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ISOLATION AND STUDY OF LIPASE ACTIVITY OF AN OLIVE OIL DEGRADING *THERMOACTINOMYCES* SP.

By

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A thesis submitted in partial
fulfillment of the
requirements for the degree
of Masters of Science

Microbiology-Biotechnology

Under Supervision of Dr. Fuad Hashwa

Lebanese American
University- Byblos Campus

May 2002

Lebanese American University

Graduate Studies

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ABSTRACT

ISOLATION AND STUDY OF LIPASE ACTIVITY OF AN OLIVE OIL DEGRADING *THERMOACTINOMYCES* SP.

By Rami Al Khudary

Chairperson of the Supervisory Committee: Dr. Fuad Hashwa
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A filamentous, Gram-Positive, spore forming filamentous bacterium was isolated from soil (Al Koura - Lebanon) on rhodamine agar plates at 60^0C. The isolate, HRK-1 produced large quantities of an extra cellular thermostable lipase that degrades olive oil.

It can be primarily classified as *Thermoactinomyces putidus* due to filamentous structure of its molecules that bear one spore each on an un-branched sporophore, the resistance of its spores to boiling, utilization of sucrose as a carbon source and production of dark pigments.

The isolate grew optimally at a temperature of 60^0C, a pH of 7.3, and an orbital shaking of 250 r.p.m.

It showed an efficient olive oil degrading ability. No traces of triolein were detected after 36 hours of cultivation. A concentration of 10%(v/v) olive oil did not inhibit its growth.

Lipase production was constitutive, and did not depend on the presence of olive oil. The optimal concentration of olive oil for lipase activity was 1%

(v/v), and activity was not enhanced at higher concentrations, but on the contrary, a decrease in enzyme activity was recorded.

The lipase HRKL-1 was bio-chemically characterized. It has a molecular weight of 80 k-Daltons and an optimal activity at 60^0C and pH 8.0. This lipolytic enzyme showed resistance to boiling and to a wide range of metallic ions and inhibitors. It was only slightly inhibited by PMSF (Phenylmethylsulfonyl fluoride), while its activity increased in the presence of Ca^{++} ions.

The Km value for HRKL-1 (0.021mM) indicated high affinity of the enzyme to its substrate.

The formation of this heat-stable lipase started in the early exponential growth phase, while maximal extracellular enzyme activity was detected at the end of the decline phase, when most of the cells appeared as spherical spores.

The exceptionally high activity of lipase (2.37 U/ml) produced by HRK-1 measured in the cell free supernatant clearly indicated the commercial importance of this isolate, especially after it showed great stability at elevated temperatures.

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ACKNOWLEDGMENTS

I would like to express deepest gratitude to Dr. Fuad Hashwa for his generous, continuous and enthusiastic assistance in every aspect of this research.

My sincere thanks are due to the members of the committee, Dr. Muhammad Mroueh, Dr. Constantine Daher, and Dr. George Baroody for their useful suggestions.

Special Thanks to Dr. Muhammad Mroueh for his help in the GC and HPLC experiments.

Thanks to my lab mates and to all those in the Department, who at some point provided assistance and cooperation.

Finally, my deepest gratitude to my family, for their encouragement and continuous support.

GLOSSARY

BOD. Biological oxygen demand

GC. Gas chromatography

HMWM. High molecular weight marker

HPLC. High performance liquid chromatography

kDa. Kilo- Daltons

Km. Michaelis-Menten constant - the substrate concentration where $v_0=1/2$ Vmax.

LMWM. Low molecular weight marker

M.W. Molecular weight

MCPM. Modified Czapek peptone medium

mM. mmole/l

MWCO. Molecular weight cut off

NaN_3. Sodium azide

OMW. Olive mill waste water

PMSF. Phenylmethylsulfonyl fluoride

p-NPP. Para-nitrophenyl palmitate

R.P.M. Revolution per minute

RBA. Rhodamine B agar

SDS. Sodium dodecyl Sulfate

SDS-PAGE. SDS Polyacrylamide gel electrophoresis

SM. Sierra medium

Chapter 1

INTRODUCTION

In recent years there has been an increase in the awareness of potentially harmful effects of olive-mill wastewater (OMW). Olive-mill wastewater is a major pollutant to surface and ground water resources in many Mediterranean countries. This serious problem occurs yearly during the olive-harvesting season and in areas (Fig. 1) with no available wastewater treatment systems. For example, in Lebanon small olive oil mills that are mostly family owned dispose their waste effluents (BOD about 50 g/l) into the surrounding land (ground pits), rivers, lakes, and the sea (Mediterranean). The waste water seeps then to the soil reaching the groundwater that constitutes the sole drinking water resource. It is estimated that around 1.6 million Liters of Zibar is produced in Lebanon per year. One of the solutions for this problem is employing microbial biotechnology.

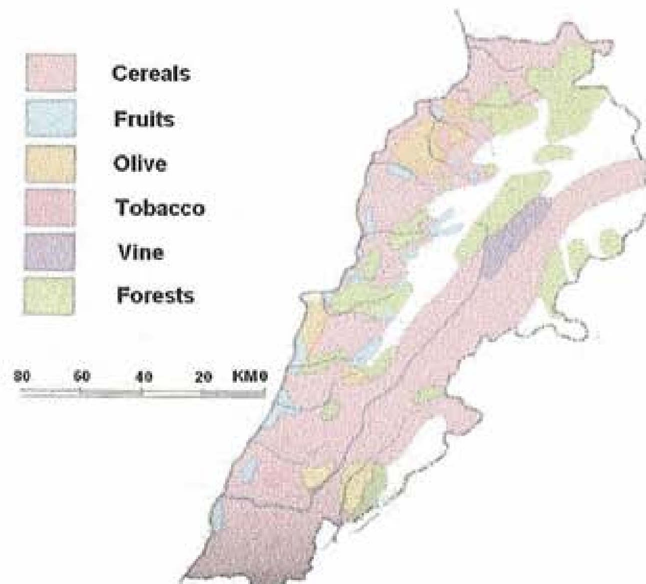


Figure 1 Olive trees distribution in Lebanon

Research carried out by Batelle and Columbus (1990) has shown that among the types of environmental impact that diminish the quality of life for groups of Inhabitants or in certain regions, the most important is produced by water pollution. One of the most polluting discharges of waste waters comes from olive-oil mills, which is a residue with a high phenol compound content with antimicrobial and phytotoxic characteristics.

The proliferation of co-operatives and industries which continuously use oil-yield processes and increasingly longer dry periods has caused OMW loads to exceed the self-purification capacity of fluvial runoffs it is emptied into. A solution to this problem has been attempted with the use of rafts (Fig. 2) on which OMW is stored, awaiting its natural evaporation. The use of these rafts usually found in locations close to urban centers, causes especially unpleasant environmental pollution (bad odors) during the spring and summer months. Another problem faced is that some oil mills do not correctly water proof the land on which the rafts are built, and this leads to underground water pollution (Hoyos et al., 2002).

Figure 2 A non-water-proofed Zibar raft in North Lebanon (Abou Samra) diluted by rain water

The increased demands and the strict jurisdiction in the realm of environment protection require the development of modern biotechnological processes for the treatment of such industrial wastes (Markosian et al. 2000).

The presence of fats and oils in waste water often causes major problems in conventional mesophilic biological treatment processes. In aerobic processes lipids may have a detrimental impact on oxygen transfer. Chao and Yang (1981) mention that wool grease, the major constituent of wool scouring waste water, reduces the oxygen transfer rates to the biological floc.

Moreover, in activated sludge systems the amount of lipids present has been shown to relate to the occurrence of filamentous actinomycete *Nocardia amarae* known to be involved in the formation of scum and stable foams (Becker et al. 1999).

In addition, long chain fatty acids, the primary hydrolysis products of fats and oils, have been demonstrated to lead to severe inhibition of the biomass in anaerobic treatment processes (Becker et al. 1999). When applied to OMW anaerobic treatment was applicable only to highly diluted OMW and their economy was further compromised by the use of chemicals from alkalinity, pH and nitrogen contents setting (Marques et al. 2001).

Moreover, lipid depositions in pipes and other plant components frequently cause operational disturbances. Especially emulsified oils and fats are still of major concern, e.g. in the food processing industry. Current physico-chemical technologies such as floatation and sedimentation are often insufficient and extremely problematic sludges are produced when using flocculating agents like polyelectrolytes or salts. Consequently, new technologies for the treatment of lipid-rich wastewaters are still demanded. The biological treatment of fats and oils under thermophilic conditions, i.e. above 60^0C, is expected to be advantageous due to favorable changes in most physical properties of these hydrophobic compounds with increasing temperature.

The melting point of fats and long chain fatty acids is often well above ambient temperatures. Above their melting temperature, i.e. in the liquid state, these substances become more accessible to microorganisms and their lipolytic enzymes. Both, diffusion coefficients and the solubility, for example, of fatty acids in aqueous media increase significantly with rising temperatures allowing for a better mass transfer. Furthermore, thermophilic processes have the advantage of hygienic operation which might be of considerable interest in sensitive areas of the food industry and if one considers to re-use the excess sludge, e.g. in agriculture (Becker et al. 1999).

Microorganisms that live under extreme conditions are defined as extremophiles. Microorganisms capable of growing optimally at temperatures between 50^{0}C and 60^{0}C are designated as moderate thermophiles. Most of theses microorganisms belong to many different taxonomic groups of eu- and pro-karyotic microorganisms such as protozoa, fungi, algae, streptomycetes, and cyanobacteria, which comprise mainly mesophilic species. It can be assumed that moderate thermophiles, which are closely related phylogenetically to mesophilic organisms, may be secondarily adapted to life in hot environments. (Bertoldo et. al. 2001)

Thermostable enzymes such as amylases, proteases and xylanases, have been investigated from thermophilic organisms belonging to various archea and bacteria. Little, however, is known on the production of heat-stable lipases. Lipases will play an increasingly important role in future enzyme markets. However, most of the thermostable lipases commercially available are produced by mesophilic bacteria or fungi. Because of the possibility of increased stability and resistance to denaturation, lipases from thermophiles are expected to play a significant role in industrial processes (Becker et al. 1997).

Most of the esterases and lipases yet reported are isolated from mesophilic microorganisms: *Pseudomonas cepacia, Pseudomonas fluorescens, Psuedomonas*

aeroginosa, Arthrobacter globiformis, Candida cylindracea, Butyrivibrio fibrisolvens, Brevibacterium linens, Acinetobacter calcoacticus, Bacillus subtilis, and *Aspergillus niger.* Although they have interesting properties, few industrial chemical processes have been developed using these enzymes. The major drawbacks limiting the industrial use of these enzymes is their instability mainly at high temperatures, in operating industrial conditions; therefore, the search for new thermostable enzymes is important for the development of new applications. Furthermore, it has been reported that the enzyme resistance to denaturation in organic solvents is correlated with their thermostability in water. For this reason, thermostable enzymes are attractive not only to be used in aqueous media but also in organic media (Kademi et al. 1999).

The major objective of this work is the isolation and characterization of a thermophilic microorganism able to produce thermostable extracellular enzymes, with special emphasis on olive oil degrading enzymes like lipases. This will be followed by the characterization of the thermostable lipase. This study includes:

Screening, isolation, selection and identification of a potential lipase producing thermophile

Optimization of growth conditions, including optimal temperature, pH and aeration rate.

Enzyme enrichment and purification

Enzyme characterization, to include; thermostability, effect of different inhibitors on enzyme activity, and molecular weight determination

Chapter 2

LITERATURE REVIEW

Relatively few investigations have been carried out on production and activity of lipases from thermophilic bacteria. With regards to actinomycetes, work on lipases of thermophilic isolates seems lacking, though there are studies on heat resistance of lipases produced from mesophilic isolates.

2.1 - Effect of carbon source on lipase production

The composition of the medium greatly affects the growth and production of extracellular lipases in bacteria. Carbon, nitrogen, sodium and potassium salts, metal ions and amino acids composition among others, are known to affect the growth of the organism and its lipase synthesis and secretion. In lipase production, the carbon source is the most important component of the medium affecting the synthesis and release of the enzyme.

Elwan et al. (1978) found that in the case of *Thermoactinomyces vulgaris* the activity of the lipolytic enzyme increased with increase of tributyrin substrate (maximum activity at 0.1%). 18 of the 28 tested substrates were hydrolyzed at variable rates: the maximum was obtained with oils from corn, peanut, caraway, bergamot, clove, lemon, and neroli and the minimum with olive oil and cholesterol stearate. The oils from bennet, lettuce, cotton seed, linseed, helba, cinnamon, fish, lavender, jasmine, and triacetin were not hydrolyzed.

Schmidt et al. (1994) working with *Bacillus thermocatenulatus* reported that 1-mono-, 1,2-di-, 1,3-di- and triolein were hydrolyzed by its lipase. Also lipase activity was measured with linseed oil, fish oil, triolein, soybean oil, and olive oil as substrates. A relative activity of 155% with linseed oil, 113% with soybean oil, 89% with fish oil, and 87% with triolein (100% with olive oil) was

observed during pH-stat assay. Schmidt et al. (1996) also showed that the shorter the chain of the acyl group (down to C_4) the higher the activity of the lipase which suggests that the shorter the chain the easier it is for the lipase to access the cleavage bonds.

Becker et al. (1997) working with lipase producing thermophile *Bacillus* sp. IHI-91 showed that, among several oils (sunflower seed, soybean, olive and fish oil), fatty acids (oleic, palmitic), Tween 80 and glycerol, olive oil is the best inducer of the lipase. Becker (1999) also noted that in a continuous culture of *Bacillus thermoleovorans* IHI-91 steady state biomass values increased linearly reflecting the stoichiometry between biomass formation and substrate consumption. However, at feed oil concentrations exceeding 4 g l^{-1} a sharp decrease in steady state cell numbers was observed. Concurrently, steady state lipid concentrations showed a dramatic increase indicating a decline in lipid degradation rates.

Lee et al. (2001) working on two lipases (BTID-A & BTID-B) of the thermophilic bacterium *Bacillus thermoleovorans* ID-1, showed that the difference of substrate specificities and positional specificities of both enzymes suggest that these distinct enzymes probably act synergistically to hydrolyze extracellular lipids to free fatty acids and glycerols.

2.2 - Pattern of substrate hydrolysis by lipase

Olive oil is composed of a variety of triglycerides of which triolein composes 40 to 45% and thus the demonstration of degradation of olive oil is done by following triolein concentration (Becker et al. 1997).

In a study done by Abia et al. (2001), it was shown that triolein composes 62%, and palmitoyl-dioleoyl-glycerol composes around 29% of virgin olive oil.

Simple lipids (fats) consist of fatty acids bonded to the C_3 alcohol *glycerol* (Fig.3). Simple lipids are frequently referred to as triglycerides because three fatty acids are linked to the glycerol molecule. Fatty acids link to glycerol by ester linkages. (Fats are esters of glycerol and fatty acids) Microorganisms utilize fats only after hydrolysis of the ester bonds, and extracellular enzymes called lipases are responsible for the reaction (Fig.3). The end result would be formation of glycerol and free fatty acids. Lipases are not highly specific and attack fats containing fatty acids of various chain lengths (Brock et al. 1994).

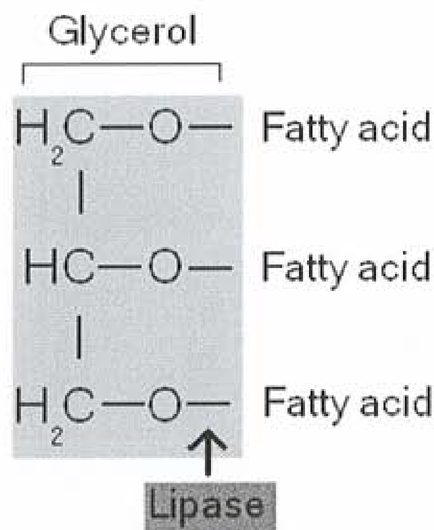


Figure 3. Lipase target

Triolein is composed of a glycerol molecule attached to three oleic acid molecules via ester bonds (Fig. 4). The complete break down of a triolein molecule would yield three oleic acid molecules (Fig. 5). Concurrently, intermittent accumulation of oleic acid at concentrations of more than 100 mg/ml was observed during batch fermentations (Becker et al. 1997).

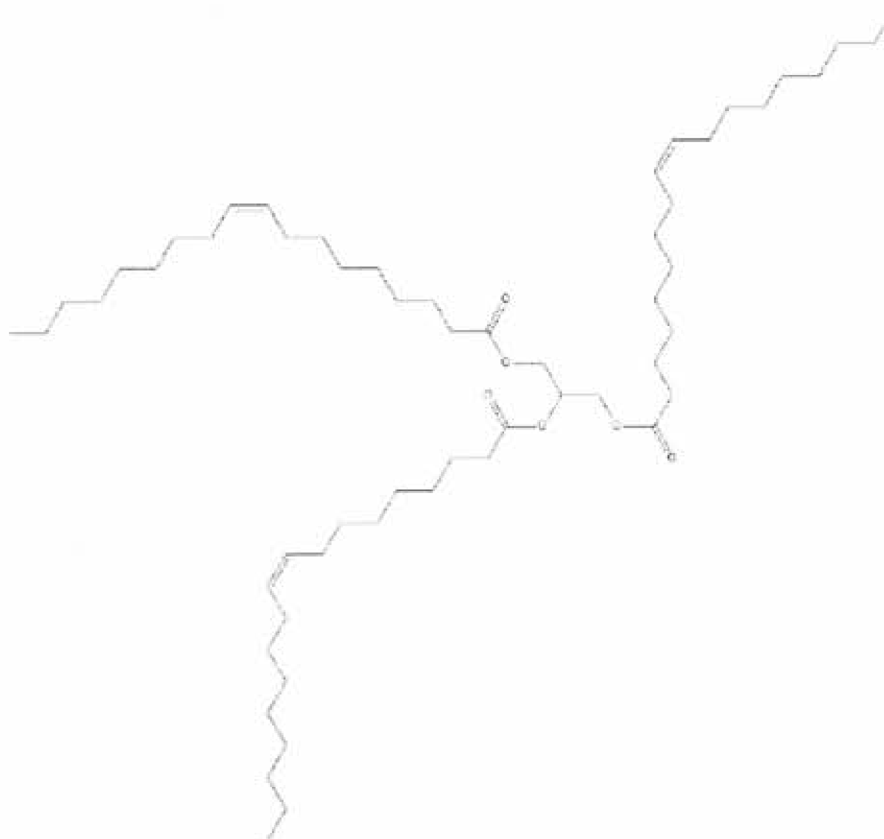


Figure 4 9-Octadecenoic acid (Z)-, 1,2,3-propanetriyl ester [122-32-7] Synonyms: Triolein; Glycerol Trioleate; Olein; trioleoylglycerol; 1,2,3-Propanetriyl tri-((E)-9-octadecenoate); Glyceryl Tri(oleate-1-13C); $C_{57}H_{104}O_6$ 885.445

Figure 5 Oleic acid [112-80-1] Synonyms: cis-Delta-9-octadecanoate; 9-Octadecenoic acid (Z)-; cis-9-Octadecenoic acid; cis-octadec-9-enoic acid; century cd fatty acid; emersol 210; emersol 213; emersol 6321; emersol 233ll; glycon ro; glycon wo; cis-delta(sup 9)-octadecanoic acid; 9-octadecenoic acid; wecoline oo; tego-oleic 130; vopcolene 27; groco 2; groco 4; groco 6; groco 5l; hy-phi 1055; hy-phi 1088; hy-phi 2066; K 52; neo-fat 90-04; neo-fat 92-04; hy-phi 2088; hy-phi 2102; Metaupon; red oil; (Z)-9-Octadecenoic acid; Octadecenoic acid; oleate; $C_{18}H_{34}O_2$ 282.4654

Lee et al. (2001) working on two lipases (BTID-A & BTID-B) of the thermophilic bacterium *Bacillus thermoleovorans* ID-1, showed that the major hydrolysis products by BTID-B were 1,2- and 1,3-diolein, whereas 2-monolein was also detected as a main product by BTID-A. These results indicated that BTID-A is likely to be a 1, 3-specific lipase and BTID-B a positional nonspecific one.

2.3 - Thermostability of Lipases

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. With respect to industrial applications, thermostable lipases are more promising, since thermostable proteins have several advantages as reviewed recently. So, there is a considerable commercial pressure to develop thermostable forms as biocatalysts in modern biotechnology (Schmidt et al.1994).

One important requirement for industrial application of enzymes is thermostability. Although many lipases from mesophiles are stable at elevated temperatures, the main focus was on lipases from thermophiles to further enhance thermostability. In addition to higher thermostability, proteins from thermophiles often show more stability toward organic solvents and show higher activity at elevated temperatures (Schmidt et al. 1996).

Recently several extracellular lipases have been reported from the genus *Bacillus* such as *B. subtilis, B. liqueniformis, B. catenulatus, and B. stearothermophilus.* Among them, two extracellular thermostable lipases have been cloned, purified, and characterized from *B. stearothermophilus* L1 and *B. thermocatenulatus.*

To date, a number of lipases from mesophiles have been extensively characterized. However, little study has been reported for the lipases from thermophiles partly due to the low level of enzyme production and the strong hydrophobic interaction with their substrates (Lee et al. 2001).

Most of the work done on thermostable lipases has shown an activity optimum at temperatures in the range of 60 to 70^{0}C.

A thermostable lipase was produced in continuous cultivation of a newly isolated thermophilic *Bacillus thermoleovorans* Strain IHI-91 growing optimally at 65^{0}C (Becker et al. 1997).

Another isolate of *Bacillus thermoleovorans* marked ID-1, isolated from the hot springs in Indonesia showed extracellular lipase activity and high growth rates on lipid substrates at elevated temperatures. The excreted lipase of ID-1 showed optimal activity at 70-75^{0}C and exhibited 50% of its original activity after 1 hr incubation at 60^{0}C and 30 min at 70^{0}C (Lee et al. 1999).

Later Lee et al. (2001) isolated two additional lipases, BTID-A & BTID-B, from the ID-1 strain. The optimal temperature of BTID-A was observed at 60-65^{0}C and that of BTID-B at 60^{0}C. When BTID-A was incubated for 30 min at 60^{0}C, its residual activity was over 75%.

Schmidt et al. (1994-6) purified and characterized two extracellular lipases from a *Bacillus thermocatenulatus* strain. The lipase BTL-1 showed optimal activity at 60-70^{0}C. Studies of lipase stability at elevated temperatures revealed no loss of activity up to 40^{0}C. However, at temperatures above 40^{0}C, the stability of the lipase decreased after 30 min of incubation in a phosphate buffer (pH 7.5). At 60^{0}C 50 % of residual activity was observed. The second lipase, BTL-2 was most active toward tributyrin at 60^{0}C and toward *p*-NPP at 60-70^{0}C at pH 9.0. However, the lipase was also inactivated at these temperatures. Below 40^{0}C, the lipase was stable for at least 30 minutes at pH

9.0, but above this temperature inactivation occurred. For example, a sample incubated at 60°C for 30 minutes retained 48.5% of the original activity.

Elwan et al. (1978) characterized a lipase of an isolate of *Thermoactinomyces vulgaris*. The optimum temperature of activity was 55°C. Pre-incubation of 2x dialysate at an increasing temperature gradient (60-100°C) showed that after 45 minutes at 80°C no activity could be detected. At 90°C and 100°C, activity ceased after 5 minutes of pre-incubation. At 60°C and 70°C, activities were recorded after 2 hours of pre-incubation. The enzyme activity lasted for 22 days at 50°C (19% loss) and 14 days at 55°C (23% loss).

Macarie et al. (1999) characterized a thermostable esterase of *Bacillus licheniformis*. The optimum temperature for activity was 45°C and the half-life was 1h at 64°C.

Kim et al. (2000) produced, purified and characterized a thermostable lipase of *Bacillus stearothermophilus*. Its optimum temperature for hydrolysis of olive oil was 68°C and it was stable up to 55°C for 30 minutes-incubation.

Finally a thermostable esterase from the moderate thermophilic bacterium *Bacillus circulans* showed maximal activity at 60°C. After 1 h incubation at 70°C and 85°C the remaining activities were 100% and 50% respectively, showing the high thermostability of this esterase activity (Kademi et al. 2000).

2.4 - Effect of hydrogen ion concentration (pH) on lipase activity and stability

Early in 1909 Sörensen demonstrated the effect of pH on enzymatic activity, since then the importance of pH on the stability of enzymes has been considered as one of the most important factors affecting their structure and catalytic activity.

Bacillus thermoleovorans ID-1 showed extracellular lipase activity and high growth rates on lipid substrates. The excreted lipase of ID-1 showed optimal activity near neutral pH (pH 7.5) (Lee et al. 1999).

The pH optima of BTID-A and BTID-B (*Bacillus thermoleovorans)* for lipase activity were 9 and 8-9, respectively. Both of them were stable at neutral pH range for 24 hrs at room temperature. Comparison of the deduced amino acid sequence and physicochemical characteristics of BTID-B with other lipases showed that it was similar to those of the lipases form *B. catenulatus* and *B. stearothermophilus* L1. High activity of the enzymes at pH 9-10 might result from the substitution of Ala for the first Gly- residue in the consensus sequence Gly- X- Ser- X- Gly (Lee et al. 2001).

The lipase (*Bacillus thermocatenulatus)* BTL-1 showed optimal activity with olive oil as substrate at pH 8.0. However, even at pH 11 (29%) and at pH 5 (19%) of the maximum activity was observed. The lipase was stable (95% residual activity) at pH 7.0 in a 0.1 M phosphate buffer for 16.5 hrs at 30^0C. Partially purified lipase, obtained after methanol precipitation with a 100 fold higher protein concentration, is stable in a broader range from pH 4.0 to 11.0. The second lipase, BTL-2 was most active toward tributyrin at pH 8.0. At pH 9.0 the activity was 84% of the maximum, while at pH 5.0, there was no activity. The lipase retained 90-100% of its activity after incubation at pH 9.0-11.0 for 12 hrs at 30^0C (Schmidt et al. 1994, 1996).

Elwan et al. (1978) characterized a lipase of an isolate of *Thermoactinomyces vulgaris.* The optimum pH of activity was 8.8.

The lipase of the isolate *Bacillus licheniformis* showed maximal activity at a pH of 8-8.5 (Macarie et al. 1999). While that of *Bacillus circulans* was in the range of 8.5-9.5 (Kademi et al. 2000).

2.5 - Effect of metal ions and inhibitors on lipase activity

Heavy metals like copper, silver, mercury… etc, act as potent poisons of enzymes, functioning even at low concentrations. Monovalent, divalent, and trivalent ions may have stimulatory or inhibitory effect on enzyme activity.

The catalytic activity of the lipase, produced by *Bacillus thermoleovorans* isolate marked ID-1, in the presence of Ca^{2+} and Zn^{2+} (1mM), was enhanced to about 120% and inhibited by SDS (Sodium Dodecyl Sulfate) and PMSF (Phenylmethylsulfonyl fluoride). Partial inactivation of the lipase by PMSF may be caused by modification of an essential serine residue that plays a key role in the catalytic mechanism (Lee et al. 1999).

The effect of various cations at a concentration of 1 mM on the activities of BTID-A and BTID-B (*Bacillus thermoleovorans*) were assessed. Ca^{2+}, Co^{2+}, Na^{2+}, and Mn^{2+} ions were found to enhance BTID-B activity. In contrast, none of the above ions could enhance BTID-A activity, and Cu^{2+} and Fe^{2+} inhibited BTID-A. BTID-A was strongly inhibited by PMSF, suggesting that BTID-A is a serine esterase. EDTA treatment strongly inhibited both enzymes, confirming that the enzymes require metal ions for their activity (Lee et al. 2001).

No influence on the lipase (*Bacillus thermocatenulatus)* BTL-1 of 1 mM and 10 mM $CaCl_2$, EDTA, $MnCl_2$ and I mM PMSF, NaN_3 (Sodium Azide) was observed after 30 minutes of incubation at 30°C. Only $MgCl_2$ reduced the lipase activity after 30 minutes of 18% (1mM $MgCl_2$) and of 24% (10 mM $MgCl_2$). $AgNO_3$ totally inactivated the lipase after 30 minutes of treatment. The second lipase, BTL-2 was not affected by treatment with 10 mM $CaCl_2$, 10 mM EDTA, 1 mM NaN_3, 1mM PMSF and 10 mM MgCl2, for 0.5 hrs at 30°C. But 1 mM $AgNO_3$ reduced the lipase activity after 30 minutes 28 % and 10 mM $MnCl_2$ 31% (Schmidt et al. 1994, 1996).

The lipase produced by *Bacillus licheniformis* was not affected by metal ions or sulfyhdryl reagents. Surprisingly, the enzyme was only slightly inhibited by PMSF (Macarie et al. 1999).

The activity of the lipase of *Bacillus stearothermophilus* increased in the presence of calcium ions. This calcium-dependent thermostability was confirmed by the tryptophan fluorescence emission kinetics showing that the enzyme starts to unfold at 66^{0}C in the presence of calcium ions but at 58^{0}C in the absence of calcium ions, implying that the calcium ions bind to the thermostable enzyme and stabilize the protein tertiary structure even at such high temperatures (Kim et al. 2000).

2.6 - Lipase Production

Biocatalysts are chosen in the industry as a good alternative for inorganic catalysts where specific products are obtained (Sarkar et al.1998). A number of thermophilic microorganisms produce thermoactive lipases and esterases (Markosian et al. 2000).

In batch cultures, and under optimum conditions, *B. thermoleovorans* IHI-91 had a maximum activity of soluble lipase of 0.3 U/ml in the cell free supernatant (i.e. soluble lipase) after 7-8 hrs (Becker et al. 1997). *B.thermoleovorans* ID-1 showed maximum lipase activity of 0.52 U/ml after only 3.5 h cultivation due to its rapid specific growth rate (Lee D. et al. 1999). After 40 h of cultivation at pH 6.5, a maximum of 0.36 U/ml lipase activity was produced by *B. thermocatenulatus* (Schmidt-D. et al. 1994). Finally, *Bacillus circulans* showed a maximal lipase activity of only 0.04U/ml (Kademi et al. 1999).

Chapter 3

MATERIALS AND METHODS

3.1 - Screening, isolation, and purification of lipase producers

Lipase producing bacteria were isolated on agar plates supplemented with either 1% Tween™ 80 or 3% olive oil; and a final pH of either 5.0 or 7.0.

Soil samples were collected from various locations including olive orchards (North Lebanon, Al Koura), and olive oil mills (KfarAkka). Jift (olive dry pulp) samples were collected from olive oil mills (in KfarAkka). Liquid samples were collected from fresh Zibar (KfarAkka) and old Zibar (Byblos (one year).

Enrichment cultures of soil samples (1% w/v), jift samples (1% w/v) and of Zibar samples (1% v/v), were performed at 60^0C and an initial pH of 5.0 & 7.0 with shaking (200rpm) in the following defined salt medium:

MA medium: (Becker et al. 1997)

3.0 g/l NaCl, 0.8 g/l $K_2HPO_4.3H_2O$, 0.6g/l KH_2PO_4, 1.0g/l $(NH_4)_2SO_4$, 0.2 g/l $MgSO_4.7H_2O$, 0.05g/l $CaCl_2.2H_2O$, 0.1 ml/l $FeCl_3$ (1%), 10ml/l trace element solution.

After enrichment, 0.1 ml innocula of the appropriate dilutions were spread on the agar plates and incubated at 60^0C for 18 hrs under humid atmosphere. The plates were sealed with plastic bags to minimize dryness of the medium.

Lipase producing thermophiles were screened on Sierra Medium Plates (SM) and Rhodamine B (Sigma) agar plates (RBA). Positive colonies would produce a white precipitate in the first medium, and would emit orange fluorescence when subjected to UV (350 nm) on the second medium (Fig. 6 & 7).

Positive colonies were picked up and purified by using the 13-streak method. Pure isolates were stored on nutrient agar slants at 4^0C.

3.2 - Selection of potential lipase producers

Selection of high lipase producers was performed after testing the ability of the isolates to produce lipolytic enzymes on two different media.

Sierra medium plates: (Fig. 6) (Atlas 1993)

Composition per Liter:

Agar 15.0g, Peptone 10.0g, NaCl 5.0g, $CaCl_2.H_2O$ 0.1g, Tween^{TM}80 10.0 ml PH 7.4 at 25^0C.

Add components, except Tween^{TM}80, to distilled/deionized water and bring volume to 990.0 ml. Mix thoroughly. Gently heat and bring to boiling. Autoclave for 15 minutes at 15 psi pressure-121^0C. Cool to 45-50^0C. Separately autoclave Tween^{TM}80. Aseptically add 10.0ml of sterile Tween^{TM}80. Mix thoroughly, pour into sterile Petri dishes.

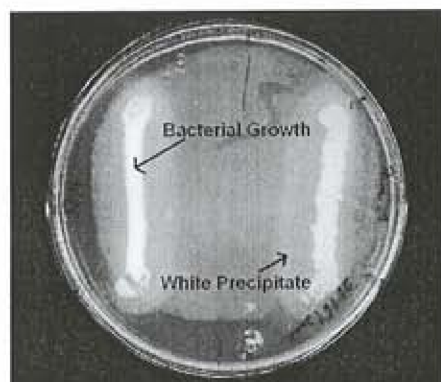


Figure 6 Sierra Medium Plate showing a white precipitate as an indication of lipase activity

Rhodamine B agar plates: (Fig. 7) (Kouker and Jaeger 1987)

Rhodamine B (1mg/l; Sigma Chemical Co.) was dissolved in distilled water and sterilized by filtration using Millex-HA Syringe driven filter unit (0.45μm, 25 mm, Sterile) filters.

Composition per liter:

Nutrient broth 8g, sodium chloride 4g, and agar 10g

The medium was adjusted to pH 7.0, autoclaved, and cooled to about 60°C. Then 31.25 ml of olive oil (Sigma Chemical Co.) and 10 ml of rhodamine B stock solution was added, and the whole mixture was emulsified using an ultra sonicater. 20 ml of medium was poured into each plastic Petri dish.

Lipase production was monitored by observing the white precipitate formed in the Sierra plates, and the orange fluorescence produced by Rhodamine B plates upon irradiation with UV light at 350 nm.

Figure 7 Rhodamine B agar plate showing orange fluorescence as an indication of lipase activity

3.3 - Characterization of the selected lipase producers

The isolates were gram stained and observed under regular light microscope. Pure colonies were streaked on SM plates and incubated at different temperatures (ambient, 37, 45, 50, 60, & 70 ^{0}C) to observe the range of optimal growth conditions.

3.4 - Confirmation of the thermophilic character of the selected lipase producer

A suspension of the isolate was boiled for 15 minutes and streaked on an SM plate to test for resistance of the spores, which is a characteristic of the *Thermoactinomyces* species - According to Bergey's, Group 28, Thermoactinomyces (Holt et al. 1994).

3.5 - Lipase production systems

The following medium was used for cultivation:

Modified Czapek peptone medium (MCPM) (DSMZ list of Media)

(Czapek peptone medium contains 30g/l sucrose. In this modified medium sucrose was removed)

Composition per liter:

3.0g/l $NaNO_3$, 1.0g/l K_2HPO_4, 0.5g/l $MgSO_4.7H_2O$, 0.5g/l KCl, 0.01g/l $FeSO_4.7H_2O$, 2.0g/l Yeast Extract and 5.0 g/l peptone.

1% olive oil was used as the only substrate in most of the cultivations.

Adjust pH to 7.3.

Sterilize by autoclaving for 15 minutes at 121^{0}C.

3.5.1 - Cultivation of batch cultures was carried out in two systems:

In an orbital shaker (60°C, 250 rpm) – (NBS Innova 4400 – New Brunswick Inc. NewJersy, USA) (Fig. 8):

1000-ml Erlenmeyer flasks containing 500 ml of MCPM and 1% olive oil were inoculated with 5 ml of an 18 h preculture of HRK-1 and incubated at 60°C and at 250 rpm. Crude enzyme preparation was obtained from the supernatant after centrifugation at 25,000 x g in a Sorvall centrifuge for 30 minutes.

Figure 8 NBS Innova 4400 orbital shaker

And in a foil fermenter (60°C, 1000 rpm) – BIOENGINEERING (Wald Switzerland) fermenter (Fig. 9):

1.5 liters of MCPM with 1% olive oil were added to the fermenter. The temperature was set to 60°C and agitation to 1000 r.p.m. pH was not controlled in batch studies. The medium was inoculated with 15 ml of an 18 h preculture of HRK-1.

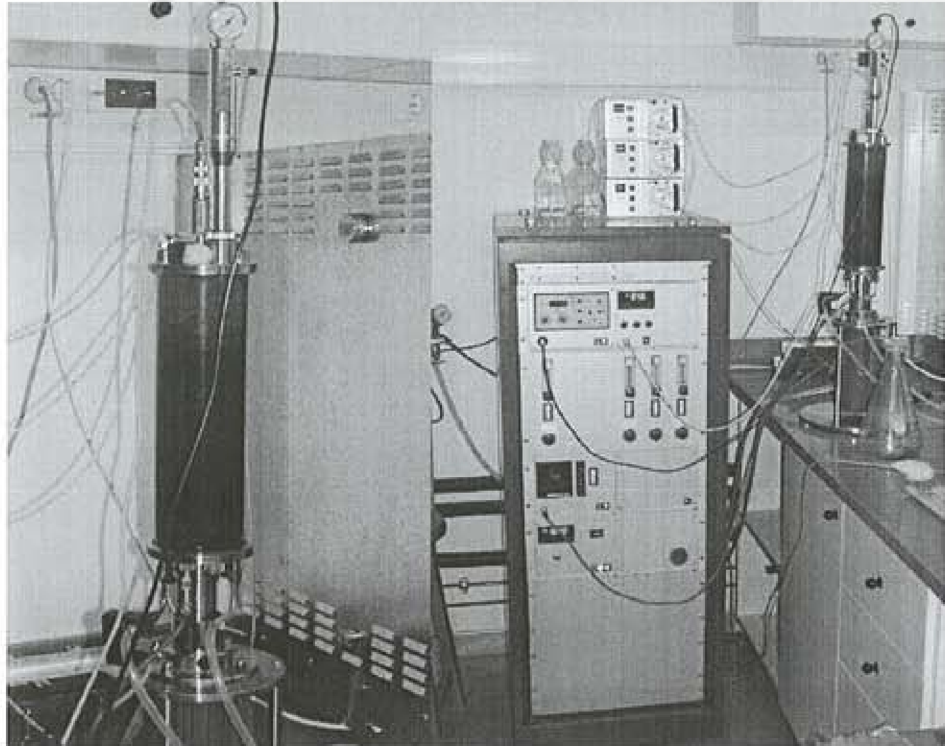


Figure 9 BIOENGINEERING (Wald Switzerland) fermenter general overview – right, with a close up look on the reactor –left

3.6 - Determination of biomass concentration

Determination of biomass concentration is usually done using one of three methods.

1. Direct microscopic enumeration of the cells in a Neubauer chamber.
2. Measurement of optical density of the bacterial culture at 540 nm.
3. Dry cell weight determination.

Due to the fact that the microorganism that is dealt with in this work is a filamentous bacterium, the first two methods were not suitable. Filaments can not be counted, and their mode of growth does not give accurate readings of optical density.

Thus the third method was the only one suitable for this work.

Millipore membrane filters (MILLIPORE 0.45μm, white 47 mm gridded, Sterile) were used. Each filter was weighed before being used. 5 ml sample of the bacterial culture was taken, filtered and then the filter was placed in an incubator at 60°C and left to dry overnight. The filter was weighed again and the difference between the values before and after filtration corresponded to the dry biomass.

3.7 - Optimization of growth conditions

3.7.1 - Optimal temperature:

Two 1000-ml Erlenmeyer flasks containing 500 ml of modified Czapek medium and 1% olive oil, pH 7.3 were inoculated with an 18 hrs preculture of the isolate HRK-1 and incubated at the desired temperature in the orbital shaker at 250 r.p.m.

Aliquots (10 ml) were collected for dry biomass analysis at 6 hours intervals from each flask.

3.7.2 - Optimal hydrogen ion concentration (pH):

Five 1000-ml Erlenmeyer flasks containing 500 ml of modified Czapek medium and 1% olive oil were inoculated with an 18 h preculture of the isolate HRK-1 and incubated at 60°C in the orbital shaker at 250 r.p.m.

Each of the flasks was buffered by a 0.2 M phosphate buffer system. The pH values tested were: 6.0, 6.5, 7.0, 7.5, and 8.0.

Aliquots (10 ml) were collected at 24 h intervals from each flask for pH and dry biomass determination.

3.7.3 - Optimal aeration rate

Optimal aeration rate was calculated as for the optimal temperature. The following speeds were tested: 150 and 250 r.p.m. in the orbital shaker. Optimal aeration rate and the effect of high agitation on filaments' growth were also tested in the fermenter. Two speeds were tested 500, and 1000 r.p.m. with maximal air flow.

3.8 - Ability to hydrolyze olive oil

Two 250-ml Erlenmeyer flasks, each containing 100 ml modified Czapek medium and 1% olive oil were inoculated with an 18 h preculture of the isolate HRK-1 and incubated at 60^0C in the orbital shaker at 150 r.p.m. 10 ml samples were taken every 24 hours.

To demonstrate the ability of the lipase to hydrolyze olive oil, GC analysis of the samples was carried out to detect oleic acid concentration which is the major product of olive oil hydrolysis.

Another set of two 1000-ml Erlenmeyer flasks, each containing 500 ml modified Czapek medium and 1% olive oil were inoculated with an 18 h preculture of the isolate HRK-1 and incubated at 60^0C in the orbital shaker at 250 r.p.m. 10 ml samples were taken every 6 hours.

This set was used to demonstrate the ability of the lipase to hydrolyze triolein, which is olive oil main component (62%), HPLC analysis of the samples was carried out to detect triolein concentration.

3.8.1 - Gas chromatography

Oleic acid concentration was followed by gas-chromatography analysis. 10 ml Samples were acidified with HCl (1:1) and extracted with hexane/ether (1:4). The combined extracts were volume reduced under a stream of nitrogen gas and the residues were dissolved in 2 ml hexane/acetone (7:3). The compounds were derivatized by the addition of 500 µl *N*-methyl-*N*-trimethylsilyl-heptafluorobutyramide, purchased from Macherey-Nagel, Duren, Germany and incubation at 60^0C for 10 minutes. (Becker et al. 1997)

Finally measurements were performed using a GC-17A Gas Chromatograph, Shimadzu- Japan, equipped with a Supelcowax™- 10 capillary column (Fused Silica) 30m x 0.25mm x 0.25µm film thickness.

The following settings were used for the readings:

Carrier gas: N_2 **Split ratio**: 1:20, **Temperatures:** Column oven: (set 150, max 400), Injection port: (set 280, max 470), Detector: (set 280, max 470), Column Oven temperature program: (Initial 150, Final 280 at 12^0C/ min), **Column pressure:** 100 kPa, **Column flow:** 2.82546 ml/min, **Linear velocity:** 52.3771 cm/s, **Total flow:** 62 ml, **Column length:** 25 m, **Column diameter:** 0.32 mm.

3.8.2 - High performance liquid chromatography

Triolein concentration was followed by HPLC analysis. 10 ml Samples were acidified with HCl (1:1) and extracted with hexane/ether (1:4). The combined extracts were volume reduced under a stream of nitrogen gas and the residues were dissolved in 2 ml hexane/acetone (7:3).

The obtained samples were analyzed using a Waters Spherisorb® 10µm ODS-2 4.6x 250 mm analytical column (Ireland) using acetone/acetonitrile (1:1 v/v) (Plattner et al. 1977; Hiol et al. 2000; Perona et al. 1998; Najera et al.

1999). A Rheodyne (Cotati- California) manual injector with a maximal capacity of 200μl was used. A Waters 510 pump was set at a flow rate of 2ml/min. the triolein peaks were detected using a Waters 2410 Refractive Index Detector attached to a Waters 746 data module (Chart speed 1cm/min). The detector temperature was set at 30°C with a sensitivity value (1).

3.9 - Effect of olive oil concentration on the biomass and the activity of lipase

To detect the effect of various olive oil concentrations on the biomass and the activity of lipase, five 250-ml Erlenmeyer flasks, each containing 100 ml modified Czapek medium were inoculated with an 18 h preculture of the isolate HRK-1 and incubated at 60°C in the orbital shaker at 150 r.p.m.

The olive oil concentration in each of the flasks was; 0, 1, 3, 5, & 10% (v/v)

Samples (3 ml) were collected at 24 hours intervals from each flask to measure dry biomass and lipase activity.

3.10 - Cellular morphology and changes during growth

Changes of cellular morphology of each of the cultures were studied using a Nikon Eclipse TE300 inverted light microscope (Nikon Corp. Japan), equipped with a Sony Power HAD DXC-970MD digital camera used to take photographs of the wet mount preparations.

3.11 - Standard assay of lipase

A spectrophotometric assay with *p*-nitrophenyl laurate (Sigma) as substrate was performed to determine lipase activity. Lipase activity was measured after filtration in the cell-free extract (referred to as *soluble lipase* - Only extracellular

lipase activity was determined). A volume of 25 μl sample was dissolved in 725 μl phosphate buffer (50mM, pH 7.2). The enzymatic reaction was carried out under shaking at 65^0C after addition of 100 μl substrate solution (25mM *p*-nitrophenyl laurate in absolute ethanol). After 10 minutes of incubation, 250 μl 100mM Na_2CO_3 was added and the mixture was centrifuged at 4^0C (10min, 13 000 rpm). The absorbance of liberated *p*-nitrophenol was measured at 420 nm. One unit (U) of lipase activity is defined as the amount of enzyme that causes the release of 1μmol *p*-nitrophenol/min under test conditions. The molar absorption coefficient of *p*-nitrophenol at 420 nm is 13.5 l/mol/cm. (Becker et al. 1997; Maurich et al. 1991; Winkler and Stuckmann 1979)

3.12 - 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 $CuSO_4$. $5H_2O$, 1 g $Na_3C_6H_5O_7$. $2H_2O$, ad distilled water to 100ml

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

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

Solution D, (20 ml): 10 ml Folin-Ciocalteu 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}.

A standard curve was plotted using bovine serum albumin (Fig. 10).

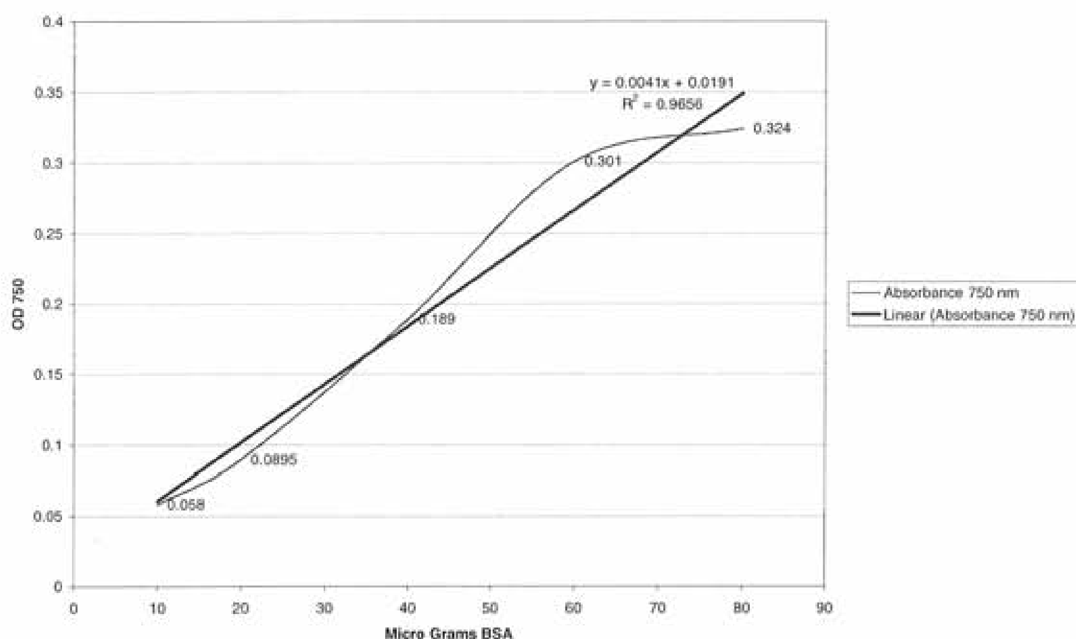


Figure 10 Lowry's Assay standard curve used for protein concentration determination (BSA mass vs. optical density at 750 nm)

3.13 - Characterization of the lipase

3.13.1 - Ultra filtration

Concentration of the lipase was achieved in the following way. 250 ml of the cell free broth were placed in an ultrafiltration unit equipped with a 10,000 molecular weight cut off (MWCO) membrane.

The filtration chamber was pressurized by a stream of nitrogen gas for 12 hrs. The residual 15 ml were divided into 5 aliquots and placed in deep freeze (-85^{0}C).

3.13.2 - Electrophoretic pattern of the lipase

Gel electrophoresis under non-denaturing conditions (Bollag et al. 1996)

Non denaturing gel electrophoresis is commonly run with high pH buffers (pH 8.8). At this pH, most proteins are negatively charged and migrate toward the anode.

- Working solutions:

Solution A (Acrylamide stock solution), 100 ml

30% Acrylamide, 0.8% Bis-acrylamide

a. 30 g Acrylamide

b. 0.8 g bis-acrylamide

Add distilled water to make 100 ml and stir until completely dissolved. Work under hood and keep acrylamide solution covered with Parafilm until acrylamide powder is completely dissolved.

Solution B (4x separating buffer), 100 ml

1.5 M tris-HCl (pH 8.8)

a. 18.2 g tris in 40 ml H_2O

b. Add HCl to pH 8.8

c. Add H_2O to 100 ml

Solution C (4x stacking buffer), 100 ml

0.5 M tris (pH 6.8)

a. 6.0 g tris in 40 ml H_2O

b. Add HCl to pH 6.8

c. Add H_2O to 100 ml

10% Ammonium Persulfate, 5 ml

a. 0.5 g ammonium persulfate

b. 5 ml H_2O

Electrophoresis Buffer, 3 liters

a. 9.0 g Tris (25 mM)

b. 43.2 g glycine (192 mM)

c. H_2O to 3 liters (final pH should be 8.8)

5 x sample buffer, 10 ml

a. 3.1 ml 1 M tris-HCl (pH 6.8) --- 312.5 mM

b. 5 ml glycerol --- 50%

c. 0.5 ml 1% bromophenol blue --- 0.05%

d. 1.4 ml H_2O

Pouring the Separating Gel (for an 18 x 16 cm x 1.5 mm gel size)

One 8% separating gel (need to prepare 30 ml)

14.4 ml H_2O

8.1 ml Solution A

7.5 ml Solution B

---

150 µl 10% Ammonium persulfate

5 µl TEMED

Do not prepare until following the numbered instructions below.

a. Assemble gel sandwich

b. Combine solutions A & B & water in a small Erlenmeyer flask. Acrylamide in solution A is a neurotoxin, so plastic gloves should be worn at all times.

c. Add ammonium persulfate and TEMED, and mix by swirling or inverting container gently. Work rapidly at this point because polymerization will be under way.

d. Introduce solution into gel sandwich using a pipette.

e. When the appropriate amount of separating gel solution has been added, gently layer about 1 – 5 mm of water on top of the separating gel solution.

f. Allow gel to polymerize (30 – 60 minutes). When the gel has polymerized, a distinct interface will appear between the separating gel and the water, and the gel mold can be tilted to verify polymerization.

Pouring the stacking gel (for an 18 x 16 cm x 1.5 mm gel size)

One 5 % stacking (Need to prepare 8 ml)

4.6 ml H_2O

1.34 ml Solution A

2.0 ml Solution C

---

60 µl 10% Ammonium persulfate

10 µl TEMED

Do not prepare until following the numbered instructions below.

a. Pour off water covering the separating gel.

b. Combine Solution A, C & water in a small Erlenmeyer flask.

c. Add ammonium persulfate and TEMED, and mix by gently swirling or inverting the container.

d. Carefully insert comb into gel sandwich.

e. Pipette stacking gel solution onto separating gel until solution reaches top of plate.

f. Allow stacking gel to polymerize (about 30 minutes).

g. After stacking gel has polymerized, remove comb carefully (making sure not to tear the well ears).

h. Place gel into electrophoresis chamber.

i. Add electrophoresis buffer to inner and outer reservoir making sure that both top and bottom of gel are immersed in buffer.

Sample preparation

a. Combine protein sample (Enzyme crude preparation obtained by Ultrafiltration) and 5x sample buffer (40 µl sample + 10 µl buffer) in an Eppendorf tube.

b. Introduce sample solution into well using a disposable gel loading tip.

c. Typically 1-5 µg of protein is loaded per well, or up to 30 µg of a complex protein mixture.

Running the gel (Hoefer SE 600 series with buffer saver) (Fig. 11)

a. Attach electrode plugs to proper electrodes. Current flows towards the anode for pH 8.8 gels.

b. Turn on power supply to 100 – 200 V (constant current).

c. Electrophoresis should continue until the dye front migrates to within 1 – 5 mm of the bottom of the gel. (Around 5 h)

d. Turn off power supply

e. Remove electrode plugs from electrodes

f. Carefully remove a spacer, and inserting the spacer in one corner between the plates, gently pry apart the gel plates. The gel sticks to the lower plate.

Figure 11 Electrophoresis apparatus Hoefer SE 600 series

Staining the gel

Coomassie blue staining can detect as little as 0.1 μg of protein in a single band.

Stock solutions:

a. Coomassie gel stain, 1 liter

   1.0 g Coomassie Blue R- 250

   450 ml methanol

   450 ml H_2O

   100 ml glacial acetic acid

b. Coomassie gel destain , 2 liters

   200 ml methanol

   200 ml glacial acetic acid

   1600 ml H_2O

Staining procedure

The gel is stained in an automatic gel stainer (Amersham Pharmacia Biotech Automated staining of polyacrylamide gels with Hoefer Processor Plus). The machine is programmed to wash the gel for 5 minutes with distilled water, then with stain for 30 minutes and finally with destain overnight. The destain is changed several times.

Lipase band detection

The gel section containing the lipase bands was detected by cutting the gel into horizontal sections. (Each section contains a protein band). Each section is cut into fine parts using a blade and is placed in phosphate buffer to allow the lipase to diffuse out of the gel. The section in which lipase activity is detected is prepared again as described above to be used in the SDS-PAGE electrophoresis in the next part.

Gel electrophoresis under denaturing conditions: SDS Polyacrylamide gel electrophoresis (SDS-PAGE) (Bollag et al. 1996)
A gradient gel was prepared.

Working solutions

1) Solution A- 30 % acrylamide, 0.8% bis-acrylamide

2) Solution B- 1.5 M tris-HCl (pH 8.8), 0.4% SDS

3) Solution C – 0.5 M Tris-HCL (pH 6.8), 0.4% SDS.

4) 10% APS

5) Electrophoresis buffer, 3 liters

    a) 9 g Tris ---25 mM

    b) 43.2 g glycine ---192 mM

    c) 3 g SDS ---0.1%

    d) H_2O to make 3 liters. (pH should be approximately 8.3)

6) 5 x sample buffer, 10 ml

    a) 0.6 ml 1 M tris- HCl (pH 6.8) –60 mM

    b) 5 ml 50% glycerol --25%

    c) 2 ml 10% SDS ---2%

    d) 0.5 ml 2-mercaptoethanol ---14.4 mM

    e) 1 ml 1% bromophenol blue --0.1%

    f) 0.9 ml H_2O

Amounts of solutions for an 8 – 20% separating gel (for an 18 x 16 cm x 1.5 mm gel size)

8%	20%	
4.05 ml	10.005 ml	Solution A
3.75 ml	3.75 ml	Solution B
7.2 ml	--	H_2O
--	2.25g	Sucrose
75 µl	75 µl	10% APS
7.5 µl	7.5 µl	TEMED

Forming separating gel

a. Prepare the gel sandwich and set up the gradient maker.

b. Prepare the separating gel solutions without adding TEMED

c. Add TEMED to the separating gel solutions and mix gently. Immediately transfer the appropriate volume (15 ml) of each solution into the mixing chamber. The high concentration (20%) acrylamide solution should be added to the chamber closer to the outlet.

d. Turn on the magnetic stirrer and open the connection between the two chambers.

e. When the separating gel has been poured, gently layer about 1 to 5 mm of water on top of the gel.

f. Wash the gradient maker and tubing with water to prevent the acrylamide solution from polymerizing inside.

Pouring the stacking gel

An 8% stacking gel is prepared as described in non denaturing gel electrophoresis.

The gel section containing the lipase bands prepared earlier is placed between the sandwich plates after treatment with sample buffer. Aluminum foils are

placed to make wells for adding additional samples as shown in (Fig. 12). Stacking gel is poured and allowed to polymerize for 30 minutes.

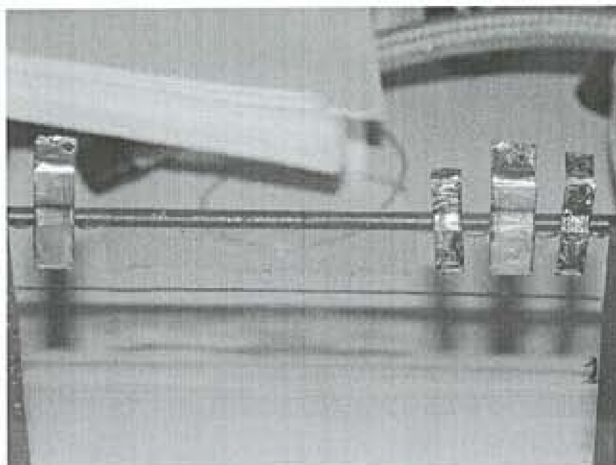


Figure 12 Stacking gel assembly

Preparing and loading samples

1) Combine protein sample and 5 x sample buffer

2) Heat at 100^{0}C for 2 – 10 minutes.

3) Spin down protein solution for 1 second in a microfuge.

4) Introduce sample solution into well using a disposable gel loading tip.

The samples prepared are high and low molecular weight markers (HMWM, LMWM), and an Enzyme crude preparation obtained by Ultrafiltration

The gel strip obtained from the non denaturing gel electrophoresis was placed in boiling sample buffer for 1 minute before placing it in the gel sandwich.

Finally the gel was run and stained as in the non denaturing gel electrophoresis method above.

3.13.3 - Enzyme Kinetics

- Effect of pH on enzyme activity was measured using *p*-nitrophenyl laurate in 50 mM phosphate buffer. A series of 50 mM phosphate

buffers were prepared with the following pHs 6.0, 6.5, 7.2, 7.5, 8.0, & 8.5, and the regular procedure for the measurement of lipase activity was followed.

- Enzyme activity as a function of temperature was studied by incubating the *p*-nitrophenyl laurate reaction vessels in different temperatures (18, 30, 40, 55, 60, 65, & 70^{0}C)

- The effect of metal ions and inhibitors 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 each of the following ions: Ca, Cu, Co, Mn, Mg, Zn, Fe^{2+}, & Fe^{3+}. Also the enzyme was incubated with 1 mM of each of SDS, EDTA, PMSF and Urea.

- Temperature stability of the enzyme was determined by preparing 6 Eppendorf tubes each containing 0.5 ml of 10x diluted lipase crude sample. The tubes were then properly sealed and placed in boiling water. One tube was removed every 10 minutes and placed in a cold water bath. lipase activity was then determined in each of the tubes.

- Michaelis-Menten kinetics: enzyme-substrate saturation curve was obtained by running the reactions using 25, 6.25, 1.5, 0.4, 0.1, & 0.025mM *p*-nitrophenyl laurate. The Km value was calculated using the Michaelis-Menten equation: $v_0= (V_{max}*[S])/ (Km+[S])$ where (S is substrate concentration, and Km the Michaelis-Menten constant, is the substrate concentration where $v_0=1/2\ V_{max}$) (Reed et al. 1998; Schlegel et al. 1988)

- Enzyme activity as a function of enzyme concentration was studied by carrying out the lipase assay using different concentrations of the crude enzyme preparation.

- Molecular weight of the enzyme was determined from the SDS-PAGE gel obtained. A standard curve of the HMWM was generated (Fig. 13) and the molecular weight of the lipase was then extrapolated from this curve.

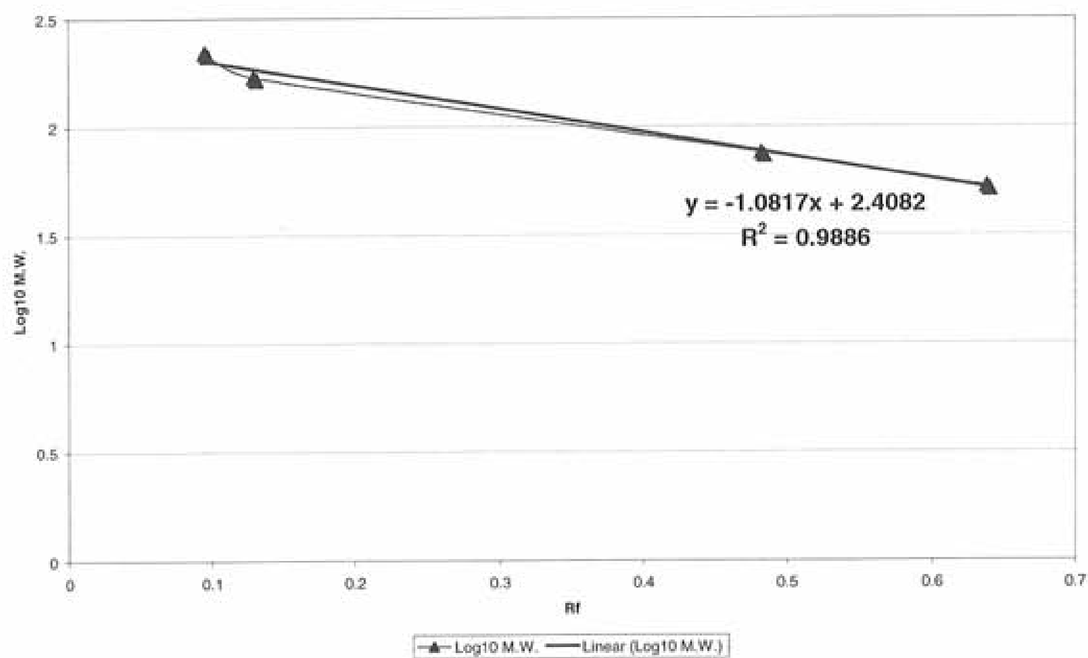


Figure 13 Graph of Log 10 of molecular weight versus relative mobility for HMWM. This graph is used to determine M.W. of the lipase.

Chapter 4

RESULTS

4.1 - Strain isolation

A total of 7 lipase producers from several soil and water samples were selected after showing positive results on Sierra and Rhodamine plates. Pure cultures were obtained using the 13 streak method. All of the isolates were taken from pH 7.0 plates. No growth was observed on pH 5.0 plates.

4.2 - Selection of potential thermophilic lipase producers

Selection of thermophilic strains producing reasonable quantity of lipase was accomplished by streaking pure cultures on Sierra and rhodamine plates and incubating the plates at different temperatures. Results are summarized in (Table 1).

All of the isolates showed positive lipase activity at temperatures suitable for their growth, but only two isolates HRK-1 & HRK-7 occurred to be strict thermophiles.

The isolate HRK-1 was found to be a filamentous actinomycete. This characteristic of isolate HRK-1 was important because most of the research done on thermophilic lipase producers was focusing on the genus *Bacillus*.

Table 1 Pattern of growth as related to temperature: (24 hours of incubation)

Source of Isolate	Maximal Lipase Activity	Temperature/ Isolate	Ambient	37^0C	45^0C	50^0C	60^0C	70^0C
Soil (Al Koura)	2.37 U/ml	HRK-1	-/-	-/-	-/-	-/+	+	+
New Zibar	0.46 U/ml	HRK-2	-	+	+	+	-/-	-/-
Old Zibar	N.D.	HRK-3	-/+	+	+	-/-	-/-	-/-
Jift	N.D.	HRK-4	-/+	+	+	-	-/-	-/-
Soil (Unknown Source)	N.D.	HRK-5	-/+	+	+	-/-	-/-	-/-
Soil (Unknown Source)	N.D.	HRK-6	-/+	+	+	-/+	-/-	-/-
Soil (Al Koura)	0.2 U/ml	HRK-7	-/-	-/-	-/-	-/+	+	+

Key:
-/- → No growth
- → Very weak growth
-/+ → Weak growth
+ → Good growth
N.D. → Not determined

N.B. Gram staining of all the isolates showed that they are gram-positive spore forming Bacilli, except for HRK-1 which turned out to be a gram-positive spore forming filamentous actinomycete.

4.3 - Characterization of the selected lipase producer

The isolate HRK-1 isolated from a soil sample in Al Koura – North Lebanon was found to be a filamentous spore forming, gram positive, obligate-thermophilic bacterium, with a minimum growth temperature of 50°C. The gram- positivity of the isolate was tested using conventional Gram's staining (Fig. 14).

The thermophile HRK-1 was stored in a deep freeze at -85°C in a solution consisting of 50% (v/v) glycerol and 50% (v/v) nutrient broth.

Morphological studies indicated that the microorganism formed spores towards the end of the filamentous stage, while short rods were formed at the beginning of the growth stage.

When the spores of the isolate were boiled for 15 minutes and then streaked again on an SM plate, vegetative growth occurred which is a characteristic of the *Thermoactinomyces* sp.

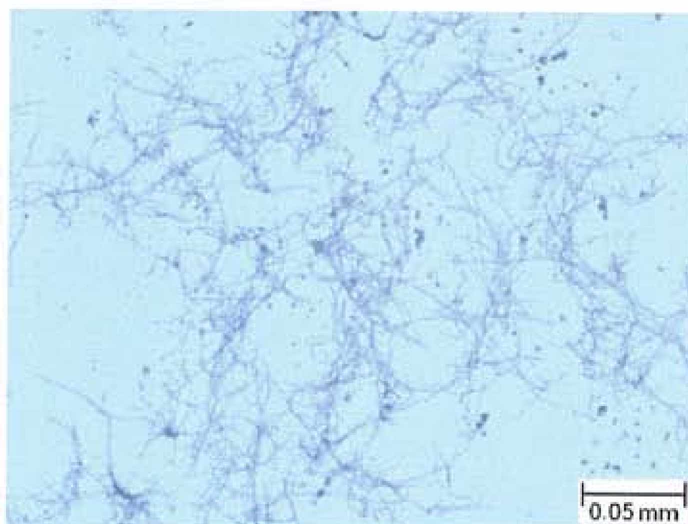


Figure 14 Gram stain of the HRK-1 Isolate

4.4 - Optimization of growth conditions

4.4.1 - Optimal temperature

The isolate HRK-1 was grown at 50, 60, and 70°C at 250 r.p.m. Maximum growth (9.01 mg/ml) occurred at 60°C after around 1.5 days (Fig. 15). Maximal biomass was 9.3% more at 60 than at 50°C, and 15.2% more at 60 than at 70°C. The growth rate at 60°C was (8.3mg/ml/day). A growth rate of 5.3 mg/ml/day was observed at 50°C and 7.82 mg/ml/day at 70°C.

4.4.2 - Optimal pH

Growth curves for isolate HRK-1 under different pH values are shown in (Fig. 16). Optimal pH appeared to be 7.3 using 0.2 M phosphate buffer. Maximum growth at this pH (9.01 mg/ml) occurred at around 1.5 days.

4.4.3 - Optimal aeration rate

Optimal speed of aeration of bacterial batch cultures was determined by growing the organism at speeds of 150, 250 r.p.m. in the orbital shaker at optimal temperature (60°C) and pH (7.3). In this range optimal speed of aeration occurred at 250 r.p.m., as reflected by the highest biomass yield (Fig. 17). (Table-2)

4.4.4 - Fermenter studies

Batch cultures were run in the BIOENGINEERING fermenter at optimal temperature (60°C) and pH (7.3), and at two different speeds (500 & 1000 rpm) using maximal air flow. No important difference was noted between the two speeds, but compared to the orbital shaker the growth rate was much higher but did not yield a high biomass due to the sheering effect of the high speed of the rotor on the bacterial filaments (Fig. 18). (Table-2)

The maximum specific growth rate μ_{max} was calculated from batch fermentation data (1000 r.p.m.). During the exponential growth phase, μ_{max} is equal to the slope in a plot of (ln) of (Biomass mg/ml) versus time and was

determined to be $0.85h^{-1}$ (Fig. 19). Accordingly Doubling time (t_d) was determined: $t_d = \ln 2 / \mu = 0.81$ hrs = 48.93 min.

Table 2 Comparison of biomass changes in the orbital shaker and fermenter at different agitation speeds.

	Revolutions per minute (rpm)	Maximal growth rate recorded (mg/ml/hr)	Maximal biomass recorded (mg/ml)
Orbital Shaker	150	0.062	9.125
	250	0.970	9.450
Fermenter	500	3.56	7.75
	1000	4.19	7.06

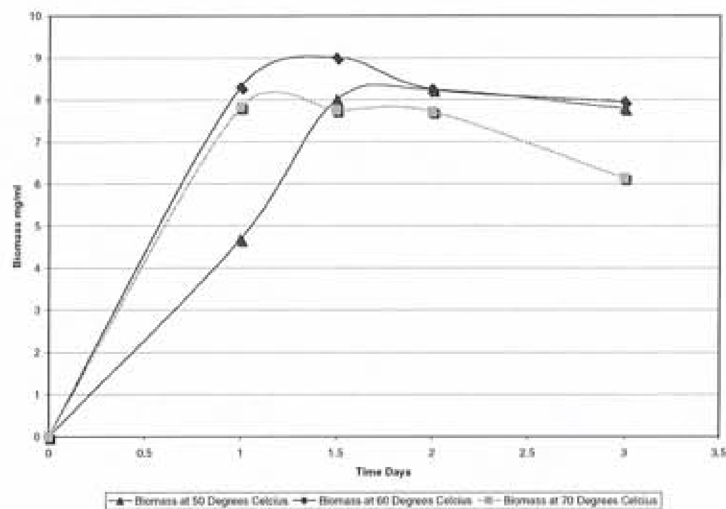


Figure 15 Growth of isolate HRK-1 at different temperatures when cultivated using modified Czapek medium and 1% olive oil at 250 r.p.m.

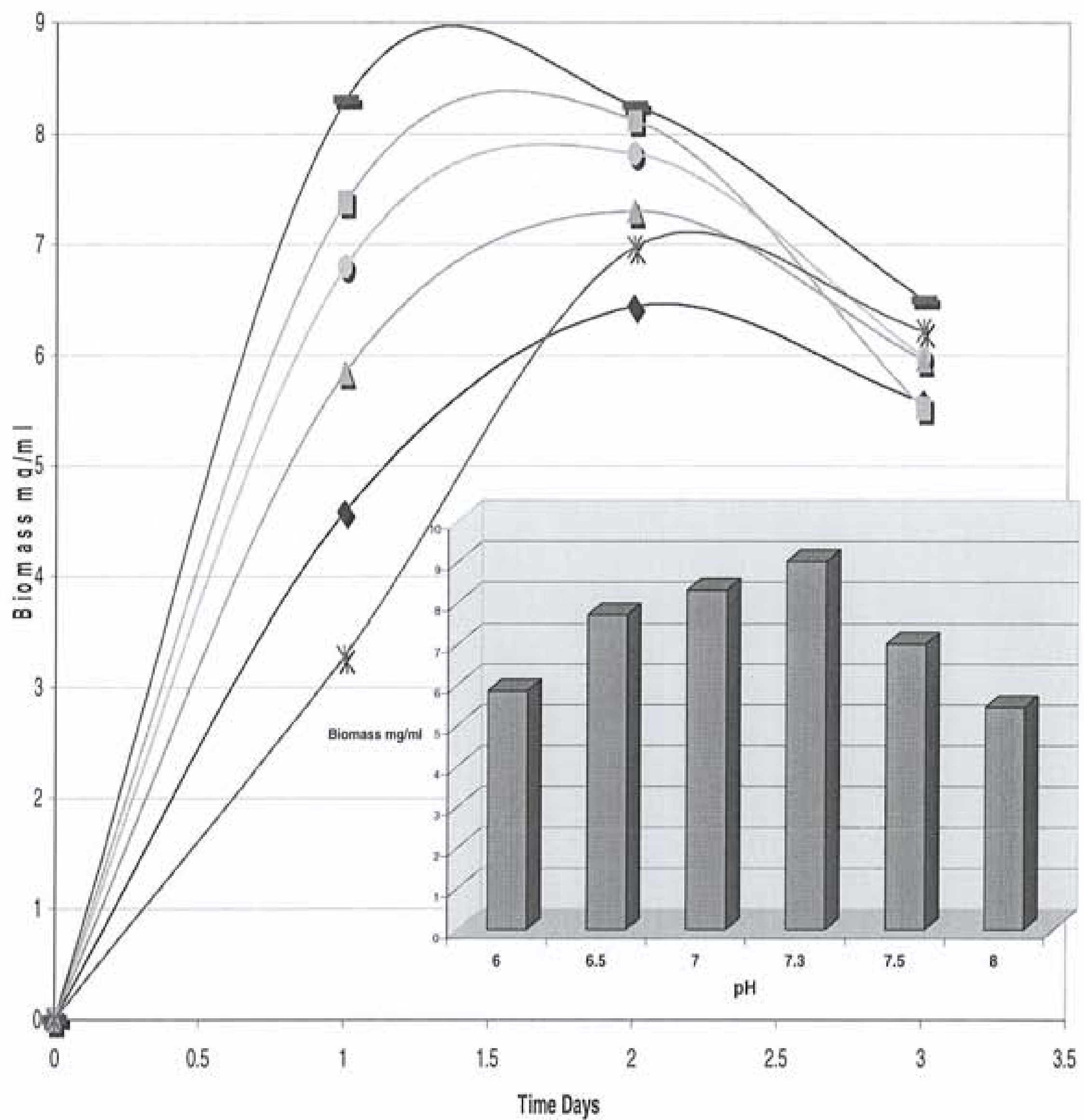


Figure 16 Optimal pH for growth of isolate HRK-1 with bars showing biomass differences at day 1.5 when cultivated using modified Czapek medium and 1% olive oil at 250 r.p.m.

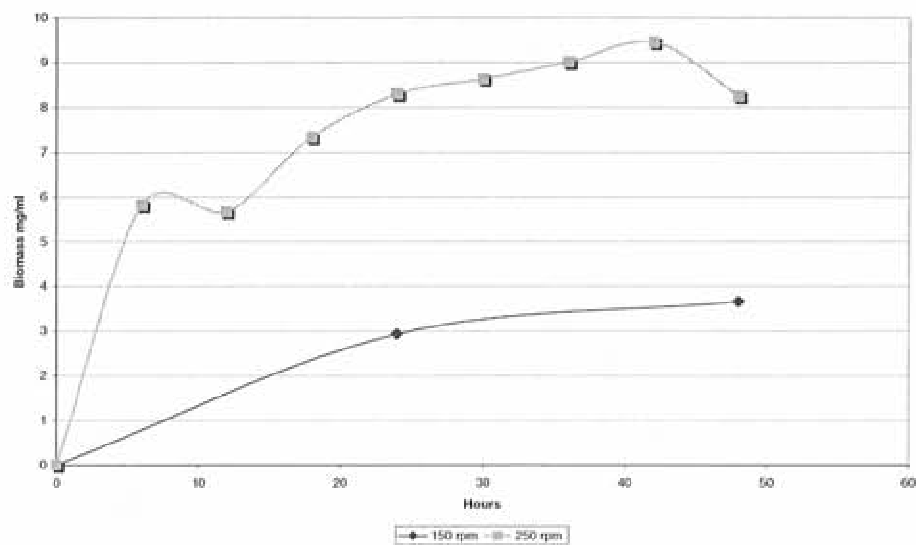


Figure 17 Growth of isolate HRK-1 at different agitation rates (150 & 250 rpm) in an orbital shaker when cultivated using modified Czapek medium and 1% olive oil

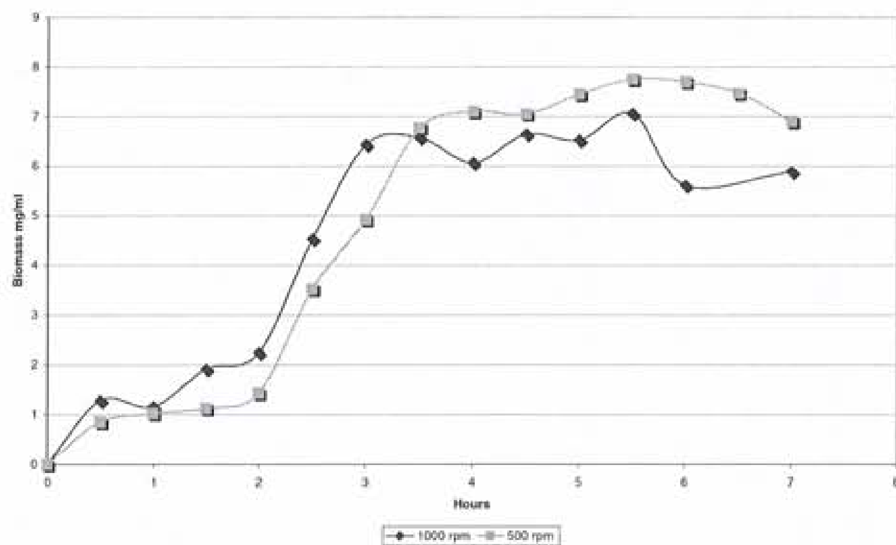


Figure 18 Growth of isolate HRK-1 at different agitation rates (500 & 1000 rpm) in a fermenter under maximal air flow when cultivated using modified Czapek medium and 1% olive oil

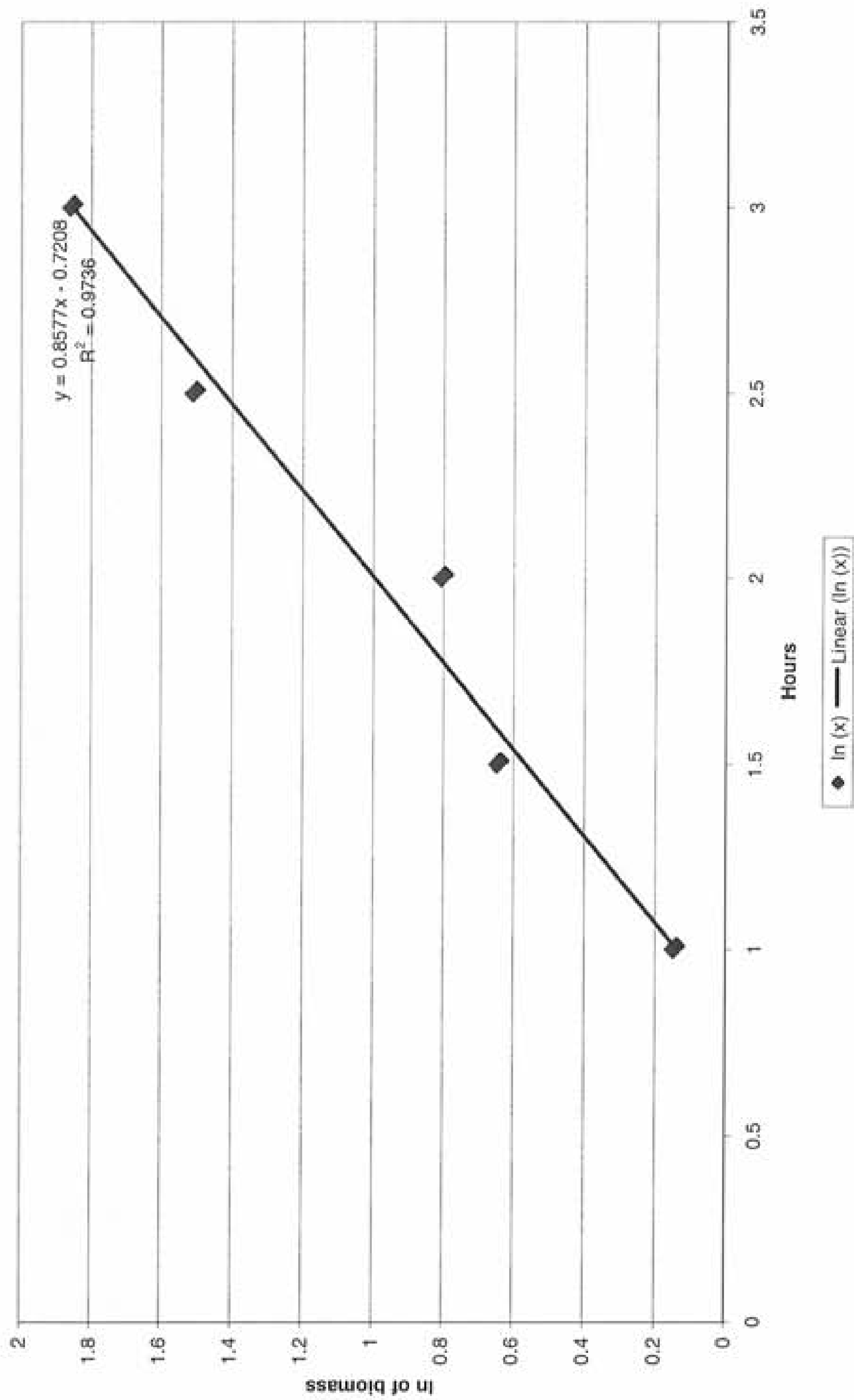


Figure 19 A plot of ln (biomass) vs. Time to determine μ max (slope of the linear trend line)

4.4.5 - Growth under optimal conditions

Optimal growth conditions of the isolate HRK-1 were: temperature, 60°C; pH, 7.3 with 0.2 M phosphate buffer, and an aeration speed of 250r.p.m. in the orbital shaker (Fig. 20).

Growth at optimal conditions appeared to be biphasic. It showed a very high rate exponential phase (0.97mg/ml/hr) followed by a relatively short stationery phase followed by a second exponential phase (0.126mg/ml/hr) and finally a slow decline phase (Fig.20). It is noteworthy to mention that light microscopic examination of cells taken at different growth phases showed that the vegetative cells were transformed to spherical spores during the decline phase (Discussed later).

4.5 - Ability of the isolate to hydrolyze olive oil

To demonstrate the ability of the isolate HRK-1 to hydrolyze olive oil GC analysis was carried out to detect changes in the level of oleic acid. Due to the presence of many products that masked the oleic acid peak, exact determination of oleic acid concentration by reading the area under the peak was not possible. Yet, determination of the change of the level of oleic acid was possible by reading the height of the peaks.

An oleic acid standard was used to determine the position of the oleic acid peak which appeared at around 8.13 minutes.

Oleic acid peak increased smoothly with the increase of biomass (Fig. 21). This indicates the ability of HRK-1 isolate to hydrolyze triolein into oleic acid and glycerol molecules.

Another experiment (HPLC) was carried out to show the decrease in triolein concentration. A triolein standard was used. Triolein peak showed a retention time of 21 minutes (Fig 22). Triolein concentration started decreasing from

3.3 mg/ml at the beginning of the cultivation and disappeared after 36 hours of cultivation (Fig. 23, 24).

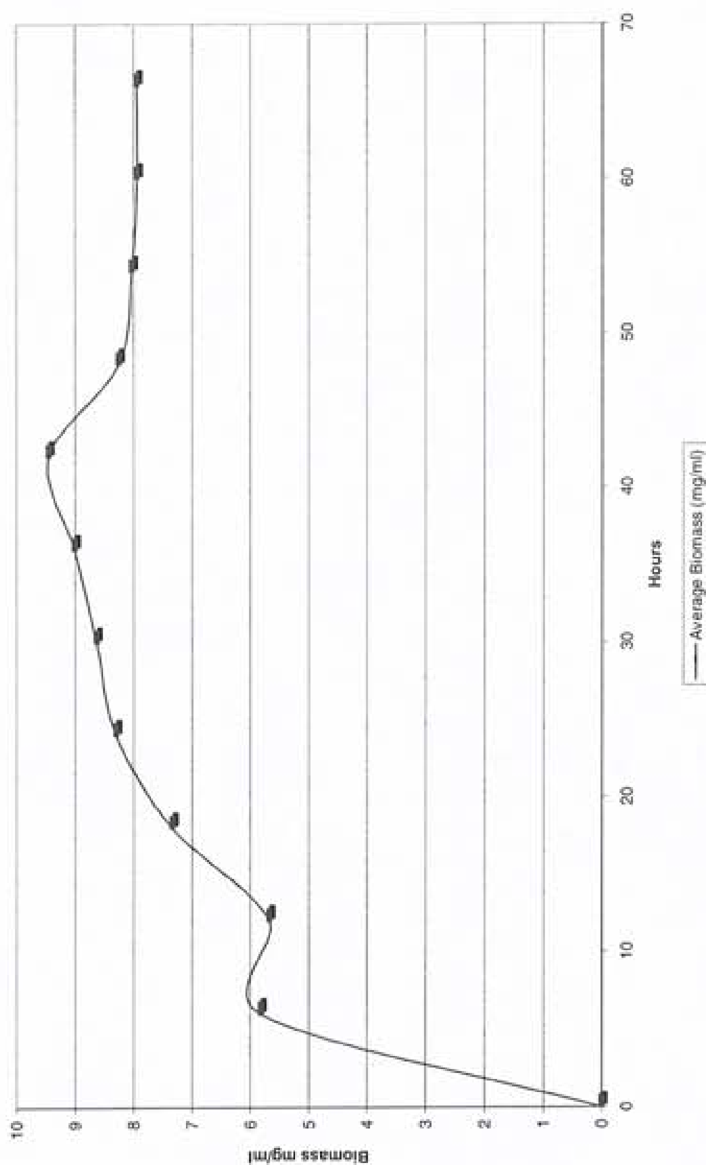


Figure 20 Growth curve of isolate HRK-1 under optimal conditions. 1% olive oil, Temperature; 60°C, pH; 7.3, and agitation rate; 250 r.p.m.

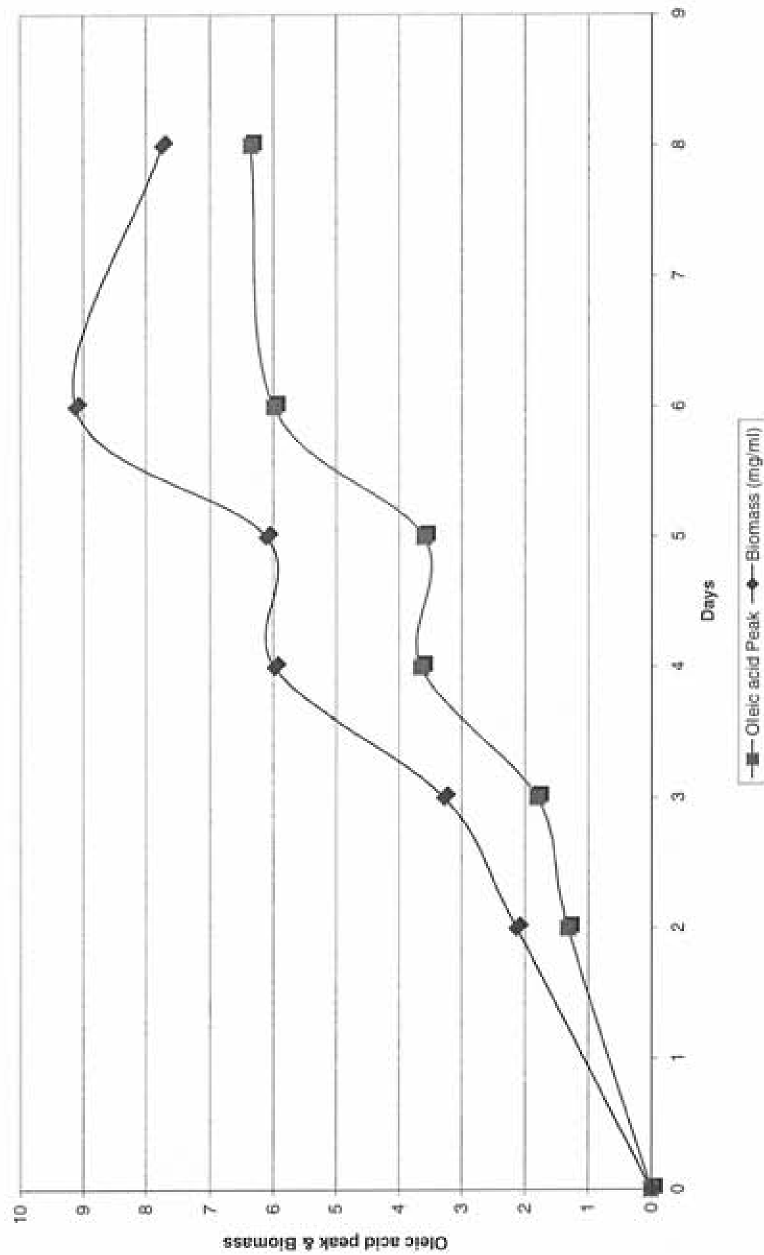
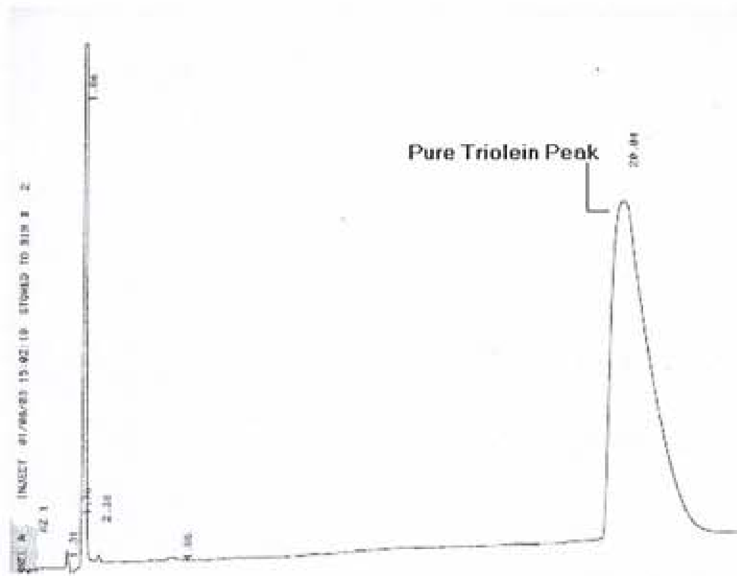


Figure 21 Growth curve of isolate HRK-1, Temperature: 60ºC, pH: 7.3, and agitation rate: 150 r.p.m. (orbital shaker) using 1% olive oil in modified Czapek medium and the increase in oleic acid peaks as an indication of triolein hydrolysis as determined by GC experiments



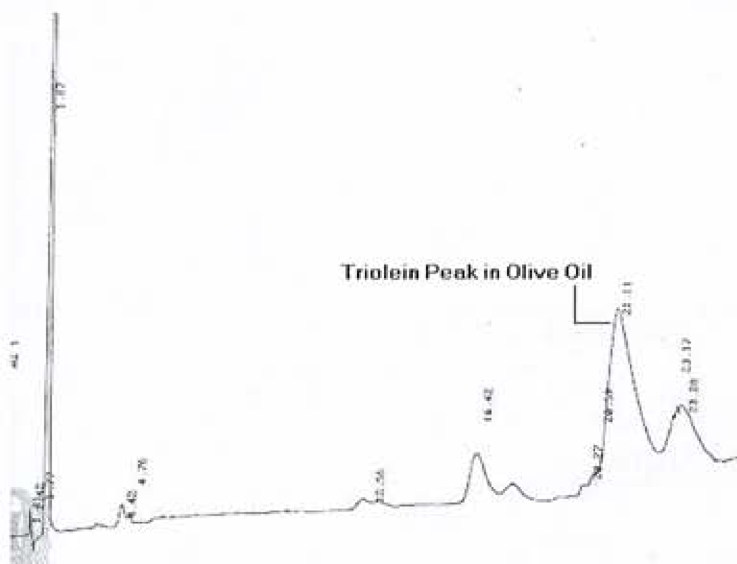


Figure 22 HPLC chromatogram of triolein, in a pure sample (standard) and in pure olive oil, showing a retention time of 21 min

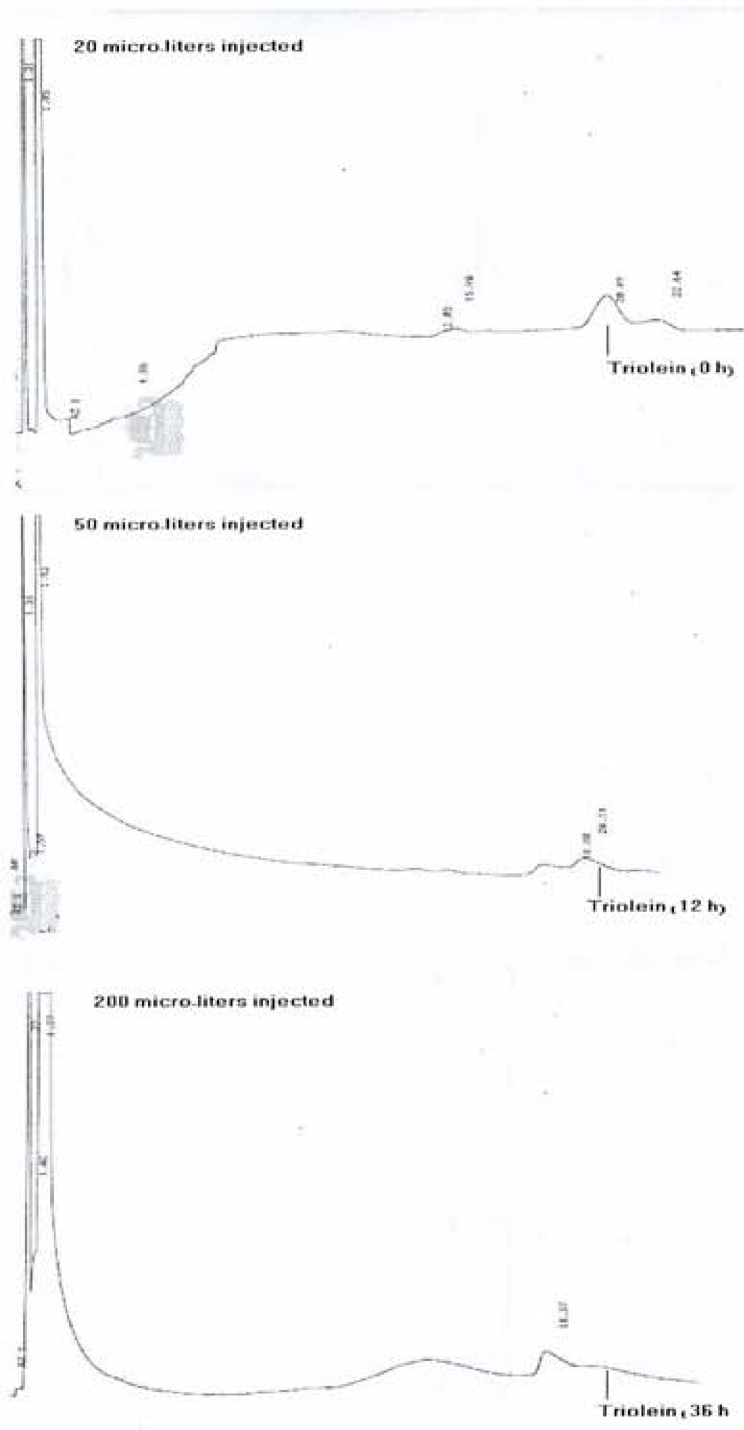


Figure 23 HPLC chromatograms – (Chart speed 1cm/min) showing decrease in the area of triolein peak as an indication of triolein hydrolysis during cultivation of HRK-1 at 60^{0}C, pH 7.3, and agitation of 250 r.p.m.

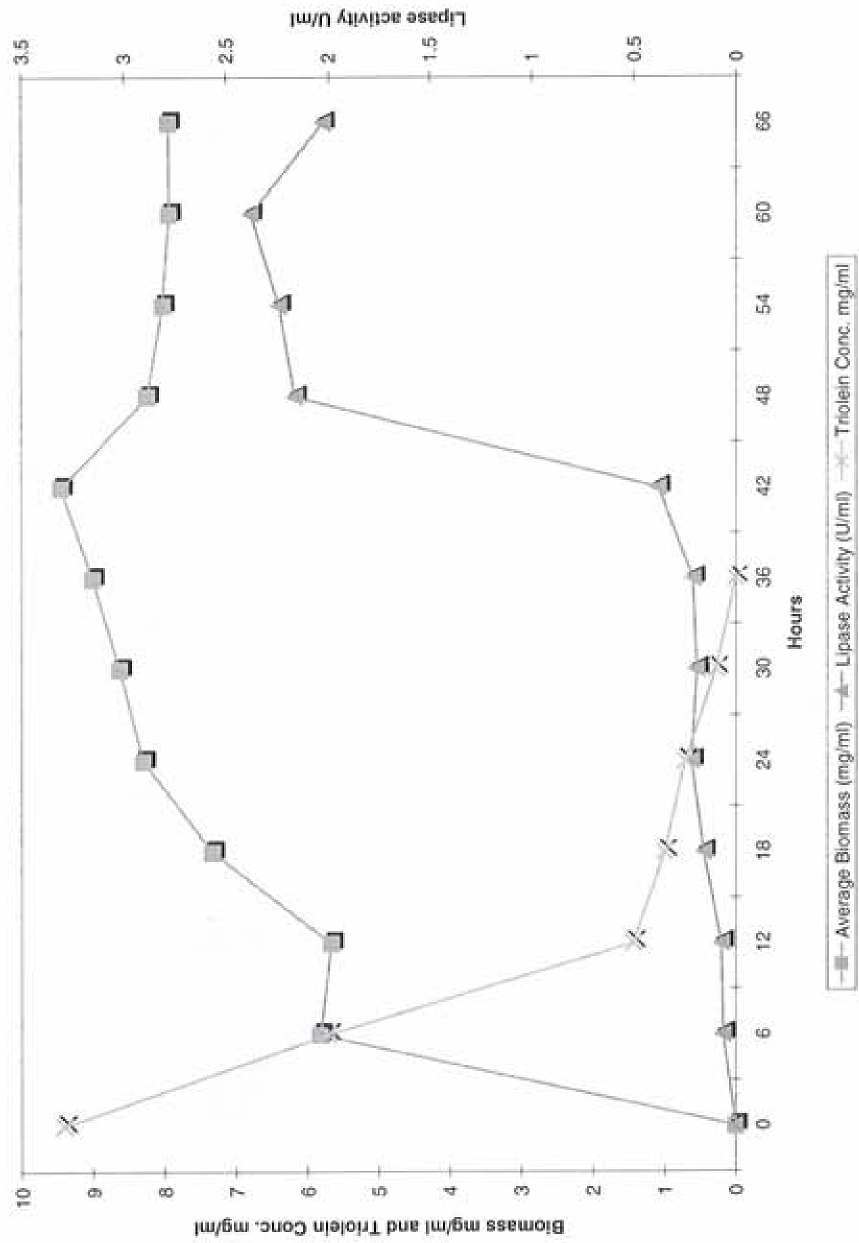


Figure 24 Growth curve of isolate HRK-1, Temperature: 60ºC, pH: 7.3, and agitation rate: 250 r.p.m. (orbital shaker) using 1% olive oil in modified Czapek medium and the decrease in triolein conc. as an indication of triolein hydrolysis as determined by HPLC experiments

4.6 - Time course of lipase activity

The results obtained for lipase activity in growth experiments in modified Czapek medium with 1% olive oil at 60°C, pH 7.3, and 250 r.p.m in the orbital shaker are represented in (Fig. 25).

Maximal bacterial growth was reached after 42 hours and then it started to decrease. Lipase activity shooted when biomass started to decrease which, suggests the release of intracellular lipase from cells upon their bursting. At this phase lipase productivity reached a maximum of 296.83 U/l/h.

The pH of the broth decreased slightly from 7.3 to 7.05 during the first 6 hrs of growth and then increased again until it reached 9.0 at the end of the decline phase.

Extracellular lipase activity increased slightly during the early hours of growth and then increased exponentially at 42 h to reach a maximum at the beginning of the bacterial death phase when the percentage of filaments was lowest.

4.7 - Effect of olive oil concentration on biomass and lipase activity

Investigations were conducted to study the influence of olive oil concentration (0% to 10%) on biomass and lipase activity (Fig. 26, 27 & 28).

Maximal lipase activity was obtained with 0 & 1% olive oil. Lipase activity was not enhanced at olive oil concentrations higher than 1%; on the contrary, a decrease in enzyme activity was recorded (Fig. 26).

Maximal biomass obtained showed a constant increase with the increase of olive oil concentration. Minimal biomass was achieved by using 0% olive oil (1.76mg/ml in 72 h). Maximal biomass was achieved by using 10% olive oil (10.16 mg/ml in 120 h) (Fig. 27 & 28).

It was noticed that olive oil concentrations of more than 3% cause a temporary slight inhibition at the beginning of the growth phase which is followed by an exponential growth (Fig. 27).

The maximal biomass reached with 10% olive oil at 150 r.p.m. (10.1 mg/ml) was equivalent to the maximal biomass reached with 1% olive oil at 250 r.p.m. (9.45 mg/ml)

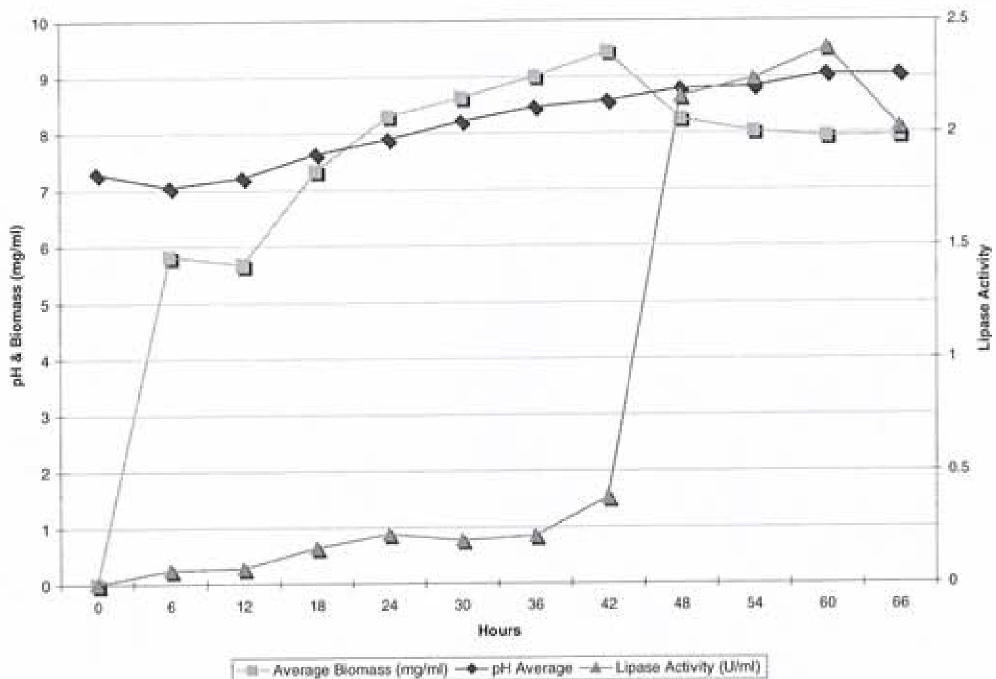


Figure 25 Time course of lipase activity and pH changes during growth of strain HRK-1 on 1% olive oil in modified Czapek medium at optimal conditions of temperature, 60ºC, pH, 7.3, and agitation, 250 r.p.m.

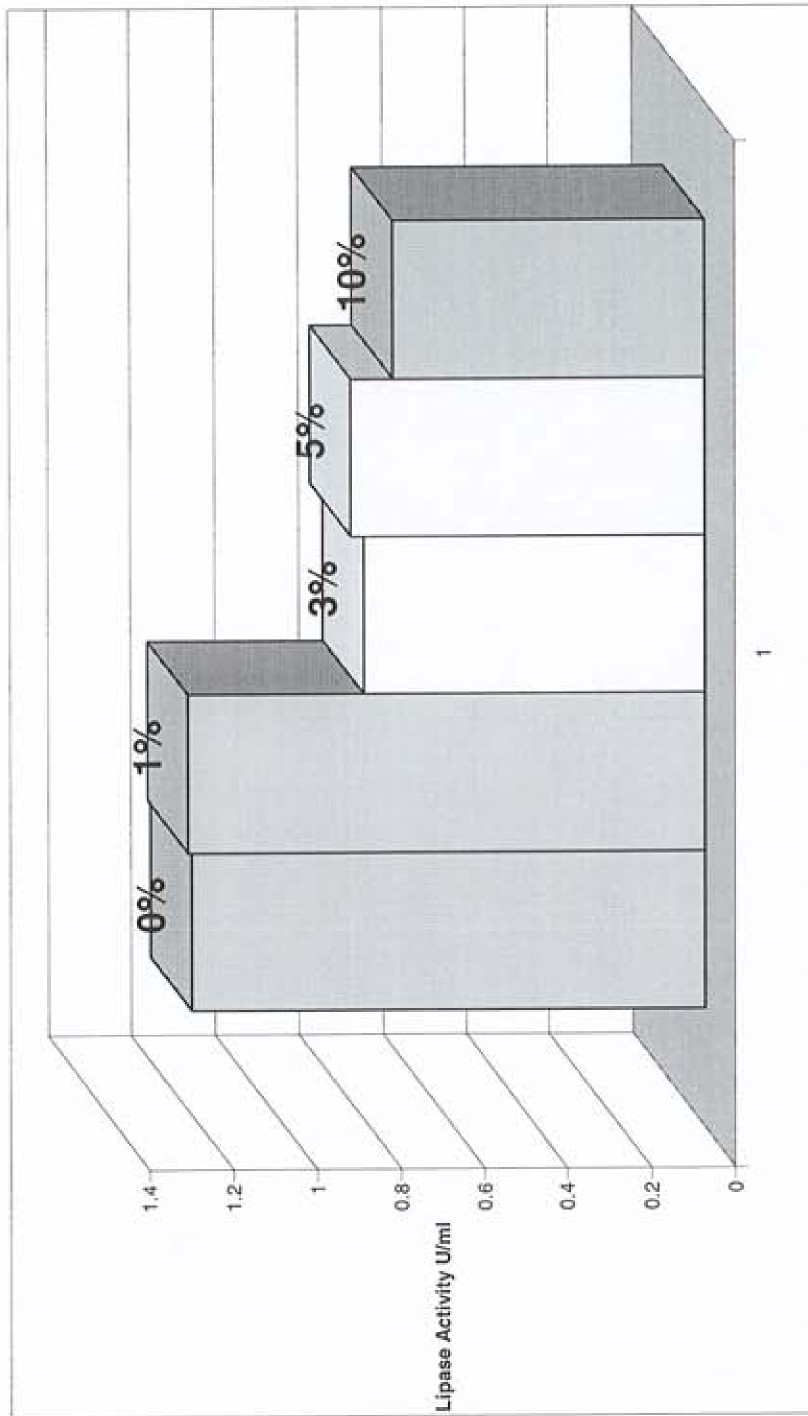


Figure 26 Effect of olive oil concentration on lipase activity

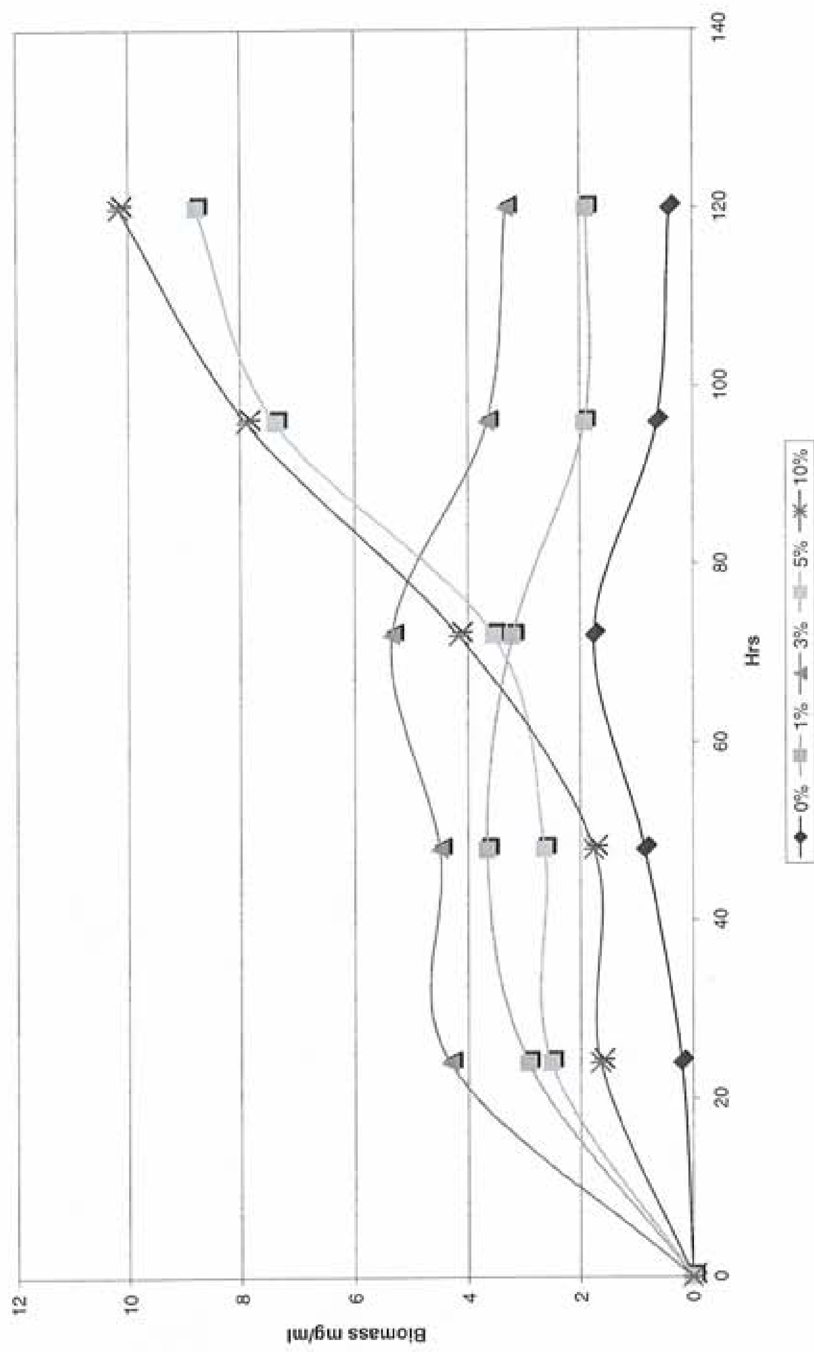


Figure 27 Biomass change during the cultivation of HRK-1 on different olive oil concentrations in modified Czapek medium at 60ºC, pH 7.0, and agitation 150 r.p.m.

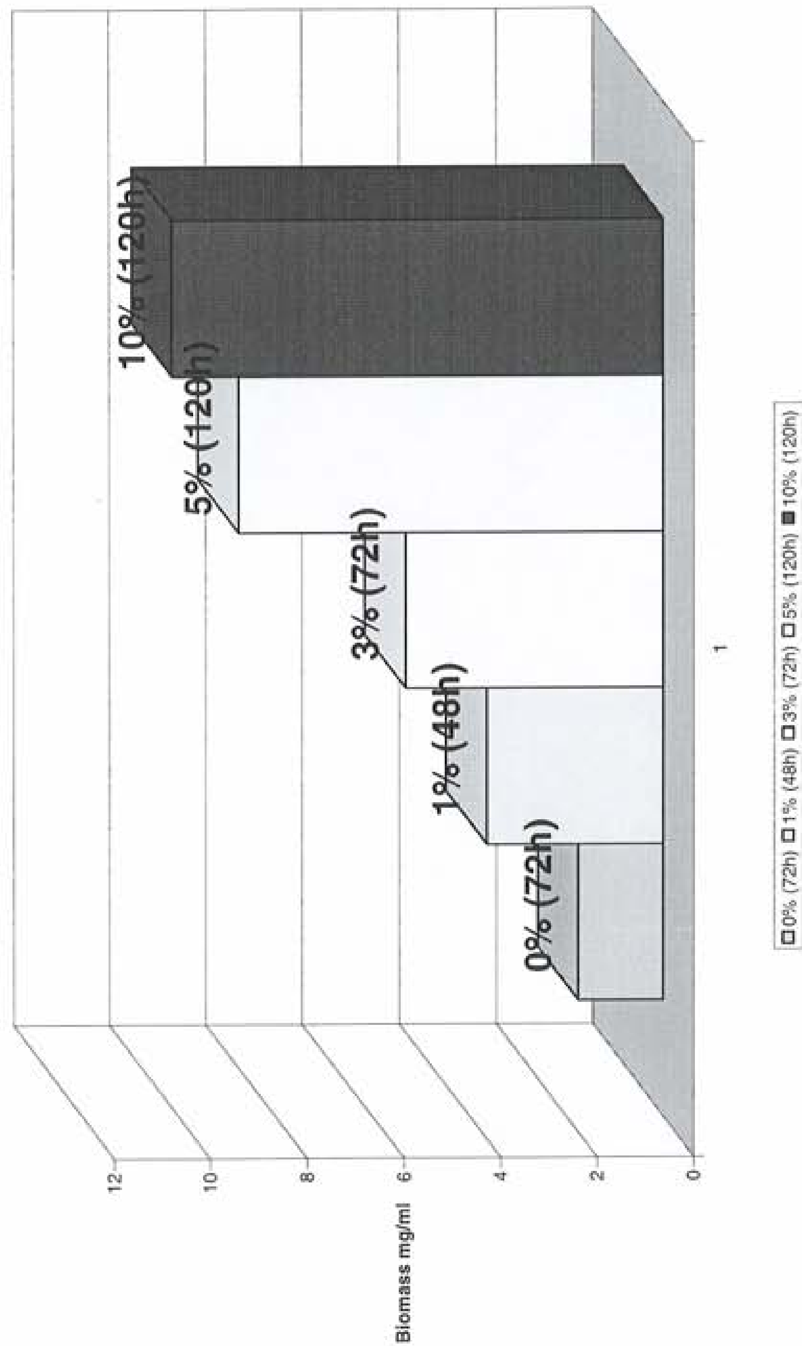


Figure 28 Effect of olive oil concentration on maximal biomass obtained. Bars showing time needed to obtain maximal biomass at different olive oil concentrations

4.8 - Morphological changes during growth on 1% olive oil

In the following paragraph morphological changes of the isolate HRK-1 are described during cultivation in a 1000-ml Erlenmeyer flask filled with 500 ml of modified Czapek medium and 1% olive oil at a temperature of 60°C, and a pH of 7.3 and an aeration rate of 250 r.p.m. in the orbital shaker (Fig. 29).

- During the early 6 hours the innoculum start proliferating into small short rods with a single spore at the end of each.
- After 12 hours some short filaments start to appear.
- At 18 hours clusters of filaments start to appear.
- At 24 hours filament clusters start to grow up and increase in size.
- At 36 hours filament clusters start to decrease in size.
- At 48 hours only some filaments attached to oil droplets can be seen and the rest of the culture is full with spores.
- Finally, at 54 hours only spores can be seen in the culture.

A morphological feature that was observed only when HRK-1 was cultivated in liquid cultures at low (6.0) or high (8.0) pH was the formation of spherical colonies with filaments projecting on the surface (Fig. 30).

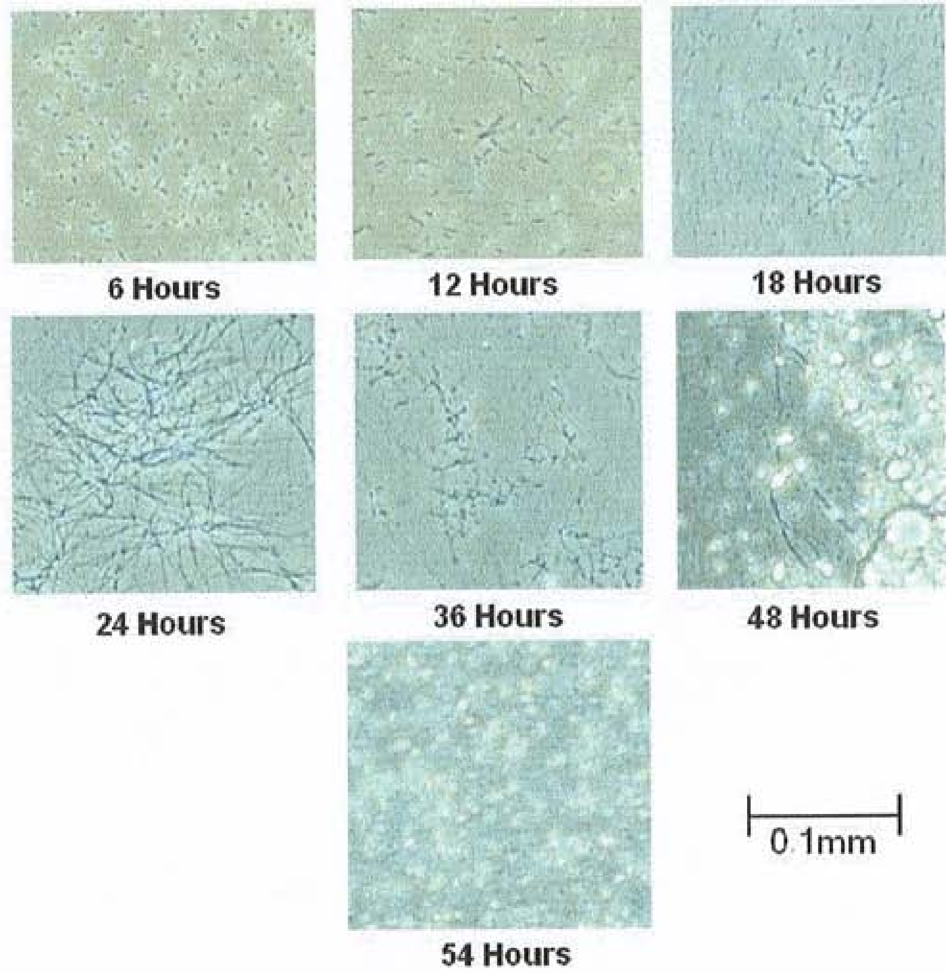


Figure 29 Morphological changes of HRK-1 during growth on 1% olive oil under optimal orbital shaker conditions

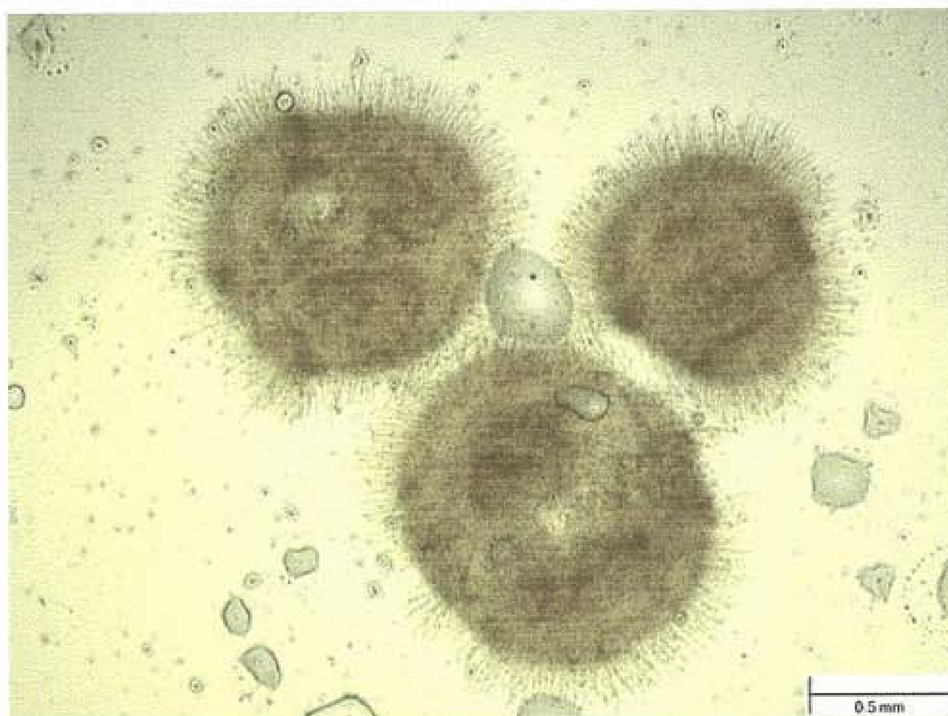


Figure 30 Spherical filamentous colonies observed when HRK-1 is cultivated under low or high pH values.

4.9 - Electrophoretic pattern of the Lipase

Electrophoretic mobility profiles of the lipase are shown in (Fig. 31).

The enzyme crude extract obtained by ultrafiltration was run on a nondenaturing polyacrylamide gel. Only one lipase (HRKL-1) band was identified after extraction of different bands from the gel and testing for lipase activity (Fig. 31).

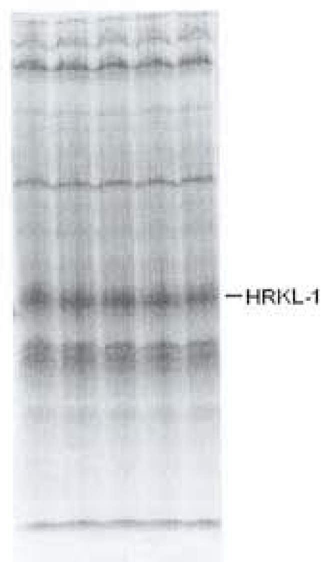


Figure 31 HRKL-1 pattern under native gel electrophoresis

The identified lipase band was then run on a gradient denaturing SDS-PAGE along with HMWM and LMWM and a lipase crude sample as a control (Fig.32).

The Rf value of the HRKL-1 band was determined to be 0.46667.

By extrapolation from (Fig. 13) the molecular weight of HRKL-1 turned out to be 80.0583 k Daltons.

The HRKL-1 band appears displaced and a little bit higher in position than the corresponding band in the crude sample (Fig. 32). This is due to an expected error that may result by using this technique. The crude control sample used however compensated for the error produced.

Another band was generated of molecular weight 26.0296 kDa which is most likely a monomer produced by the denaturing effect of SDS on HRKL-1

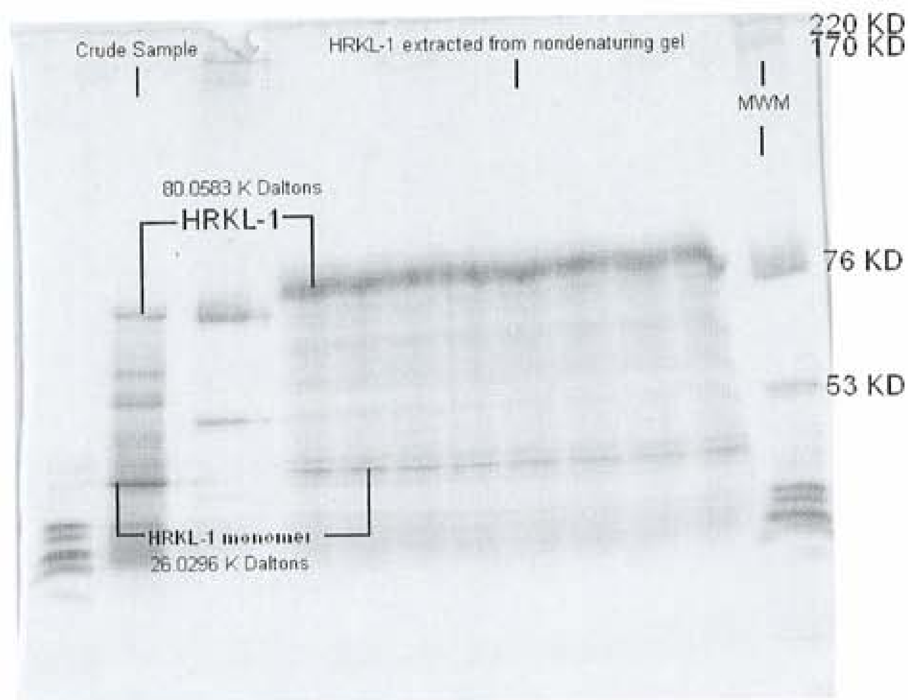


Figure 32 SDS-PAGE of HRKL-1

4.10 - Temperature and pH optima of the lipase HRKL-1

The optimal temperature and pH for the activity of HRKL-1 were determined under standard conditions. The optimal temperature for the activity of the lipase was 60°C (Fig. 33) and the residual enzyme activities measured at 65 and 70°C were 56 and 49 % respectively.

The lipase HRKL-1 exhibited a pH optimum of 8.0. The effect of different pHs on lipase activity is given in (Fig. 34).

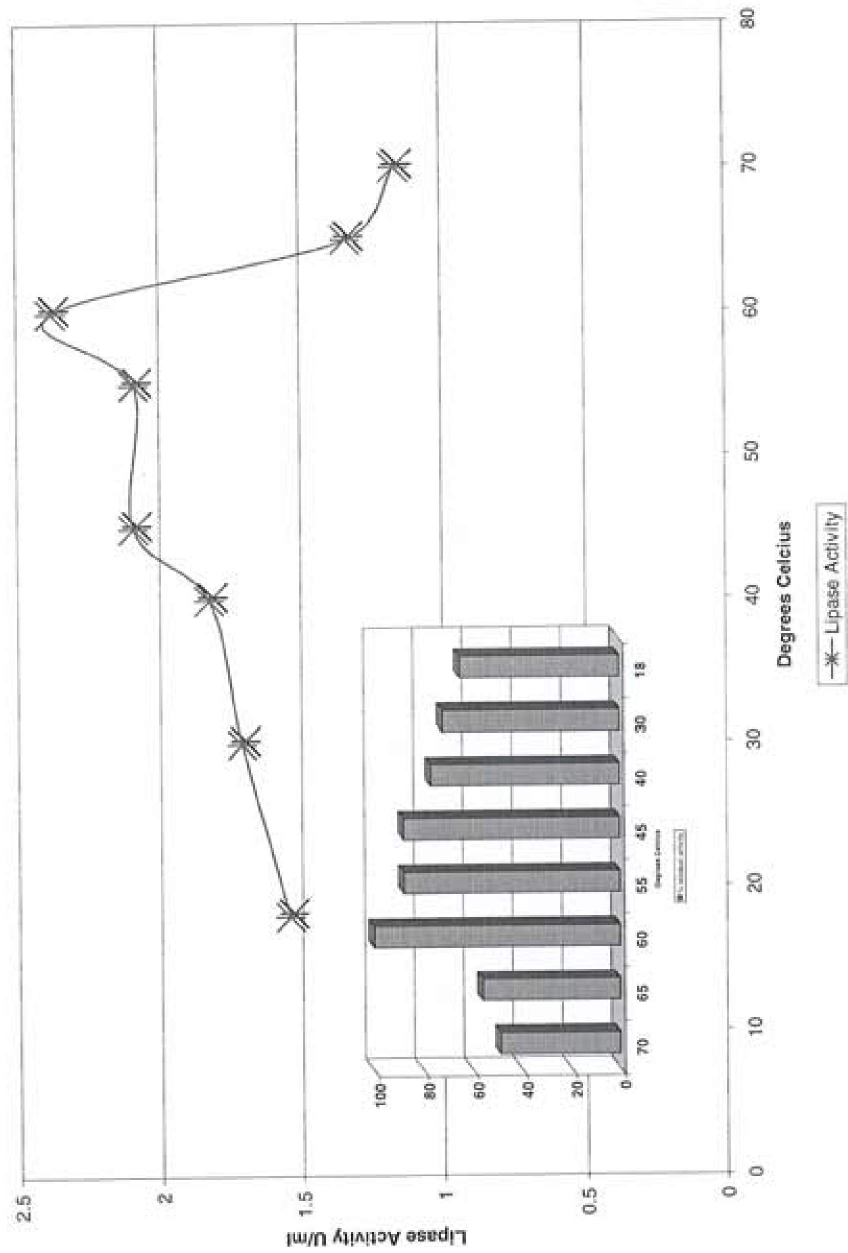


Figure 33 Effect of temperature of assay on HRKL-1 activity with insert showing % residual activity at different temperatures

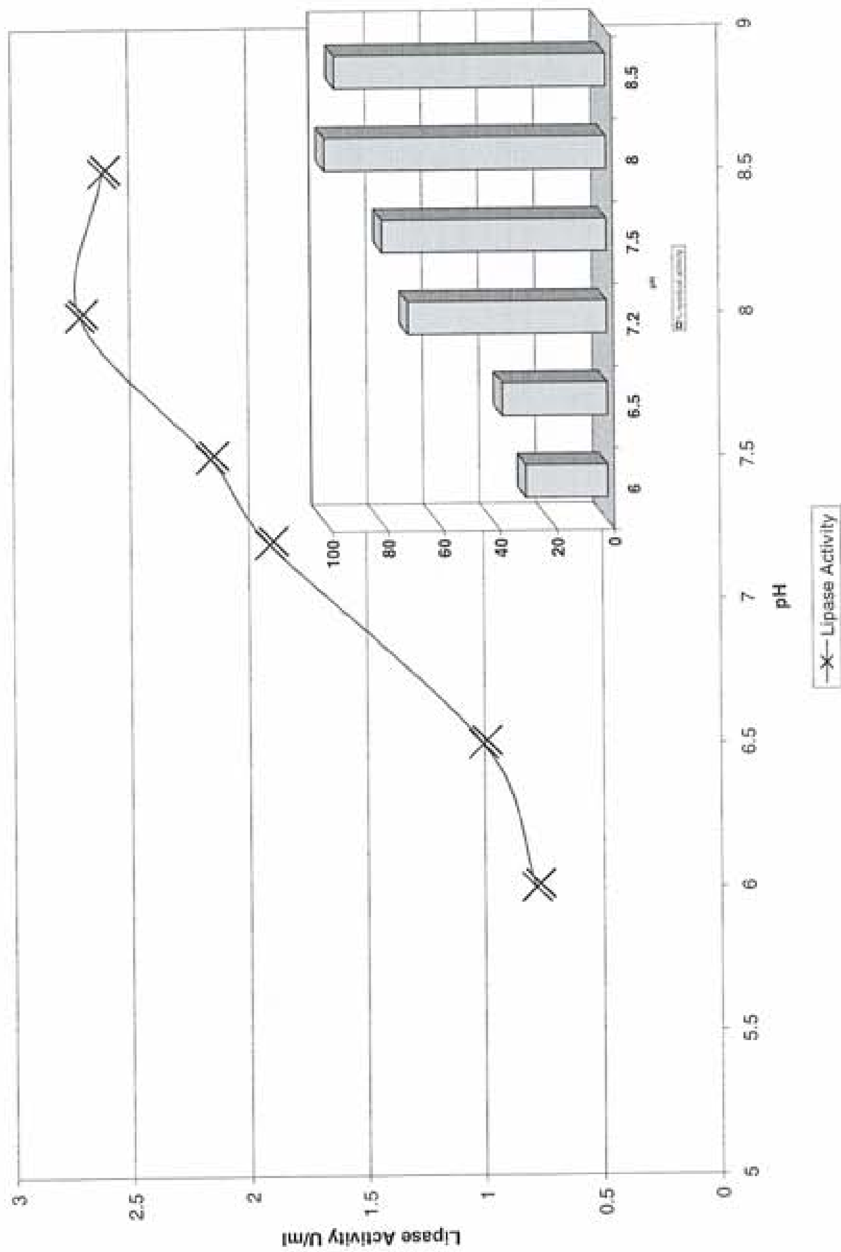


Figure 34 Effect of pH of assay on HRKL-1 activity with insert showing % residual activity at different pH values

4.11 - Thermostability of HRKL-1 upon heat treatment

The enzyme showed a very high stability upon treatment with very high temperatures. No loss of activity was detected even after boiling for 1 hour (Fig. 35).

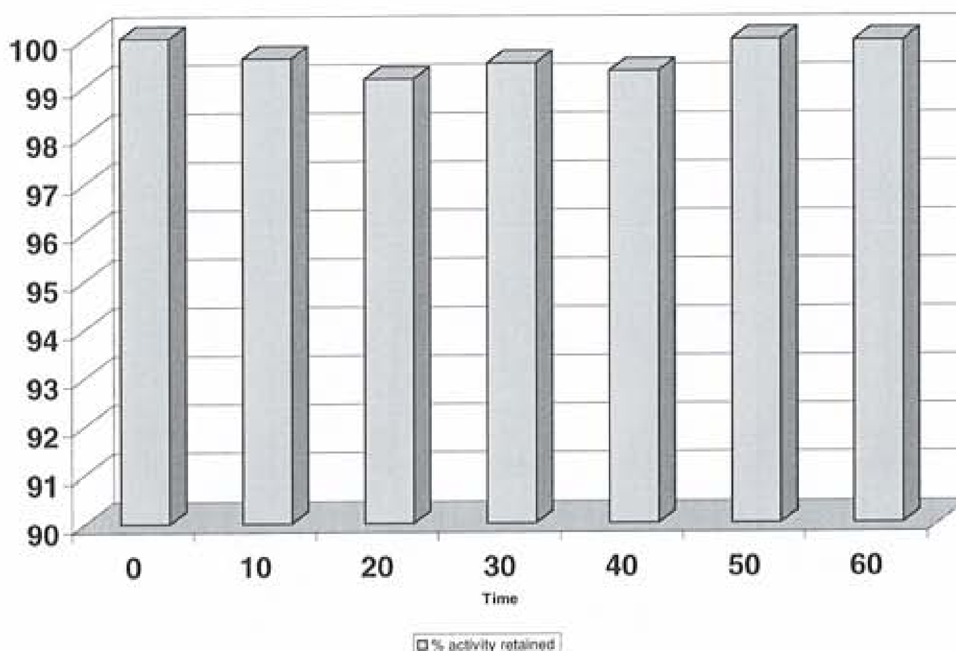


Figure 35 Effect of prolonged boiling on lipase activity with bars showing % residual activity

4.12 - Determination of Km value and Vmax

To obtain the Km value of HRKL-1, initial reaction rates were measured at different substrate (*p*-nitrophenyl laurate) concentrations within the range of 25mM – 0.025mM. From the data for the reaction rates for each substrate concentration, a straight line was obtained with the Lineweaver-Burk plot (Fig. 36). From this straight line, the Michaelis constant and the maximum

reaction rate were calculated. The following values were obtained: Km = 0.021 mM and Vmax = 0.062 U/mg protein.

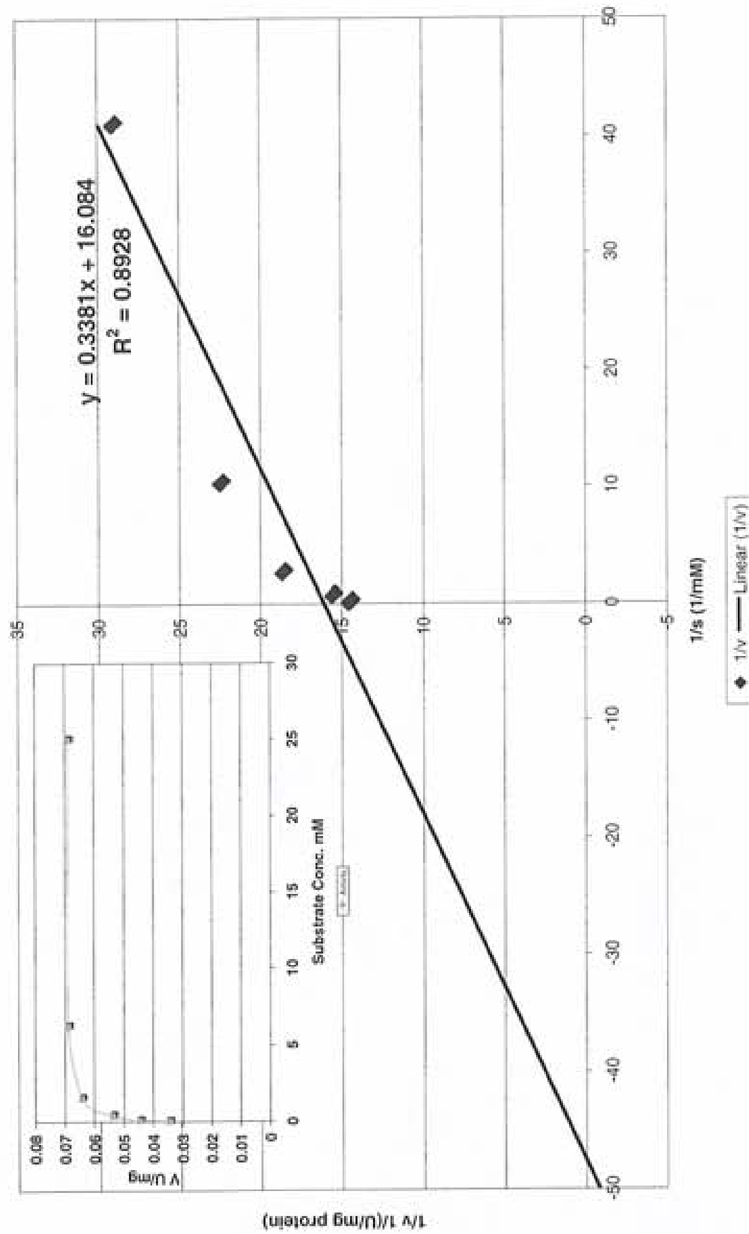


Figure 36 Lineweaver-Burk plot of reaction rates. Michaelis-Menten plot in the insert

4.13 - Enzyme activity as a function of enzyme concentration

(Fig. 37) shows the effect of different enzyme concentrations on enzyme activity. Upon diluting the enzyme 2, 4, 8, 16, & 32 X a corresponding decrease in enzyme activity was observed.

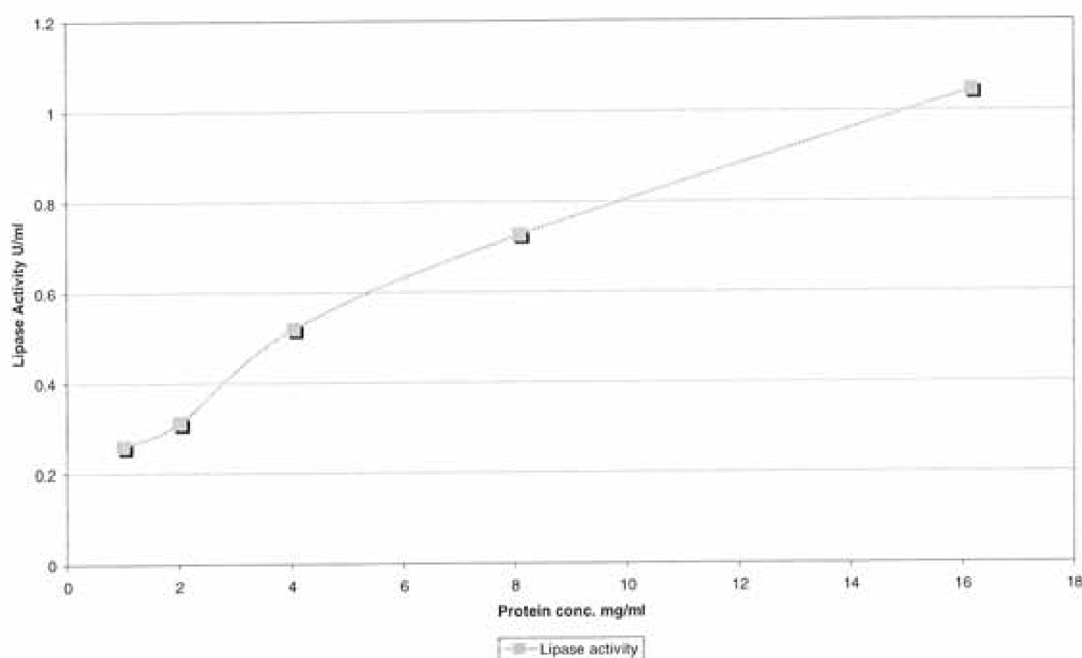


Figure 37 Enzyme activity as a function of enzyme concentration

4.14 - Effect of metal ions and inhibitors on lipase activity

The effect of metal ions and inhibitors was studied using the standard *p*-nitrophenyl laurate assay. The results are summarized in (Fig. 38 & 39) Lipase activity was enhanced by one mM of each of Fe^{++} (106%), Fe^{+++} (125%), & Ca^{++} (124%) ions. Lipase activity was slightly affected by 1 mM of SDS (93%), and Urea (85%).

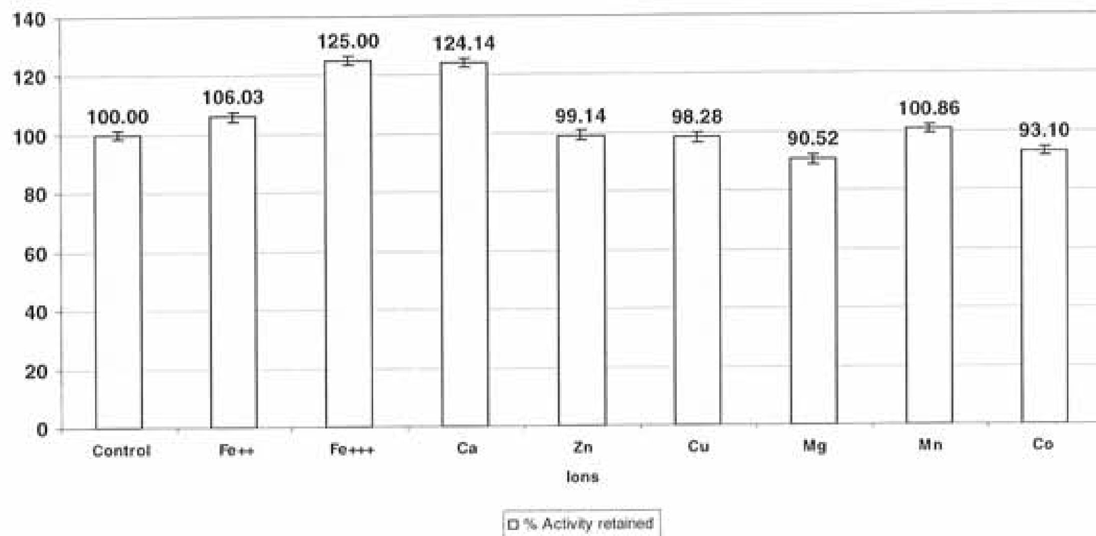


Figure 38 Effect of metal ions on activity of HRKL-1with bars showing % residual activity

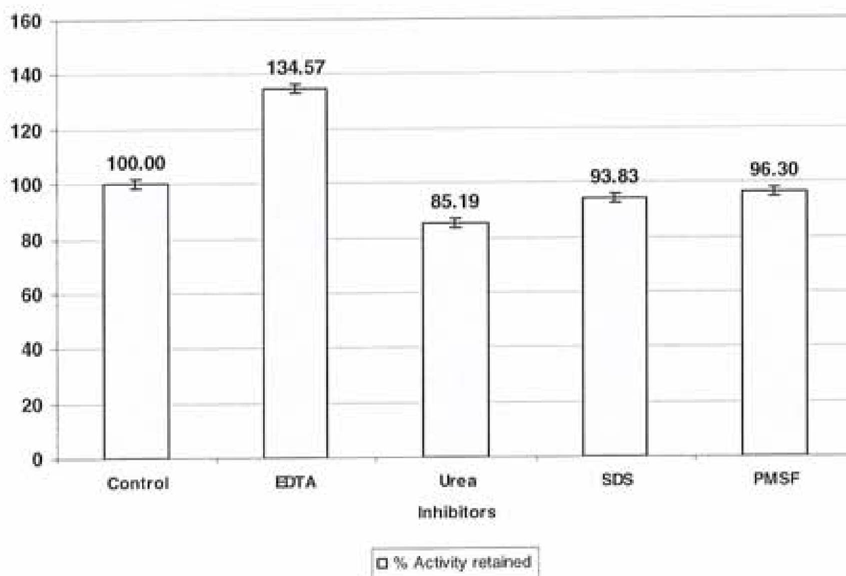


Figure 39 Effect of inhibitors on activity of HRKL-1 with bars showing % residual activity

Chapter 5

DISCUSSION

The industrial application of biocatalysts began in 1915 with the introduction of the first dehairing (pancreatic) enzyme by Dr. Rohm. Since that time enzymes have found wider application in various industrial processes and production. There are more than 3,000 enzymes known to date that catalyze different biochemical reactions among the estimated 7,000. The world market for industrial enzymes, which includes enzymes for research and diagnosis, is estimated to be around 1 billion US$. The products derived form these enzymes are estimated to represent a value of more than 100 billion US$. For various industrial applications there is a great demand for enzymes of high specificity and stability. Extreme environments provide a unique resource of microorganisms and novel biocatalysts (Bertoldo et. al. 2001).

Lipases are widely distributed in nature. Although lipases have been found in many species of animals, plants, bacteria, yeast and fungi, the enzymes from microbial sources are currently receiving particular attention because of their actual and potential application in industry mainly in the detergents, oils, fats, dairy, and pharmaceutical industries (Sarkar et. al. 1998).

Apart form their general biological significance; lipolytic enzymes play an increasingly important role in biotechnology. For the biochemist, perhaps the most important and fascinating aspect of lipolytic enzymes is the unique physicochemical character of the reactions they catalyze. These enzymes are perfectly water soluble and act very efficiently on water insoluble lipidic substrates which spontaneously self organize in water as monomolecular films, bilayers, liposomes, emulsions, or micelles. This catalysis is essentially occurring at the lipid/water interface (Verger et al. 1992).

In particular, lipases from thermophiles are expected to play a significant role in industrial processes, since they are thermostable and resistant to chemical denaturation (Lee et al. 1999).

Most of the esterases and lipases yet reported are isolated from mesophilic microorganisms: *Pseudomonas cepacia, Pseudomonas fluorescens, Psuedomonas aeroginosa, Arthrobacter globiformis, Candida cylindracea, Butyrivibrio fibrisolvens, Brevibacterium linens, Acinetobacter calcoacticus, Bacillus subtilis*, and *Aspergillus niger.* Although they have interesting properties, few industrial chemical processes have been developed using these enzymes. The major drawbacks limiting the industrial use of these enzymes is their instability mainly at high temperatures, in operating industrial conditions; therefore, the search for new thermostable enzymes is important for the development of new applications. Furthermore, it has been reported that the enzyme resistance to denaturation in organic solvents is correlated with their thermostability in water. For this reason, thermostable enzymes are attractive not only to be used in aqueous media but also in organic media (Kademi et al. 1999).

There are few reports in the literature involving thermostable lipases originating from thermophiles. In addition, reports on lipases originating from thermophilic actinomycetes seem lacking. This study is a report on the isolation and characterization of an aerobic, filamentous, Gram-positive, spore forming, thermophilic actinomycete that degrades olive oil and secretes large amounts of a highly active and thermostable extracellular lipolytic enzyme. In the absence of DNA sequencing, this soil isolate, designated as HRK-1, can be classified as *Thermoactinomyces putidus* - According to Bergey's manual of determinative bacteriology (Holt et al. 1994) due to the filamentous structure of its molecules that bear one spore each on an un-branched sporophore, the resistance of its spores to boiling, utilization of sucrose as a carbon source and production of dark pigments. HRK-1 grew optimally at 60^{0}C and a pH of 7.3 (Fig. 15 & 16).

Studies by Unsworth et al. (1977) were done on archeological samples deposited 1950 to 2750 years ago, showed that they contain viable Thermoactinomycete endospores. These studies confirm the extreme longevity of Thermoactinomycete endospores and suggest that these organisms may be usefully employed in future archeological and palaeoecological studies.

Thermoactinomyces putidus was included in the IJSB list in Oct. 1989 (List No. 31) which validates the name and thereby makes it available in bacteriological nomenclature.

Based on DNA relatedness data the type strains of *Thermoactinomyces intermidus, Thermoactinomyces putidus, Thermoactinomyces dichotomicus,* and *Thermoactinomyces peptonophilus* were considered to be distinct species of the genus *Thermoactinomyces* (Yoon et al. 2000).

In the present work, 0.2% of yeast extract served both as nitrogen source and growth factor. The requirement of yeast extract for growth at 60^0C is indicative of loss of synthetic abilities, reported to occur in certain bacterial thermophiles (Elwan et al. 1978).

The morphological changes of HRK-1 during batch fermentations were studied using light microscopy. HRK-1 grew during the first early hours of cultivation into small rods with a single spore at the end of each. Later filaments started to appear. The density of the filaments increased until maximal biomass was reached after which the filaments started to disappear and spores started to appear. Finally only spores were seen at the end of cultivation (Fig. 29). A distinct morphological feature of HRK-1 was its ability to form spherical filamentous colonies when subjected to extreme pH values (Fig. 30). This formation might help to protect the bacterial cells from the harsh pH conditions.

Results obtained in growth experiments, indicated that growth of HRK-1 was closely related to olive oil utilization where maximal biomass values reached increased with the increase of olive oil concentration in batch cultures (Fig. 26, 27).

When olive oil concentration was increased beyond 3% in shaking liquid cultures a temporary and slight inhibition of biomass was detected at the beginning of the growth phase; then it was followed by an exponential growth phase (Fig. 27). This inhibition at the beginning of growth could be due to the sealing effect of high olive oil concentrations that prevent oxygen from mixing efficiently with the liquid at low r.p.m. (150).

Interestingly no inhibition of growth was observed even when olive oil concentration was increased to 10%.

Maximal growth rate recorded for the HRK-1 isolate at optimal growth conditions and at 1000 r.p.m. in the fermenter was 2.143 mg/ml/hr and the corresponding maximal specific growth rate was $0.85h^{-h}$ (Fig. 19). At similar conditions (agitation between 750 and 1750 r.p.m.) the specific growth rate of *Bacillus thermoleovorans* IHI-91 was 1.0^{-h} (Becker et al. 1997). However, the isolate ID-1 showed a higher rate of 2.50 h^{-1} (Lee et al. 1999).

Maximal lipase activity and release of the enzyme was detected during the decline phase of growth, which coincides with the lysis of the filaments to leave a layer of spores (Fig. 25). This suggests that the cells lyse to release the spores and the intracellular lipase which could reflect a lipase release problem. This was not observed in batch cultures of thermophilic bacilli where lipase activity correlated with cell density over the first hours of growth, and then declined after cessation of growth due to thermal inactivation (Becker et al. 1997).

When the amount of extracellular lipase produced by HRK-1 was compared to values found in the literature, important differences were noted. The

maximal extracellular amount of lipase produced by HRK-1 (2.37 U/ml), measured in cell free supernatant, was 59 folds higher than the one produced by *Bacillus circulans* (Kademi et al. 1999), 7 to 8 folds higher than the one produced by *Bacillus thermoleovorans* IHI-91 (0.3U/ml) (Becker et al. 1997) and *Bacillus thermocatenulatus* (0.36U/ml) (Schmidt et al. 1994), and 3 to 4 folds higher than the one produced by *Bacillus thermoleovorans* ID-1 (0.7U/ml) (Lee et al. 1999). Thus, the above values clearly indicate that HRK-1 produces large amounts of an extracellular lipase, a property required for microorganisms involved in industrial production of this enzyme.

In lipase production the carbon source is the most important component of the medium affecting the synthesis and release of the enzyme. The lipase of HRK-1 is a constitutive enzyme since it was produced in the absence of specific substrates. The investigation conducted to study the influence of olive oil concentration on lipase activity of the isolate HRK-1, revealed that the optimal concentration was 1%. Activity was not enhanced by concentrations higher that 1%, but on the contrary a decrease in enzyme activity was recorded. The lipase activity decreased 30 to 40% upon increase of olive oil concentration to 3, 5, and 10% (Fig. 26). However this decrease in activity does not necessarily reflect a decrease in enzyme production. This decrease could be due to aggregate formation and due to the reaction and adsorption of the lipase at non-polar interfaces. Thus only lipase molecules at the surface of aggregates are available for substrate and the determined activity will not display the real amount of lipase (Schmidt et al. 1994).

The ability of HRK-1 to degrade olive oil was demonstrated by gas chromatography and high performance liquid chromatography. It was shown that oleic acid, the major product of the degradation of olive oil, increased with the increase of bacterial biomass (Fig.21). Similar results were obtained by Becker et al. (1997) where accumulation of oleic acid at concentrations of more than 100mg/l was observed during batch fermentations. Also, Triolein concentration decreased from 3.3 mg/ml at the beginning of the cultivation

to disappear after 36 hours of cultivation. Close results were reported by Becker et al. (1997) where from an initial concentration of 3.7 mg/ml 0.25 g/l were detected at the end of batch fermentation (27 hours). Thus it is obvious that HRK-1 is more powerful in degrading triolein. In addition, the increase of maximal biomass reached with the increase of olive oil concentration reveals the ability of HRK-1 to degrade olive oil and use it as a carbon source (Fig. 28).

When proteins are exposed to extremes of heat or pH, or to certain chemicals which affect their folding properties, they are said to undergo denaturation (Brock et al. 1994). Thus, enzymes are heat labile, and heat denaturation results in a gradual loss of their activity. The lipase HRKL-1 produced by the isolate HRK-1 was extremely stable. When boiled for one hour no loss of activity was detected (Fig. 35). This makes HRKL-1 much more stable than the excreted lipase of *Bacillus circulans* which exhibited 100% and 50% residual activity after 1 h incubation at 70°C and 85°C respectively (Kademi et al. 2000). The lipase produced by *Bacillus thermoleovorans* ID-1 exhibited 50% of its original activity after 30 minutes incubation at 70°C (Lee et al.1999), and another lipase BTID-A excreted by the same strain retained 75% of its activity when incubated for 30 minutes at 60°C (Lee et al. 2001). The lipase produced by *Bacillus thermocatenulatus* retained only 50% of its activity after 30 minutes of incubation at 60°C (Schmidt et al. 1994). The lipase produced by *Thermoactinomyces vulgaris* lost all activity after 5 minutes of incubation at 90°C (Elwan et al. 1978). The esterase produced by *Bacillus licheniformis* had a half life of 1 h at 64°C (Macarie et al. 1999). In addition the lipase produced by *Bacillus stearothermophilus* was stable up to 55°C for 30 minutes incubation (Kim et al. 2000). This clearly shows that HRKL-1 is by far much more stable than any of the lipases reported in the literature.

The optimal temperature for activity of HRKL-1 was 60°C (Fig. 33). This value is close to the optima reported for *Bacillus thermoleovorans* ID-1 lipases (60-70°C) (Lee et al. 1999-2001), for *Bacillus thermocatenulatus* lipases (60-70°C)

(Schmidt et al. 1994-1996), for the lipase produced by *Thermoactinomyces vulgaris* (55^{0}C) (Elwan et al.1978), for the lipase produced by *Bacillus circulans* (60^{0}C) (Kademi et al 2000), and for the lipase produced by *Bacillus stearothermophilus* (68^{0}C) (Kim et al. 2000)

The optimal pH for activity of HRKL-1 was 8.0 (Fig. 34). This value is close to the optima reported for *Bacillus thermoleovorans* ID-1 lipases (8.0-9.0) (Lee et al. 1999-2001), for *Bacillus thermocatenulatus* lipases (8.0-9.0) (Schmidt et al. 1994-1996), for the lipase produced by *Thermoactinomyces vulgaris* (8.8) (Elwan et al.1978), for the lipase produced by *Bacillus circulans* (8.5) (Kademi et al. 2000), and that produced by *Bacillus licheniformis* (Kim et al .2000).

HRKL-1 was slightly inhibited by Mg^{++} and Co^{++} (Fig. 38). This appears in agreement with the results reported for the lipases BTL-1 of the *Bacillus thermocatenulatus* (Schmidt et al. 1996), and BTID-A of the *Bacillus thermoleovorans* ID-1 (Lee et al. 2001). Fe^{++} and Fe^{+++} enhanced the activity of HRKL-1 which is a characteristic not reported for any of the lipases of thermophilic origin (Fig. 38) (Lee et al. 1999-2001; Schmidt 1994-1996). HRKL-1 appears to be a calcium-metallo enzyme which is a characteristic of highly thermo-stable enzymes like BTID-B (Lee et al. 2001), and the lipase produced by *Bacillus stearothermophilus.* Calcium ions seem to bind to the thermostable enzyme and stabilize the protein tertiary structure (Kim et al. 2000). Zn^{++}, Cu^{++}, and Mn^{++} had no effect on activity of HRKL-1. On the contrary these ions reduced the activity of the lipases of *Bacillus thermoleovorans* ID-1 (Lee et al. 2001), *Bacillus thermocatenulatus* (Schmidt et al. 1996) and *Thermoactinomyces vulgaris* (Elwan et al. 1978). The activity of HRKL-1 was inhibited by 15% upon pre-incubation with urea and slightly inhibited upon treatment with SDS (Fig. 39). This has already been noted for BTID-A (Lee et al. 2001) and the lipase produced by *Thermoactinomyces vulgaris* (Elwan et al. 1978). EDTA increased the activity of HRKL-1 a characteristic never reported before. This might be due to the presence of inhibiting ions in the crude enzyme sample used for the assay. EDTA was reported to have no

effect on the lipases of *Bacillus thermocatenulatus* (Schmidt et al. 1996). Phenylmethylsulfonyl fluoride (PMSF) acts in the same manner as *p*-hydroxymercuriobenzoate, and *p*-chloromercuriobenzoate, as an S-H (Serine group) inhibitor. HRKL-1 was not inhibited by PMSF which indicates that the S-H groups of the enzyme are not essential for its activity (Fig. 39). Similar results were observed for the lipases produced by *Bacillus thermocatenulatus* (Schmidt et al. 1994-6), for the BTID-B produced by *Bacillus thermoleovorans* ID-1 (Lee et al. 2001) and for the lipase produced by *Bacillus licheniformis* (Macarie et al. 1999).

The results obtained from the Electrophoretic pattern of the lipase HRKL-1 appear to indicate that it is a multimeric enzyme made of several subunits of a monomer of M.W. 26.03 kDa (Fig. 32). The molecular weight of the multimeric unit of HRKL-1 was 80.06 kDa. Molecular weights of lipases in the literature ranged from 16 kDa to 81 kDa (Schmidt et al. 1994-6; Lee et al. 1999-2001; Macarie et al. 1999).

Km and Vmax values of HRKL-1 were determined in *p*-nitrophenyl laurate reaction vessels. Vmax is the maximum velocity of the reaction (at infinite substrate concentration). It is a function of the amount of enzyme and is the appropriate rate to use when determining the specific activity of a purified enzyme. Km, the Michaelis constant, is the substrate concentration where v_0=1/2 Vmax. The Michaelis constant is expressed in terms of substrate concentration (mol/l) and is independent of enzyme concentration. Km provides a measure of the affinity of an enzyme for the substrate and this is an important characteristic of each particular enzyme. Thus an enzyme with a large Km usually has a low affinity for its substrate, while an enzyme with a small Km usually has a high affinity (Reed et al. 1998). Km was determined to be 0.021mM and Vmax 0.062 U/mg (Fig. 36). When compared to values form the literature it was shown that HRKL-1 has a very high affinity to its substrate. Km of the lipase produced by *Bacillus licheniformis* was reported to be

0.52 mM (Macarie et al. 1999), and that for the lipase produced by *Bacillus circulans* was reported to be 0.24 mM (Kademi et al. 2000).

In conclusion, this work succeeded in the isolation of an aerobic, filamentous, Gram-positive, spore forming, strict thermophilic bacterium, which produces large quantities of an extracellular thermostable lipase. The thermophilic character along with its property to degrade olive oil and secrete high amounts of a thermostable extracellular lipase makes this bacterium an attractive candidate for industrial applications.

Chapter 6

SUMMARY AND CONCLUSIONS

This study reports on the isolation and characterization of an aerobic, filamentous, Gram-positive, spore forming, thermophilic bacterium that degrades olive oil and produces a thermostable extracellular lipolytic enzyme.

This soil isolate, designated as HRK-1, can be classified as *Thermoactinomyces putidus* due to the filamentous structure of its molecules that bear one spore each on an un-branched sporophore, the resistance of its spores to boiling, utilization of sucrose as a carbon source and production of dark pigments.

Optimal growth conditions of the isolate HRK-1 were found to be: temperature, 60^0C; pH, 7.3 with phosphate buffer, and an aeration speed of 250 rpm.

The highest activity and release of thermostable lipase by HRK-1 was detected during the decline phase of the growth which coincided with the lysis of the filamentous cells and release of spores.

The extracellular amount of lipase (2.37 U/ml) produced by HRK-1, measured in the cell free supernatant clearly indicated the commercial importance of our isolate, especially after it showed great stability at elevated temperatures.

The optimal concentration of olive oil for lipase activity was 1% (v/v), and activity was not enhanced at concentrations higher than 1%, but on the contrary, a decrease in enzyme activity was recorded.

It showed a good olive oil degrading ability. No traces of triolein were detected after 36 hours of cultivation. And even 10% olive oil did not inhibit its growth.

The optimal temperature for activity of HRKL-1 was 60^0C, and optimal pH was 8.0.

HRKL-1 was slightly inhibited by Mg^{++} and Co^{++}. Fe^{++} and Fe^{+++} enhanced the activity of HRKL-1 which is a characteristic not reported for any of the lipases of thermophilic origin. HRKL-1 appears to be a calcium-metallo enzyme. The activity of HRKL-1 was inhibited by 15% upon pre-incubation with urea and slightly inhibited upon treatment with SDS. EDTA increased the activity of HRKL-1 a characteristic never reported before. This might be due to the presence of inhibiting ions in the crude enzyme sample used for the assay. HRKL-1 was not inhibited by PMSF which indicates that the S-H groups of the enzyme are not essential for its activity.

The Electrophoretic pattern of HRKL-1 indicates that it is a multimeric enzyme of molecular weight 80.06 kDa made of monomers of molecular weight 26.03 kDa.

Km value for HRKL-1 (0.021mM) indicated high affinity of the enzyme to its substrate.

In conclusion this work succeeded in the isolation of an aerobic, filamentous, Gram-positive, spore forming, strict thermophilic bacterium, which produces large quantities of an extracellular thermostable lipase. The thermophilic character along with its property to degrade olive oil and secrete high amounts of a thermostable extracellular lipase makes this bacterium an attractive candidate for industrial applications.

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