,000 rpm for 30 min.

3.3 Standard lipase assay

A spectrophotometric assay with p-nitrophenyl laurate (Sigma Aldrich) as the substrate was performed to determine lipase activity. Lipase activity was measured after centrifugation (only extracellular lipase activity is determined). 25 μl of the sample was dissolved in 725 μl phosphate buffer (50 mM, pH 7.2). The mixture was incubated at 65°C in a shaking water bath after the addition of 100 μl substrate solution (25 mM of p-nitrophenyl laurate in absolute ethanol). Following the incubation, 250 μl of 100 mM $Na_2 CO_3$ was added to stop the reaction and the mixture was centrifuged at 4 °C for 10 min at 13,000 rpm (Becker et al., 1997). The absorbance of liberated p-nitrophenol was measured at 420 nm. One unit (U) of lipase activity was defined as the amount of enzyme that caused the release of 1 μmol p-nitrophenol/min under test conditions. The molar absorption coefficient of p-nitrophenol at 420 nm was 13.5l/mol/cm (Becker et al., 1997).

3.4 Protein concentration determination

The concentration of the lipase was determined according to Lowry assay (Lowry, 1951), in order to determine the specific activity of the lipase.

Relative activity (U/ml)= $\dfrac{\text{Absorbance x 1000}}{13.5 \text{ x } 10}$

Specific activity (U/mg)= $\dfrac{\text{Relative activity (U/ml)}}{\text{Protein concentration (mg/ml)}}$

3.5 Lipase activity assay with different substrates

3.5.1 Organic acids:

Substrate specificity towards different p-nitrophenyl esters (p-NPE) was analysed spectrophotometrically. The substrates p-NP acetate, p-NP propionate, p-NP butyrate, and p-NP caproate were dissolved in acetonitrile to a final concentration of 10 mM. One milliliter of acetonitrile containing p-NPE was mixed with 4 ml of ethanol and 95 ml of 50 mM Tris-HCl buffer, pH 8.0. An appropriate amount of the lipase was added to 1 ml of the freshly prepared solution. After 3 min of incubation at 60°C, the optical density was measured at 405 nm (Kim et al., 1998).

3.5.2 Fatty acids:

Alternatively, with p-NP myristate, p-NP palmitate and p-NP stearate, *ortho*-NP stearate, p-NP laurate and o-NP laurate 20 μl of lipase solution was added to 880 μl reaction buffers containing 50 mM Tris-HCl (pH 8.0), 0.1% Arabic gum, and 0.2% deoxycholate. After 3 min of incubation at 60°C, the reaction was started by adding 100 μl of 8 mM substrate solubilized in isopropanol.

11

The reaction was stopped by the addition of 0.5 ml of 3 M HCl. Following centrifugation, 333 µl of the supernatant was mixed with 1 ml of 2 M NaOH and the OD_{420} was determined (Kim et al., 1998).

3.5.3 Triglycerides:

The lipase activity using different triacylglycerols was determined spectrophotometrically relying on copper soap formation for the detection of free fatty acids. The substrate solution consisted of triglycerides (10 mM), which was emulsified in distilled water and gum arabic (0.2 mM) using a homogenizer at a maximum speed for 2 min. Copper(II)-acetate-1-hydrate aqueous solution (Sigma Aldrich) (90 mM) adjusted to pH 6.1 with pyridine (Sigma Chemical Co.), was used as copper reagent. The chromogenic reagent contained diethyldithio carbamic acid (Sigma Aldrich) (5.8 mM) dissolved in absolute ethanol. The reaction was strarted by addition of 0.1 ml substrate solution to 0.9 ml of enzyme solution in 50 mM potassium phosphate buffer (pH 7.5). The mixture was incubated for 20 min at 60°C. Immediately after incubation, 0.45 ml of the reaction mixture was transferred to a test tube containing 0.25 ml of 3 M HCl. Fatty acid were subsequently extracted by the addition of 3 ml n-hexane and centrifugation at 5000 rpm for 2 min. The organic phase (2.5 ml) was transferred to a fresh test tube filled with 0.5 ml copper reagent. The mixture was vortexed for 1.5 min and phase separation was achieved by centrifugation. Then, the organic phase (2 ml) was mixed with 0.4 ml of the chromogenic reagent and the absorption was measured at 430 nm (Lee et al., 1999).

3.6 Enzyme purification

3.6.1 Ammonium sulphate precipitation:

Three different fractions were obtained according to the following procedure:

Fraction 1: 25%

Fraction 2: 50% (25-50%)

Fraction 3: 75% (50-75%)

The beaker containing the protein fraction was placed on top of a magnetic stirrer within another beaker containing water-ice slurry. While agitating gently on the magnetic stirrer, the appropriate amount of ammonium sulphate was slowly added for each fraction. The adding of ammonium sulphate was completed in 5-10 min, but stirring continued for 10-30 minutes after that salt was added. The solution was spinned at 10,000 xg for 10 minutes or at 3,000 xg for 30 min. Then, the supernatant was decanted and the precipitate was resuspended in 1-2 pellet volumes of buffer.

3.6.2 Desalting with Sephadex G-25 Gel filtration chromatography

The protein pellet obtained after saturation with ammonium sulphate was dissolved in   50 mM Tris-HCl buffer, and loaded onto a column of Sephadex G-25 (Sigma Aldrich) equilibrated with Tris-HCl, pH 7.2 (Adinarayana et al, 2003). Fractions were collected and used for all further studies including ion exchange chromatography (IEC), molecular mass determination, enzyme activity and thermostability.

3.6.3 DEAE cellulose Ion Exchange chromatography:

The lipase rich fractions collected after gel chromatography were pooled and applied to a column of DEAE cellulose (Sigma Aldrich), which was pre-equilibrated with 10 mM of sodium phosphate buffer at pH 7.2. The column

was washed thoroughly with the same buffer to remove the unabsorbed material, and was also eluted in the same buffer using a linear gradient of NaCl (0.01 to 1 M). The eluate was collected in 5 ml fractions (Abdou, 2003).

3.7   Characterization of the lipase

3.7.1   Molecular mass determination

Native and denaturing Polyacrylamide Gel Electrophoresis (PAGE) were carried out as shown in appendix II. For non-denaturing PAGE and SDS-PAGE, a 5 to 20% gel was prepared. All gels were run at 120 mA for 240 min proteins were stained using the Coomassie blue staining procedure.

After bands were detected on native gel, they were cut and homogenized with Tris-HCl buffer and were loaded on denaturing gel after the addition of sample buffer.

The molecular mass of the lipase was calibrated using the wide-range-molecular-mass calibration kit (Amresco, Solon, Ohio) containing myosin (212 KDa), β galactosidase (MW= 116 kDa), phosphorylase B (MW= 97 kDa), serum albumin (MW= 66 kDa), egg albumin (MW= 45 kDa), carbonic anhydrase from bovine (MW= 31 kDa), trypsine inhibitor from soybean (MW= 21 kDa) and lysozyme from egg white (MW= 14 kDa).
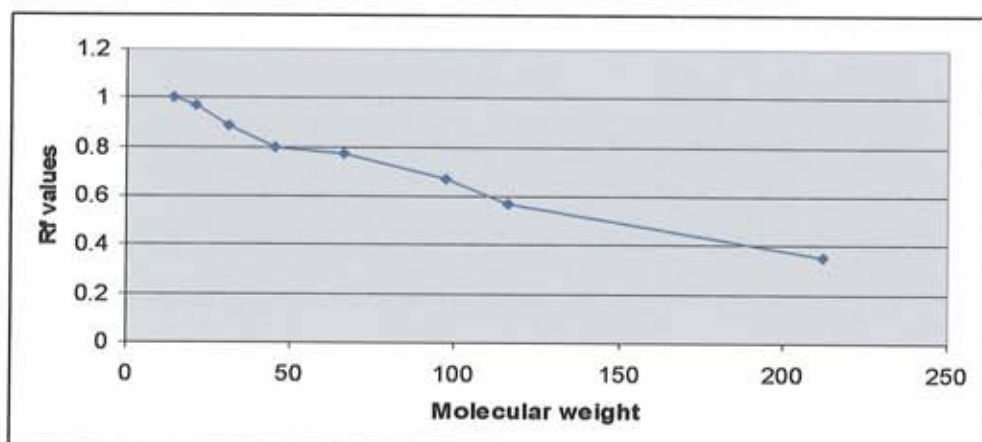

Figure 1: Rf values

3.7.2 Purified enzyme characterization

- Enzyme activity as a function of temperature was studied by incubating the protein at different temperatures (room temperature, 30, 40, 50, 60, 70, 80 and 90°C)

- Temperature stability was determined using either the crude enzyme, fraction collected from gel chromatography or fraction collected from ion exchange chromatography. For each extract, a set of 5 tubes was used each containing 1ml sample. The tubes were properly sealed and placed in boiling water. The first set of tubes from different fractions were removed after 1 minute, the second after 5 minutes, the third after 20 minutes, the fourth after 40 minutes and the last after 60 minutes. Following this step, all tubes were incubated at 60°C to determine lipase activity.

- The effect of metal ions was determined as follows: the p-nitrophenyl laurate test was carried out in 50 mM phosphate buffer after pre-incubating the enzyme samples for 30 minutes in phosphate buffer containing 1 mM of one of the following ions: Ca^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+} and Fe^{3+}.

- Shelf life:
Purified enzyme sample was stored at 4°C and at ambient temperature. Lipase activity was determined for 4 consecutive days using both samples.

RESULTS

Lipase assays described in the literature employ different substrates, with nitrophenyl being the most common. The two physiologically active forms are *para* and *ortho-* nitrophenyl laurates.

The lipase enzyme from *Thermoactinomyces* sp. was tested with *para* and *ortho* nitrophenyl laurate in order to choose the substrate with the highest affinity. With the chosen substrate, optimal temperature for the lipase activity and the enzyme thermostability were determined using each of the purified fractions (crude extract, gel filtration chromatography, and ion exchange chromatography).

Other substrates were also tested and the effect of metal ions on the enzyme activity was studied using the purified fraction of the enzyme.

The shelf life of the enzyme at room temperature and after storage at around 4°C and the molecular mass of the enzyme were also determined.

4.1   Screening and isolation of lipase producers

Bacteria growing on Mac Conckey Agar (MA) medium were Gram positive. The growth on Sierra medium (SM) showed a white precipitate (Figure 2) and the growth on Rhodamine B agar plate showed fluorescence at a wavelength of 350 nm (Figure 3).
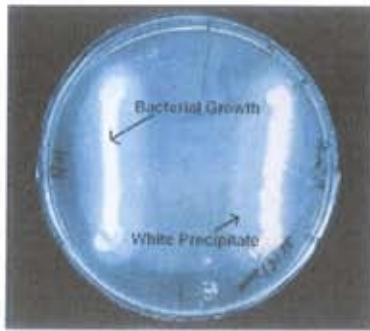
Figure 2: Growth on SM medium
showing white precipitate
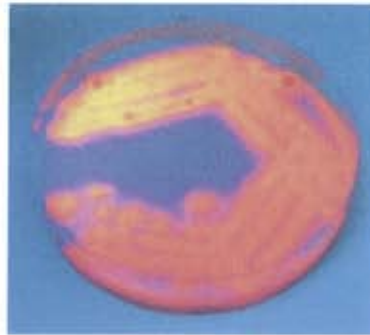
Figure 3: Growth on Rhodamin B
showing fluorescence

4.2   Affinity studies using nitrophenyl laurates

The lipase was tested with *ortho* and *para-* nitrophenyl laurate on crude and desalted extracts.

4.2.1   Test on crude extract:

Figure 4 shows the lipase affinity using *para* and *ortho-* nitrophenyl laurate as substrates at different temperatures.

The same pattern of activity was observed with both substrates. The optimum activity was at 60°C. However, the measured specific activity using *o*-nitrophenyl laurate was 50% lower compared to that with *p-* nitrophenyl laurate Thus, the lipase had a higher affinity for *p*-nitrophenyl laurate than for the *o*-nitrophenyl laurate.
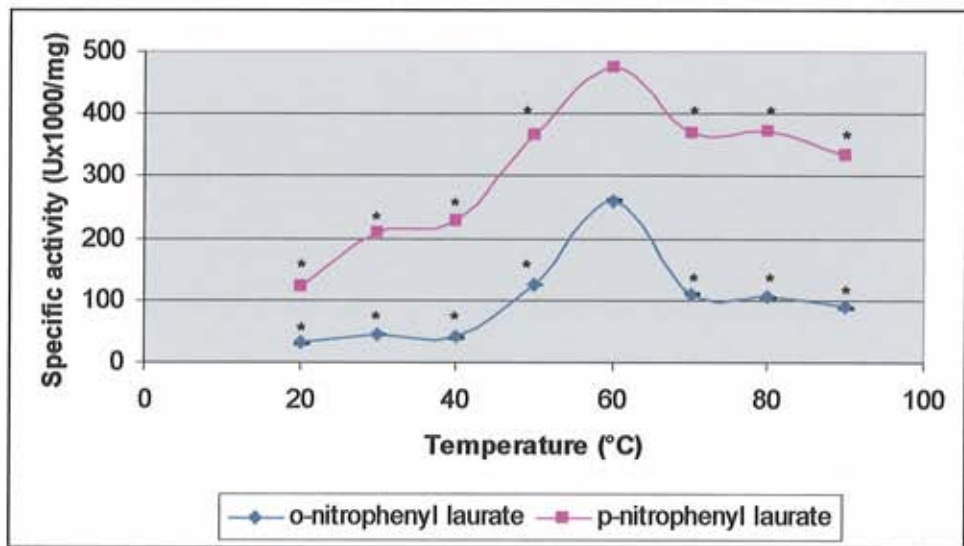
Figure 4: Enzyme activity tested with crude extract using *ortho* and *para* nitrophenyl laurate at different temperatures ranging from 20- 90°C.
*: Significance for p<0.05 with respect to optimum temperature. The ± SEM values didn't show because they were very small.
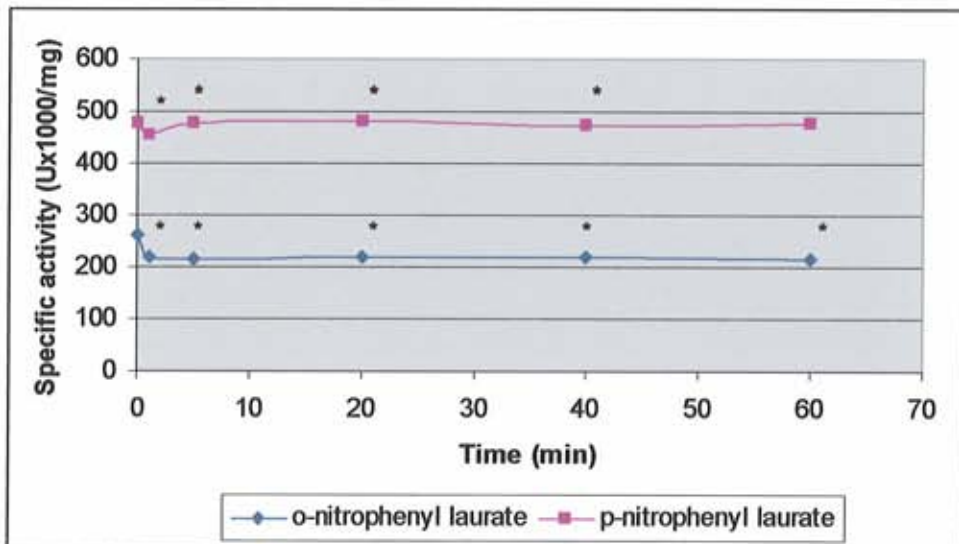
Figure 5: Enzyme stability measured under optimal temperature and upon boiling for one hour using *ortho* and *para*- nitophenyl laurate. *: Significance for p<0.05 with respect to optimum temperature. The ± SEM values didn't show because they were very small.

18

4.2.2 Crude extract thermostability

Figure 5 shows enzyme activity at boiling temperature for 1 hour using the *para* and *ortho*- nitrophenyl laurate.

The enzyme was boiled and samples were collected after 1, 5, 20, 40 and 60 min. The specific activity was determined for each time interval. The enzyme retained its activity even after boiling for 60 minutes. A higher specific activity was detected again using the *p*-nitrophenyl laurate.

4.2.3 Ammonium sulphate precipitation

Three ammonium sulphate fractions were prepared: Fraction 1 (25%), Fraction 2 (50%) and Fraction 3 (75%).

The enzymatic activity of each fraction was determined using *o*- nitrophenyl laurate as a substrate (Table 1).

Table1: Enzymatic activity in the different fractions

	0-25%	25-50%	50-75%
Relative activity (U/ml)	0.185	0.733	0.837
Specific activity ($Ux10^3$/mg)	7.34	24.35	37.7

The highest activity was detected in the 50%- 75% ammonium sulfate fraction, with a quite high activity being detected in the 25%- 50% fraction.

19

4.2.4 Optimum temperature for enzyme activity

Optimum temperature for activity was determined using the 50-75% ammonium sulphate fraction. Figure 6 shows the activity using the *para* and *ortho*- nitrophenyl laurate upon incubation at different temperatures.
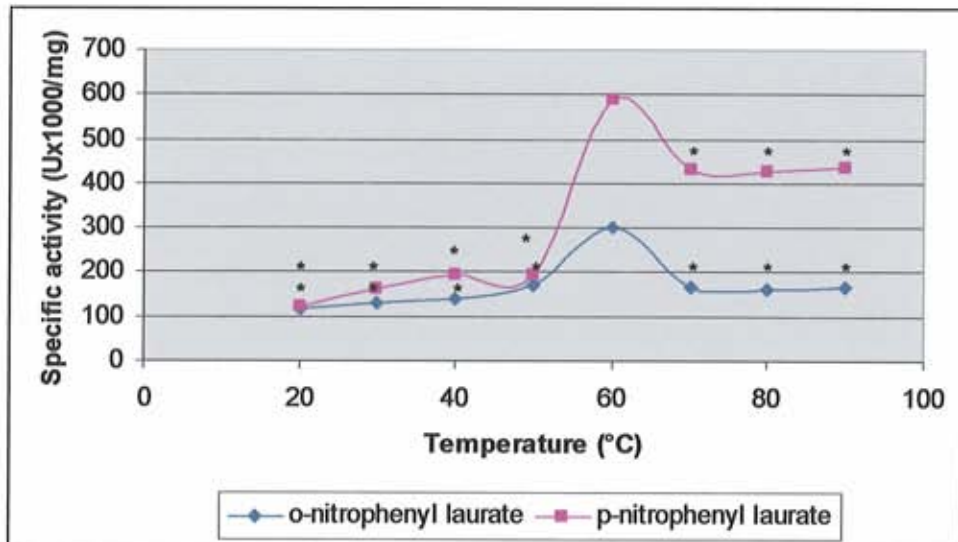

Figure 6: Enzyme activity tested with desalted ammonium sulphate 50- 75% fraction using *ortho* and *para* nitrophenyl laurate at different temperatures ranging from room temperature to 90°C. *: Significance for $p < 0.05$ with respect to optimum temperature. The ± SEM values didn't show because they were very small.
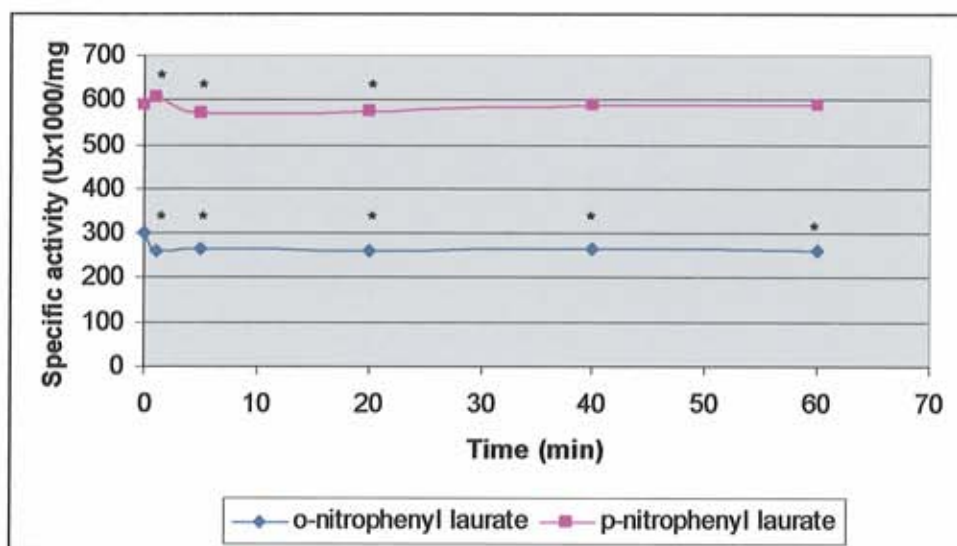
Figure 7: Enzyme stability at optimum temperature and upon boiling for one hour tested with desalted ammonium sulphate 50-75% fraction using using *ortho* and *para*-nitrophenyl laurate. *: Significance for $p < 0.05$ with respect to optimum temperature. The ± SEM values didn't show because they were very small.

4.2.5   Enzyme stability

Heat stability of the lipase was determined using 50-75% ammonium sulphate fraction (Figure 7). The activity was determined using both *para* and *ortho*-nitrophenyl laurate. The enzyme retained activity even after boiling for one hour. The specific activity of the enzyme measured using the desalted fraction was almost 20% higher than that of the crude extract.

4.3   Specific activity of the lipase in the different fractions

The optimal temperature was determined using the crude extract, 50- 75% ammonium sulphate desalted fraction and the fraction from ion exchange chromatography using *p*-nitrophenyl laurate as a substrate (Figure 8).

The optimum temperature was 60°C in all the fractions tested with the highest activity being detected in the fraction purified by ion exchange chromatography.

21

Purification fold of each fraction

Fraction	Specific activity $(U \times 10^3/mg)$	Total protein(mg)	U	% yield	Purification fold
Crude extract	475	896	435.6	100	1
50-75% ammonium sulphate (desalted)	587	33.6	19.72	4.63	1.23
Ion exchange chromatography	1520	3.73	5.67	1.33	3.2

Figure 8: Comparison between fractions (crude extract, 50-75% ammonium sulphate desalted extract and purified extract from ion exchange chromatography) of enzyme activity at different temperatures ranging from 20 to 90°C using p-nitrophenyl laurate. *: Significance for $p < 0.05$. The ± SEM values didn't show because they were very small.
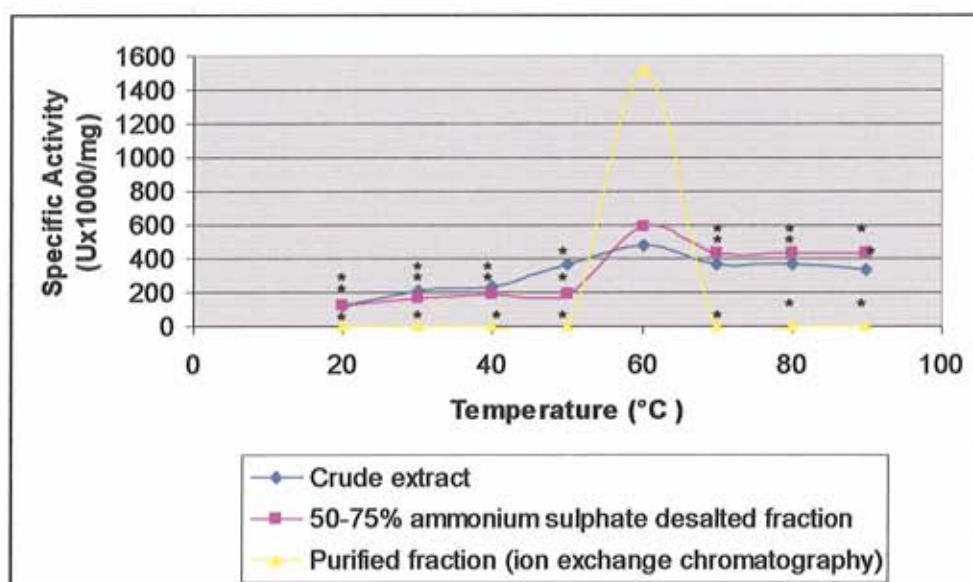
4.4 Lipase activity with different substrates

The enzyme activity was measured using the Sephadex G-25 fraction in the presence of different substrates (Figures 9, 10 and 11).

4.4.1 Organic acids

Figure 9 shows the enzyme activity when the following fatty acids were used as substrates: p-NP acetate, p-NP propionate, p-NP butyrate and p-NP caproate.

p-NP caproate and p-NP propionate had a higher activity to the lipase than the other organic acids and the absorbance was 0.566 and 0.374, respectively.

4.4.2 Fatty acids

Figure 10 shows the enzyme activity when the following fatty acids were used as substrates: p-NP myristate, p-NP palmitate, p-NP stearate, o-NP stearate, p-NP laurate and o-NP laurate.

Free fatty acids were released mainly from the reaction of lipase with p-NP palmitate (A= 1.57) and p-NP laurate (A= 1.5). The lipase didn't have high affinities to the other substrates, and accordingly, the measured absorbance was very low.

4.4.3 Triglycerides

Figure 11 shows the enzyme activity when the following triacylglycerols were used as substrates: tribehenin, triarachidin, tripalmitin, tricaproin, trierucin, tripetroselenin, trielaidin, triolein, 1,2 diolein, 1,3 diolein, tributyrin, 1-oleoyl-rac-glycerol and glyceryl tridecanoate.

Triacylglycerols were released after the reaction of the lipase with triarachidin (A= 0.247), 1-oleoyl-rac-glycerol (A= 0.501) and glyceryl trideaconate (A= 0.124). However, enzymatic reaction was not detected in the case of tribehenin, tripalmitin, trierucin, trielaidin and triolein.
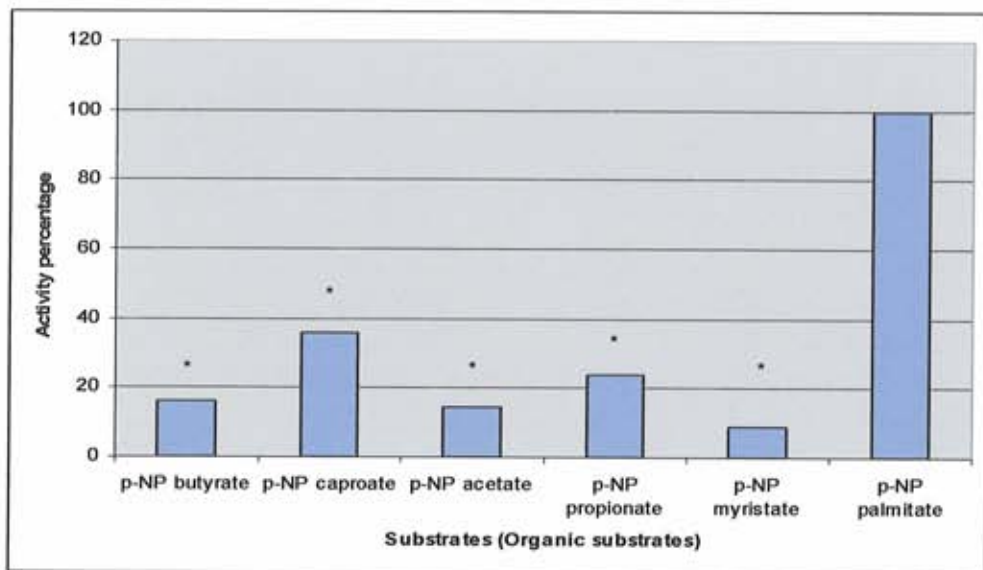
Figure 9: Enzymatic activity was measured after gel filtration chromatography using *p*-NP acetate, *p*-NP propionate, *p*-NP butyrate and *p*-NP caproate as organic substrates. *p*-NP palmitate was used as control. *: Significance for $p < 0.05$ with respect to optimum temperature. The $\pm$ SEM values didn't show because they were very small.
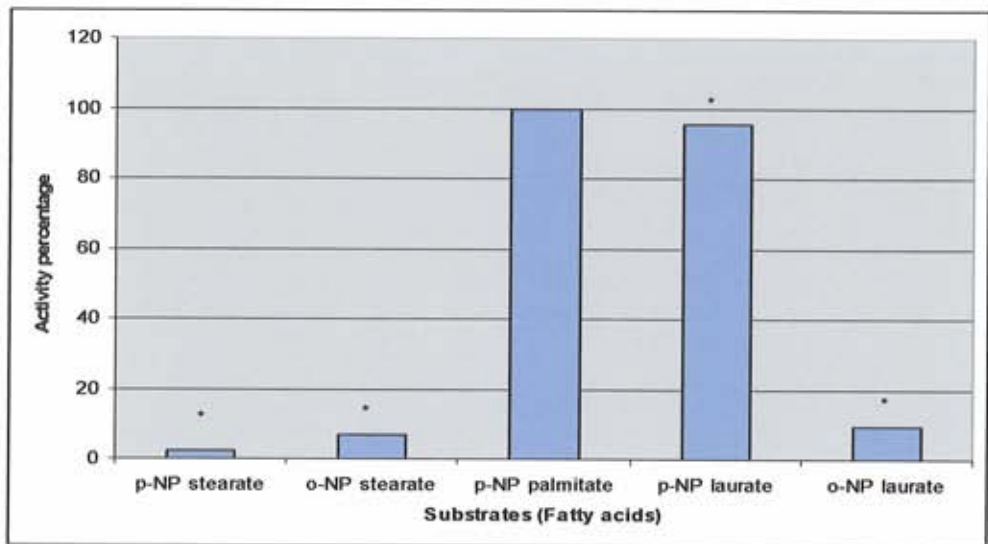
Figure 10: Enzymatic activity was measured after gel filtration chromatography using when the following fatty acids were used as substrates: *o*-NP laurate, *o*-NP stearate, *p*-NP laurate, *p*-NP myristate, *p*-NP palmitate and *p*-NP stearate. *p*-NP palmitate was used as control. *: Significance for p<0.05 with respect to optimum temperature. The ± SEM values didn't show because they were very small.
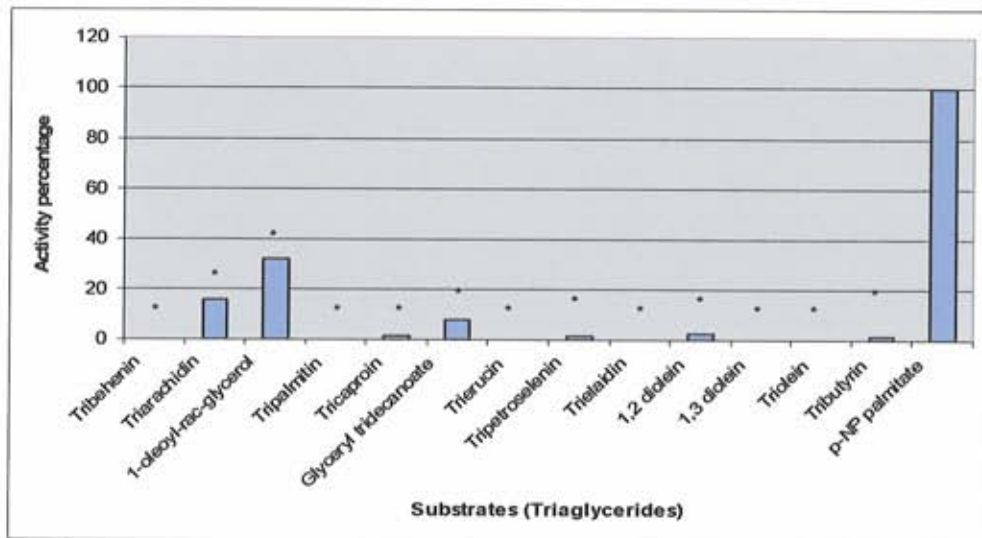
Figure 11: Enzymatic activity was measured after gel filtration chromatography when the following triacylglycerides were used as substrates: Tribehenin, Triarachidin, 1-oleoyl-rac-glycerol, Tripalmitin, Tricaproin, Glyceryl tridecanoate, Trierucin, Tripetroselenin, Trielaidin, 1,2 diolein, 1,3 diolein, Triolein and Tributyrin. *p*-NP palmitate was used as control. *: Significance for p<0.05 with respect to optimum temperature. The ± SEM values didn't show because they were very small.
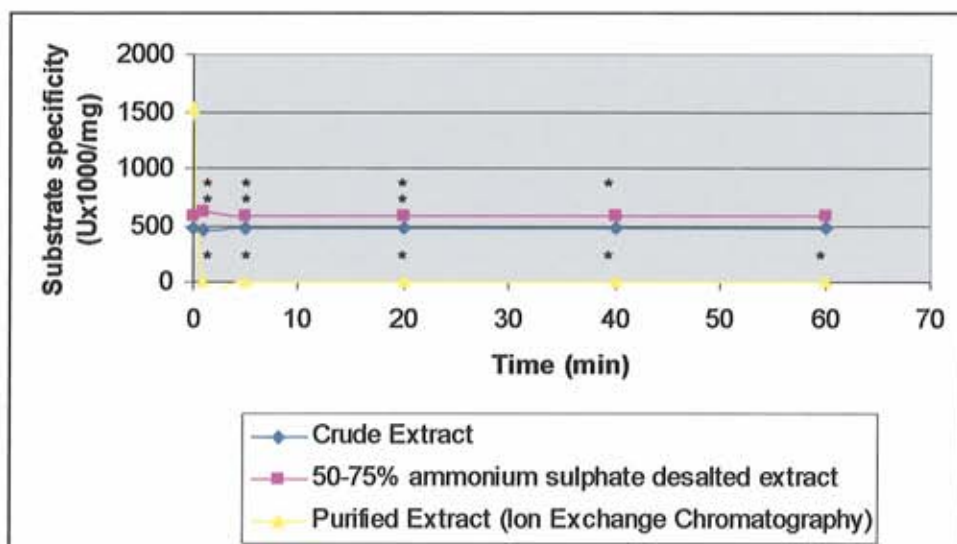
Figure 12: Thermostability of the lipase in the different fractions (Crude extract, 50-75% ammonium sulphate desalted extract, and purified extract from ion exchange chromatography) upon boiling for one hour using *p*-nitrophenyl laurate.
*: Significance for p<0.05 with respect to optimum temperature. The ± SEM values didn't show because they were very small.

4.5   Enzyme thermostability

The thermostability of the lipase from the different fractions was compared. Lipase activity in the crude extract and after gel filtration (Sephadex G-25) was thermostable even after one hour of boiling. In contrast, no activity was detected in the ion exchange fraction after one hour of boiling (Figure 12).

4.6   Effect of metal ions on activity

The effect of metal ions was studied using the standard *p*-nitropheny laurate assay after desalting. The results are summarized in Figure 13.

26
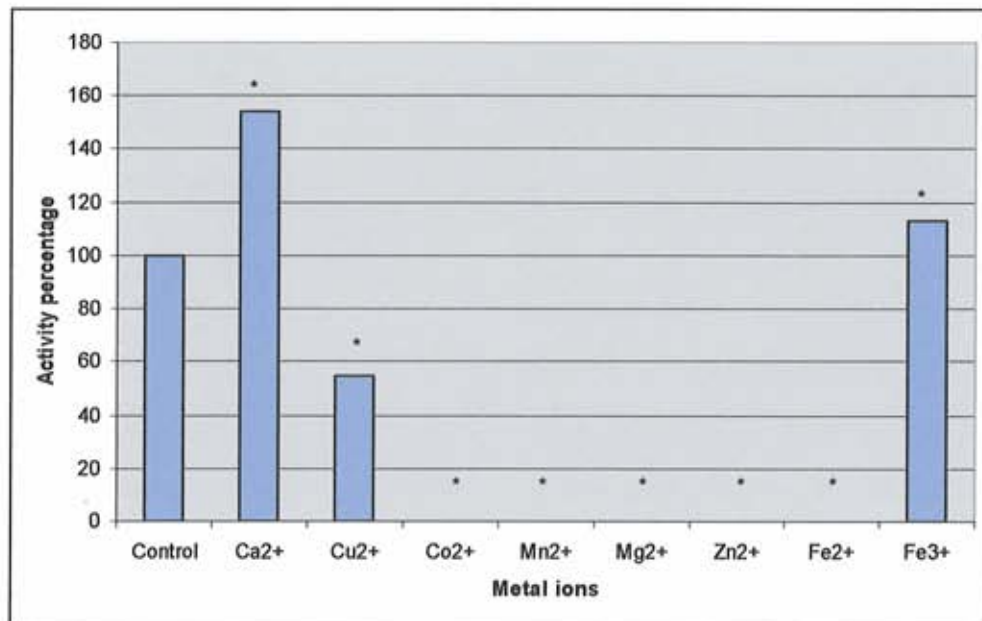
Figure 13: Effect of metal ions (Ca^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+} and Fe^{3+}) on lipase activity using p-nitrophenyl laurate as substrate after purification with ion exchange chromatography. *: Significance for $p<0.05$ with respect to optimum temperature. The $\pm$ SEM values didn't show because they were very small.

An increase in activity was detected when the lipase was incubated with Ca^{2+} (54%) and Fe^{3+} (13%).

Lipase activity was negatively affected in the presence of Cupper (54.5%) and the enzyme was totally inhibited in the presence of Co^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Fe^{2+}.

4.7   Shelf life at room temperature

The activity was studied for several days after ion exchange chromatography, at room temperature in the presence of Ca^{2+}, in order to determine lipase's shelf life (Figure 14).

After two days, the specific activity was maintained, while on the third day, there was a gradual decrease in the activity.

4.8   Determination of the enzyme's molecular weight

SDS-Polyacrilamide Gel Electrophoresis (SDS-PAGE) was performed in order to determine the lipase's molecular weight (Figure 15)

The gel loading was according to the following sequence:

Lane 1: Molecular weight markers

Lane 2: Crude exctract

Lane 3: Lipase concentrated with Ammonium Sulphate

Lane 4 & 10: Desalted enzyme (Sephadex G-25)

Lanes 5 &11: Purified lipase (Ion exchange chromatography)

Lanes 6, 7, 8, 9, 12, 13, 14 &15: Lipase from the non-denaturing gel electrophoresis
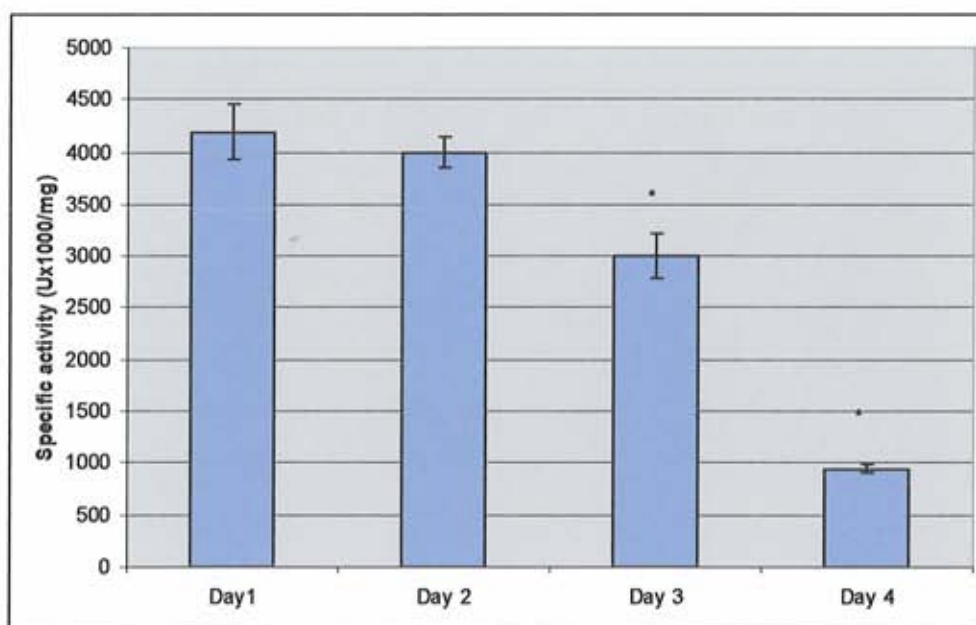
Figure 14: Shelf life of the purified thermoactinomycete at room temperature in the presence of Ca^{2+}. *: Significance for $p<0.05$ with repect to the first day of measurement. The ± SEM values didn't show because they were very small.
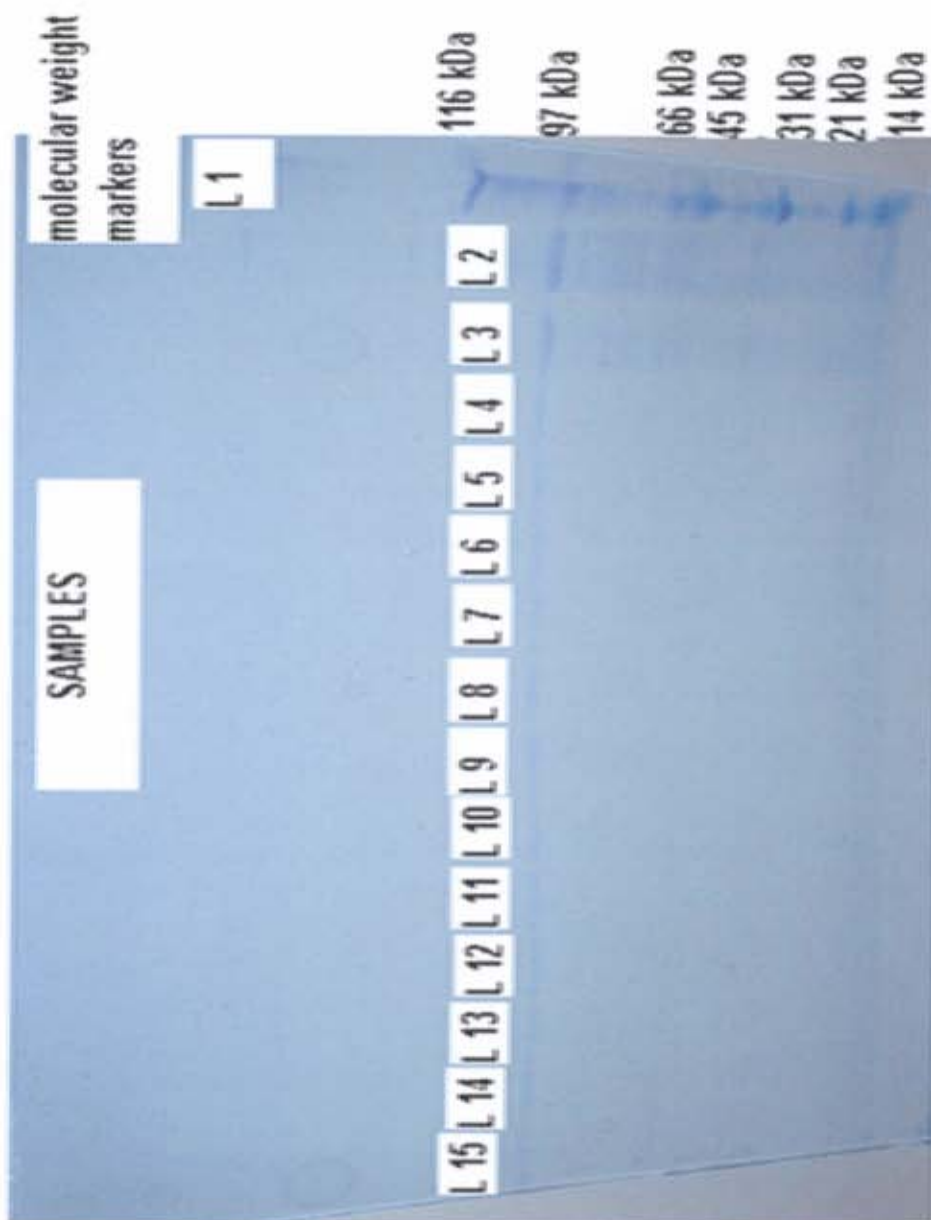
Figure 15: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis. Gel loading was according to the following sequence: Lane 1: Molecular weight markers, Lane 2: Crude exctract, Lane 3: Lipase concentrated with Ammonium Sulphate, Lane 4 & 10: Desalted enzyme (Sephadex G-25), Lanes 5 &11: Purified lipase (Ion exchange chromatography), Lanes 6, 7, 8, 9, 12, 13, 14 &15: Lipase from the non-denaturing gel electrophoresis

The bands representing the lipase (crude enzyme, salted and desalted, purified and from the non-denaturing gel) are all located at a distance equivalent to that of the phosphorylase B indicating that the molecular weight of the lipase was approximately 99 KDa.

Chapter 5

DISCUSSION

The lipase from *Thermoactinomycete* sp. that was reisolated was purified and characterized. Comparing the morphological characteristics of the *Thermoactinomycete* sp. reisolated in this study and the one isolated by Al Khudary (2002), we could observe that the morphological characteristics were the same, since in both studies, the bacteria could be grown on modified Czapeck medium, it was filamentous and rod shaped gram positive bacteria.

5.1   Thermal activity of the enzyme

The lipase was characterized in the crude extract, after desalting, and after purification using ion exchange chromatography. The enzyme activity was studied at different temperatures in the range of 20- 90°C. The results obtained working on the crude extract and on the 50-75% ammonium sluphate desalted fraction showed low enzymatic activity at temperatures ranging 20°C and 50°C (30%), and a high enzymatic activity at temperatures ranging between 70°C and 90°C (73%). A similar result was obtained by Kim et al. (1998) where lipase activity detected below optimal temperature was 20% of the optimal activity and beyond the optimal temperature, it was 60% of the optimal activity. However, Al Khudary (2002) reported that below 50°C the activity was close to the optimal activity, while beyond 60°C, it decreased exponentially. The observed differences might indicate that the reisolated microorganism was not the same as the one described by Al Khudary (2002). Also, lipases from other microorganisms were not similar to the lipase isolated in the current study in terms of enzymatic activity at different temperatures. The enzymatic activity of *Bacillus stearothermophilus* P1 (Sinchaikul et al.,

2001) decreased below (50%) and beyond (40%) the optimum temperature, and the lipase from *Bacillus thermocatenulatus* recovered 50% of optimal enzymatic activity (Rua et al., 1997) when it was exposed to the same conditions reported by Sinchaikul et al. (2001).

Furthermore, in our study and in ion exchange chromatography (IEC), there was no activity when the enzyme was incubated below and beyond 60°C. This probably indicates that the enzyme was either deactivated or denatured. A similar observation was reported with the monoacylglycerol lipase from *Bacillus* sp.   H-257 (Imamura et al., 2000) where the lipase was inactivated beyond 60°C after purification.

5.2   Thermostability:

The enzymatic activity was retained in the desalted extract even after boiling for 1, 5, 20, 40 and even after 60 min. However, this was contrary to the results obtained working with the IEC extract where no activity was detected upon boiling. The results obtained from the desalted fractions were similar to the ones reported by Al Khudary (2002). The activity in the supernatant was fully retained after 60 min of boiling. Results obtained after purification through IEC were not comparable to any other results since purified lipase didn't have any activity after boiling, since the highest temperature at which lipase from other microorganisms was stable, was 50°C (Sugihara et al., 1991; Rua et al., 1997; Lee et al., 2001).

5.3   Substrate specificity:

Lipase activity with different *p*-NP esters and triacyglycerols was determined using the optimum temperature. The lipase demonstrated wide substrate specificity with high activity being detected using the *p*-NP caproate, laurate and palmitate among *p*-NP esters. The enzyme was also active when 1-oleoyl-

rac- glycerol and triarachidin were used as substrates. No activity was detected with tribehenin, trierucin, trielaidin, tripalmitin and triolein, and a very low one with tripetroslenin, tricaproin and p-NP stearate. This study revealed that the lipase was active with short (C:2, C:3, C:4, C:6) and medium (C:12, C:16) carbon chains of p-NP esters, and long carbon chains of triglycerides (C:21, C:22). The lipase of the present study is different from other lipases isolated from *Bacillus* sp. in terms of substrate specificity. Lipase from *Bacillus stearothermophilus* (Dannert et al., 1996; Rua et al., 1997) and from *Bacillus* sp. J 33 (Nawani et al., 1999) had a high enzymatic activity when tributyrin, which is a short chain substrate (C:4), was used a substrate. Similarly, lipases from *Bacillus* sp. (Sugihara et al., 1990), *Bacillus thermoleovorans* ID-1 (Lee et al., 1999), *Bacillus thermoactenulatus* (Lee et al., 2001) and *Bacillus stearothermophilus* (Sinchaikul et al., 2001) reacted with tricaprylin, another short chain substrate (C:8). Lipases from *Bacillus stearothermophilus* (Sinchaikul et al., 2001) and *Bacillus stearothermophilus* L1 (Kim et al., 1998) reacted respectively with two relatively short chain substrates, p-NP caprate (C:10) and p-NP caprylate (C:8).

5.4   Lipase purification:

Lipase was 1.23-fold purified after gel chromatography (Sephadex G-25) and 3.2-fold purified after ion exchange chromatography using DEAE cellulose. The lipase was 3 times more pure compared to the supernatant, and therefore, could provide a higher specific activity. The purified lipase was loaded on a non-denaturing gel. After the gel was stained and destained, it showed the presence of only one band. The lipase from crude extract, desalted extract and the purified extract were also loaded on the denaturing gel. Six bands were detected with the sample from the supernatant and 4 from the desalted extract. Both purified extract and lipase extracted from non-denaturing gel showed

only one band. Saeed et al., (2005) purified a lipase from *Pseudomonas aeruginosa* using anion exchanger to 6.36 fold and it was judged pure by SDS-PAGE. Similarly, lipase from *Bacillus stearothermophilus* P1 (Sinchaikul et al., 2001) was purified to 18 fold using IEC and the pure enzyme was shown by SDS-PAGE as a single band. Kim et al., (2000) used the IEC twice to purify the lipase from *Bacillus stearothermophilus* and the lipase was also shown as a single band on SDS-PAGE.

5.5   Metal ions effect:

The effect of metal ions on lipase activity differed between one ion and another. In fact, Ca^{2+} and Fe^{3+} increased the activity of the lipase isolated from the *Thermoactinomycete* sp. while Cu^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2+} inhibited the enzyme. These results were similar to the results reported by Al Khudary (2002) indicating that these two lipases were similar with respect to the interaction of metal ions with the binding sites. The observation that lipase activity was enhanced in the presence of metal ions probably reflects the ability of these salts to react with free fatty acids adhering to the oil droplets to diminish interfacial charge effects and/or to increase droplet surface area (Saeed et al., 2005). The results also demonstrated that the lipase required divalent cations for its activity (Lopes et al., 2002). Lipases from *Pseudomonas glumae* (Noble et al., 1993), *Chromobacterium viscosum* (Lang et al., 1996) and *Pseudomonas cepacia* (Kim et al., 1997), have a calcium binding sites. The inhibitory effect of Zn^{2+} could be attributed to the direct interaction of the metal ion with the catalytic site (Saeed et al., 2005)

Ca^{2+} activated lipases from *Bacillus thermocatenulatus* (Dannert et al., 1996), *Bacillus thermoleovorans* ID-1 (Lee et al., 1999) and *Bacillus stearothermophilus* (Kim et al., 2000), while inhibiting the lipase from *Bacillus stearothermophilus* P1 (Sinchaikul et al., 2001), *Bacillus*

34

thermocatenulatus (Dannert et al., 1996), and from *Lactobacillus plantarum* (Silva Lopes et al., 2002). However, Ca^{2+} didn't have any effect on the activity of the lipases isolated from *Bacillus* sp. (Sugihara et a., 1991) and *Bacillus stearthermophilus* L1 (Kim et al., 1998). In harmony with our results, Cu^{2+} and Fe^{2+} inhibited the lipases from *Bacillus thermocatenulatus* (Dannert et al., 1996), *Bacillus thermoleovorans* ID-1 (Lee et al., 1999), *Bacillus stearothermophilus* (Kim et al.,2000), *Bacillus stearothermophilus* L1 (Kim et al., 1998) and *Bacillus* sp. (Sugihara et al., 1991). Moreover, Co^{2+} inhibited lipases from *Bacillus stearothermophilus* P1 (Sinchaikul et al., 2001), *Bacillus thermocatenulatus* (Dannert et al., 1996), *Bacillus* sp. (Sugihara et al., 1991) and *Bacillus stearthermophilus* L1 (Kim et al., 1998), while it activated the lipase from *Lactobacillus plantarum* (Silva Lopes et al., 2002).

5.6   Electrophoretic pattern:

Al Khudary (2002) showed that the enzyme was multimeric, while our results revealed the presence of only one band after purification and extraction from the non-denaturing gel. The consistency of the calculated molecular weight of the native and denatured lipase along with the single band detected, suggested that the enzyme existed as a monomer.

Thermoactinomycete sp. Strain studied by Al Khudary et al. (2000) showed a molecular mass of about 80 kDa, as compared to the 99 kDa according to the results of this study. This provides an additional support that we were working with a different strain. Similarly, lipase extracted from *Bacillus circulans* had a molecular mass of 94 kDa and showed only one band on SDS-PAGE, suggesting that the enzyme existed as a monomer (Kademi et al., 2000). The extracellular lipase however, isolated from *Lactobacillus plantarum* had a molecular weight of 97.4 kDa, and existed as a multimer (Lopes et al., 2002).

35

5.7   Shelf life study:

The lipase isolated in this study was not active after being stored for one day in the refrigerator. The purified enzyme from *Bacillus stearthermophilus* (Kim et al., 2000) was unstable upon storage at a temperature range of -20 °C and   - 70°C, whereas, the extracellular lipolytic activity of the *Lactobacillus plantarum* (Lopes et al., 2002) was active after 1 month of storage at -80°C and -4°C. Storing the enzyme at room temperature with Ca^{2+} helped in retaining the activity for 4 days after which a dramatic decrease was observed, indicating the instability of the enzyme and its short shelf life.

Chapter 6

SUMMARY AND CONCLUSIONS

In the present work, lipase from *Thermoactinomyces* sp. was purified and characterized. The lipase from supernatant, 50-75% ammonium sulphate desalted fraction and purified fraction showed a high affinity to *p*-nitrophenyl laurate.

Activity was detected at optimal temperature (60°C) and after boiling for 1 hour before purification, but it was detected only at 60°C after purification.

High activity was detected against the following substrates: *p*-NP palmitate, *p*-NP laurate, and 1-oleoyl-rac-glycerol.

The presence of Ca^{2+} and Fe^{2+} enhanced the lipase activity, while the presence of Co^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2+} inhibited the lipase activity.

The activity of the lipase was maintained in the presence of Ca^{2+} after ion exchange chromatography for 2 days, and on the third day, there was a drastic decrease.

Lipase showed one band on the SDS-PAGE with a molecular weight of approximately 99KDa.

Finally, since lipases are usually used in industrial applications and since the lipase from the present study was unstable, more work must be done in order to increase its stability.

Appendix

Appendix I

Protein determination

The protein concentration in the lipase samples used was determined using the Lowry Assay (Lowry et al., 1951)

Solutions:

Solution A, (100 ml): 0.5 g $CuSO_4.5H_2O$, 1g $Na_3C_6H_5O_7.2H_2O$, add distilled water to 100 ml.

Solution B, (1 liter): 20g Na_2CO_3, 4g NaOH, add distilled water to 1 liter

Solution C, (51 ml): 1 ml solution A, 50 ml solution B

Solution D, (20 ml): 10 ml Folin-Cicalteu phenol reagent, 10 ml distilled water

1. Bring sample solution to 0.5 ml with distilled water
2. Add 2.5 ml Solution C
3. Vortex and let stand at room temperature for 5-10 minutes
4. Add 0.25 ml solution D and vortex
5. After 20-30 minutes, read A_{750}
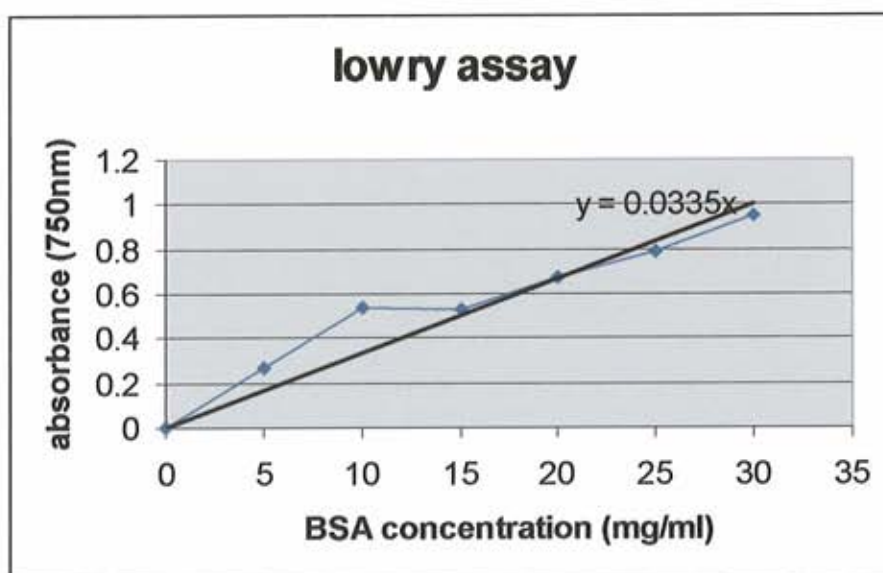
Appendix II

Ion exchange chromatography

Determining appropriate pH for ion exchange chromatography:

1. Prepare 9 test tubes, each containing 1 ml of ion exchanger (DEAE cellulose)

2. Equilibrate the first tube with 0.5 M pH 5.0 buffer containing 10mM NaCl by washing ten times with 10ml of buffer. Equilibrate the second tube with pH 5.5 buffer, and so forth until the ninth test tube is equilibrated at pH 9.0

3. Remove enough buffer so that approximately 1 ml of buffer covers the matrix

4. Add 100 μl of protein solution to each tube

5. Mix the tube and allow the matrix to settle for a few minutes

6. Test the supernatant for the protein of interest

Choosing the elution conditions:

1. Equilibrate 10 ml of DEAE cellulose ion exchanger with 50 mM Tris buffer (ph 7.9) containing 10 mM NaCl

2. Distribute 1 ml of ion exchanger into each 9 test tubes.

3. Equilibrate the second tube with 0.1 M NaCl by washing ten times with 10 ml of 50 mM Tris buffer (pH 7.9) containing 0.1 M NaCl

4. Equilibrate the third tube in the same fashion with pH 7.9 Tris buffer containing 0.2 M NaCl. Equilibrate the fourth tube with the same buffer containing 0.3 M NaCl, and continue on the with the fifth tube (0.4 M NaCl), the sixth tube (0.5 M

NaCl), the seventh tube (0.6 M NaCl), the eighth tube (0.8 M NaCl) and the ninth (1 M NaCl)

5. Remove buffer so that only about 1 ml of buffer covers the matrix

6. Add 100 µl of protein solution to each tube

7. Mix each tube and allow the matrix to settle for few minutes

8. Test the supernatants for the protein of interest.

Preparing the matrix and packing the column:

The DEAE cellulose (Sigma) used is preswollen, so the packing step was directly applied

a. Packing the column

1. Wash the matrix in a beaker with the column buffer. Swirl the slurry for a minute, then test the buffer pH. If the pH has changed from that of the original column buffer, further equilibration is needed: decant most of the buffer, add new column buffer, and repeat the slurry swirling and pH test.

2. Run the column at room temperature, degas the buffer and matrix slurry solution (apply a vacuum for one hour). This step will educe the likelihood of small air bubbles being traped in the matrix during the packing. Swirling the matrix during degassing will release air bubbles trapped in the matrix

3. Add a small amount of buffer to the column.

4. Open the column outlet to allow some of the buffer to pass, then close the outlet. This will remove air from the dead space at the bottom of the column.

5. Swirl the matrix solution, then pour into the column down a glass rod, making sure that air bubbles do not become trapped in the matrix

6. Open the column outlet and add more buffer as the matrix packs.

b. Equilibrating the matrix

After packing, the matrix material should be washed further with buffer in order to complete the packing process and to bring the matrix into a final equilibrium with the buffer pH and ionic conditions for the ensuring experiment. Washing with several column volumes is generally sufficient to achieve equilibration with most matrices.

Applying the sample:

Sample application:

1. Drain the column until the buffer reaches the surface of the matrix bed
2. With a pipet, apply the sample gently to the bed surface
3. Open the column outlet until the sample has entered the matrix, then recluse the column outlet
4. Gently apply some buffer the bed surface
5. Open the column outlet so the buffer enters the matrix, then close the outlet when the liquid reaches the bed surface
6. Again add buffer gently to the bed surface, then hook up buffer reservoir.
7. The column is now ready for washing and protein elution

Washing and eluting the protein of interest:

Step elution:

1. Wash column with 3 to 10 bed volumes of first elution buffer
2. Allow buffer to reach the bed surface before applying the new elution buffer. This will ensure that the salt concentration increases in a well defined manner
3. Apply 3 to 10 bed volumes of the second elution buffer, followed by any further elution buffer

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