Biochemical study of lipases from *Bacillus subtilis*

Faouzi Laachari[1], Fatimazahra El Bergadi[1,2], Wifak Bahafid[1,2], Adel Sayari[3], Soumya Elabed[1,2], Iraqui Mohammed[1,2], Saad Koraichi Ibnsouda[1,2]*

[1]*Laboratoire de Biotechnologie Microbienne. Faculté des Sciences et Techniques de Fès- Saïs. Université Sidi Mohamed Ben Abdellah. B.P. 2202 – Route d'Imouzzer. Fez, Morocco*
[2]*Université Sidi Mohamed Ben Abdellah, Centre Universitaire Régional d'Interface, Fez, Morocco.*
[3]*Laboratoire de Biochimie et de Génie Enzymatique des Lipases. Ecole Nationale d'Ingénieurs de Sfax. BP 3038 Sfax, Tunisia.*
**Corresponding Author: ibnsouda@hotmail.com*

Abstract

Lipases are a class of enzymes that catalyze the hydrolysis of long chain triglycerides in the lipids-water interface. Microbial lipases are currently receiving great attention to the rapid development of enzyme technology. Lipases have great potential in various industrial applications, chemical, pharmaceutical, medical, cosmetic, leather industry, paper manufacture, synthesis, biosurfactant, and agrochemicals. In this study, several bacteria have been tested and chosen for their ability to synthesize lipases. The aim of this research was to perform a screening of lipolytic strains as well as the dosage of their lipase activities. Gene which encodes the 16S ribosomal RNA for bacterial species was amplified via PCR and sequenced, the activity of lipase was examined within the pH range of 6.0-10.0 and the effect of pH on lipase stability was determined by incubating the lipase fraction in various buffer solutions ranging from 3.0 to 10.0 for 24 h at room temperature. The extracellular lipases from *B. subtilis* have a maximum production obtained after 72 hours at 50 °C, pH 8, and 40 mM of $CaCl_2$. The results showed that the use of olive oil strongly induces the lipase production from *Bacillus subtilis*.

Key words: *Bacillus subtilis*/Lipase/Olive oil.

Introduction

Lipids are a class of biological molecules soluble in organic solvents and can be distinguished from other components of living matter by their insolubility in water. They have no homogeneous structure as proteins, nucleic acids or sugars, but they are often formed by a carbon skeleton aliphatic, cyclic or polycyclic constituting the hydrophobic part, which can be attached to polar groups, constituting the hydrophilic portion. Included in lipids: fats, oils, waxes, and substances that are related (sterols, steroids, terpenes, etc.).

Lipases belong to the family of serine hydrolases acting on ester bonds of triacylglycerols insoluble in water. They are enzymes perfectly soluble in water acting on insoluble substrates. This heterogeneous biocatalysis has been decomposed into two basic steps (Verger and De Haas, 1973). The first step is adsorption of lipase to the oil / water interface and the second step is the catalysis itself. Based on the concept of heterogeneous catalysis, an interfacial enzymology has been developed (Verger and De Haas, 1976; Verger, 1980). Lipases can be of animal (Verger, 1984; Sztajer et al., 1988; Steiner and Williams 2002; Ben Bacha *et al.*, 2005;

Zouari *et al.*, 2005), microbial (Rosenstein and Götz, 2000; Sayari *et al.*, 2001; Mosbah *et al.*, 2005) or plant (Hills *et al.*, 1990; Sanz and Olias 1990; Bhardwaj *et al.*, 2001).

Materials and methods

Origin of strains

The strains used in this study were provided by the Laboratory of Microbial Biotechnology FST Fez, 40 strains respectively recorded under number 1s to 40s, were isolated from the gut of olive flies. While another 42 strains recorded under different names, are isolated from the wood of the old medina, which are isolated by the PhD Laboratory of Microbial Biotechnology FST Fez. The isolation is performed by transplanting stem from fresh cultures from glycerol stored strains in solid YPG medium (Peptone 20g; glucose 20g; yeast extract 10g; Agar 20g) and LB medium (Peptone 20g; 20g NaCl; yeast extract 10g; Agar 20g).

Abbreviations: BSL, *Bacillus subtilis* lipase; FST, (Faculté des Sciences et Techniques, Fez)

Screening of lipolytic Activity

The screening was realized on a solid medium containing 1% olive oil, 1% nutrient broth, 1% NaCl, 1.5% agar and 1% rhodamin B (pH 7). The culture plates were incubated at 30 °C, and colonies giving rise to widespread clearing around them were regarded as putative lipase producers. Among the 9 strains retained.

16S rDNA analysis: DNA extraction

The DNA extraction methods were initiated by the collection of strains cells on membrane filters (0.22µm pore size.), followed by enzymatic and chemical lysis of cells, phenol/chloform extraction, ethanol precipitation and RNase treatment.

PCR conditions

PCR conditions for *Bacillus subtilis* was performed by using a 50 µl (total volume) mixture containing 1.25 U of Taq DNA polymerase (Amplitaq Gold, Perkin-Elmer), 15 mM Tris–HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 mM, 25 pmol of each primer and 10-50 ng of the extracted DNA. The thermal cycle involved 10 min activation of the polymerase at 94 °C before 2 cycles consisting of 1 min at 94 °C, 1 min at 52 °C and 2 min at 72 °C. The annealing temperature was subsequently decreased for every second cycle until it reached to 47 °C, at which point, 30 additional cycles were carried out; finally, a 10 min extension at 72 °C was performed (Parola *et al.*, 2000). The Amplification of PCR products were visualized on a 1 % agarose gel after electrophoretic migration of 10 µl of amplified material.

Culture conditions

The bacterium was precultured during 12 h at 37 °C, stirring 200 rpm in 250 ml shaking flasks with 50 ml of medium A (17 g/L casein peptone, 5 g/L yeast extract (Difco), 2.5 g/L glucose, pH 7.4). Overnight, *B. subtilis* cultures used as inoculum were cultivated in 1000 mL shaking flasks contains 100 ml of medium A. The initial absorbance (OD) measured at 600 nm was adjusted to an approximate 0.2 value. The culture was incubated aerobically for during 72 h on a rotary shaker stirring at 200 rpm and a temperature of 37 °C. Growth was followed by measuring the OD of the cultures at 600 nm.

Lipase activity determination

Lipase activity was assayed by the spectrophotometric method (Kwon and Rhee, 1986). In short 1 mL of enzyme extract was added to 5 mL of olive oil emulsion and incubated for 30 min at 60 °C. Olive oil emulsion was prepared by mixing 20 g of olive oil and 100 mL of 0.1 M phosphate buffer pH 7.0, containing 10 mM $CaCl_2$ and 0.25 % polyvinyl alcohol (PVA) in a Homogenizer (1500 rpm for 30 min). The reaction was stopped with 1 mL of 6 M HCl and incubated in a boiling water bath for 5 min. The fatty acids released were extracted in 5 mL of isooctane by vortexing for 15 min. To 2.5 mL of organic phase, 0.5 mL of a copper acetate-pyridine solution (50 g/L, pH 6.1 adjusted with pyridine) was added, vortexed for 15 min and centrifuged at 5000 rpm for 15 min and the absorbance of the organic phase was monitored at 715 nm. A calibration curve was drawn using oleic acid as a standard. One unit of activity was defined as the amount of the enzyme, which release 1 µmol of fatty acid per minute in the assay conditions.

Preparation of the standard range of oleic acid

Oleic acid is the fatty dominant (70 %) of the triglycerides in the composition of olive oil acid. We opted for a measurement of the absorbance of oleic acid at 715 nm as a free fatty acid which is derived from the lipolytic activity on olive oil.

The effect of olive oil on the production of lipase from *Bacillus subtilis*

The effect of medium composition on the production of lipases is studied in the presence of 1% olive oil. The lipase activity was determined as described by Kwon and Rhee (1986) at different times and under the following conditions pH 7 and at a temperature of 60 °C. The ability to use a variety of substrates as the only carbon source has been studied in a liquid medium. Addition of olive oil, the substrates; tributyrin and Tween 80 were also tested as the only carbon source for bacterial strain isolated. Thus, 10 ml of a bacterial suspension of 24 h (OD = 0.1), previously prepared, inoculated into 50 mL of sterile culture medium contained in mineral baffled Erlenmeyer flasks of 250 ml. The substrate previously sterilized by filtration using a millipore filter (0.45 µm in diameter) is added to the medium at a rate of 40 ml / L. Then, the flasks were incubated for 96 hours at 37 °C under agitation (180 rpm). The evolution of lipolytic and optical density was followed every 6 hours for a period of 96 hours activity.

Effect of pH and Temperature on the Activity and Stability of BSL

The activity of lipase was examined within the pH range of 6.0-10.0 .The lipase activity was measured as described by Kwon and Rhee (1986). The effect of pH on lipase stability was determined by incubating the lipase fraction in various buffer solutions ranging from 3.0 to 10.0 for 24 h at room temperature. After the incubation period, the residual activity was determined, after centrifugation, under standard assay method Kwon and Rhee (1986). The optimum temperature for the *Bacillus subtilis* lipase activity was determined by enzyme assay at different temperatures (20 – 90 °C). The effect of temperature on lipase stability was determined by incubating the enzyme solution at different temperatures (20–90 °C) for 30 min. The residual activity was determined, after centrifugation, under standard assay method, Kwon and Rhee (1986).

Ca^{2+} dependence

Different concentrations of $CaCl_2$ were prepared from 2 to 40 mM, their effect on lipase activity was investigated by incubating the culture for 54 h to 96 h stirring at 150 rpm and a temperature of 37 °C. The lipase activity is performed according to Kwon and Rhee (1986).

Results and discussion

The detection of lipase activity

The results showed that 40 strains has no lipase activity (no fluorescence around colonies), 33 strains showed fluorescence in the medium of revelation, and 9 strains have been described as the best producers of lipase as shown in (Table 1).

Table 1. Strains with interesting fluorescence in the presence of rhodamine B

Strains	Lipase activity
Negative control (*E.coli*)	(-)
BMS_3E	++++
BMS_1A_3	++++
YMS_1A_2	++++
EMS_3C	++++
YMS_2C	+++
BHS_4C_2	+++
YMS_1E	++++
YS_3H	++++
BMS_1C_A	++++

PCR amplification of 16S rDNA

16S rDNA was amplified by PCR using two primers FD1 (5'AGAGTTTGATCCTGGCTCAG3') and Rs16 (5'TACGGCTACCTTGTTACGACTT 3) as described by Parola *et al.*, (2000). Then, the amplification products were visualized under UV after electrophoresis agarose gel 1%. Bacterial species, whose 16S rRNA gene shows high homology to YMS1A2. The alignment of the gene 16S rRNA allowed to identify YMS1A2 strain as *Bacillus subtilis* CDB86.

Production of lipase

In order to reveal the behavior of the isolated strain in the presence of olive oil used as the only carbon source, the lipase activity of the study was conducted only for the isolated bacterial strain (*Bacillus subtilis*) from samples collected from the traditional tannery. Figure 1 shows, in a first step, a maximum lipolytic activity after 72 h incubation at 37 °C (exponential growth phase) equal to 7.5 U / mL with an inoculum size of 3.10^8 cells/mL (Figure 1). The stationary phase appears to be reached from 72 hours of incubation. The mixed consortium also seems to reach this phase. Furthermore, Figure 1 shows a decrease in pH of the medium during the incubation. This decrease is the result, no doubt, to the hydrolysis of the substrate under the action of lipases and release of AG responsible for acidification of the medium.

In order to study the effect of substrate concentration on lipase production of *Bacillus subtilis*, two concentrations of olive oil have been tested: 1% and 4%. . Results obtained are illustrated in Figure 2. Apparently the use of olive oil as only carbon source at a concentration of 1% strongly induces the production of lipase and obviously bacterial growth. Indeed, Sidhu *et al.*, (1998) have shown that the rate of enzyme decreased with increasing concentration of the substrate. This phenomenon is probably due to the increase in the rate of AG accumulated during the hydrolysis of the substrate with repression of the synthesis of lipases (Sidhu *et al.*, 1998).

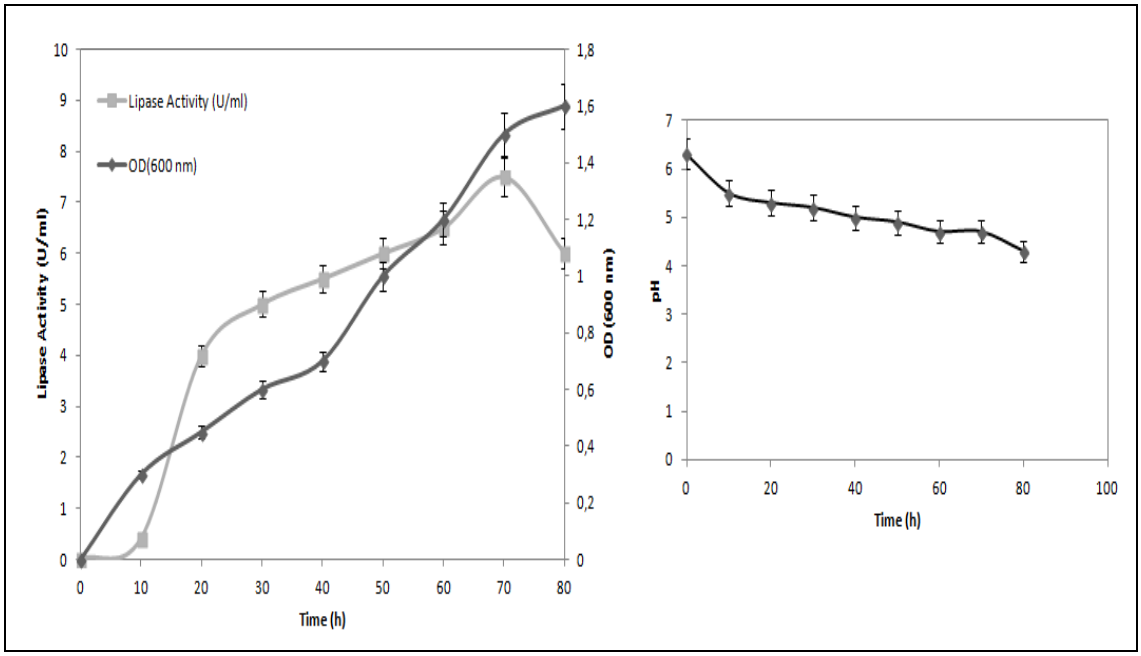

Figure 1. Production kinetics of *Bacillus subtilis* lipase.

On the other hand, the effect of the concentration of triglycerides on lipase production was also confirmed by Immanuel *et al.*, (2008). The authors found a decrease in activity observed with high concentrations of substrate (7% (v / v) olive oil) which is probably due to inhibition by the substrate itself present in excess in the medium (Immanuel *et al.*, 2008).

The results obtained from testing the study of lipolytic activity depending on the substrate made with three different substrates are shown in Figure 3. *B. subtilis*, showed their ability to use each of the three substrates added in the medium as the only carbon source. However, a higher lipase activity was obtained with tributyrin (Figure 3). The work of Cardenas *et al.*, (2001). Showed that bacterial lipases tend to reveal better hydrolytic activity against tributyrin compared to that obtained with olive oil (Cardenas *et al.*, 2001). Oleic acid accounts 70% of the composition of the olive oil. According to the established standard range (Table 2), and using the equation y = 0.009x, we can determine the concentrations of oleic acid correspondents, and therefore, the values of enzymatic activity expressed in U/mL (Figure 4).

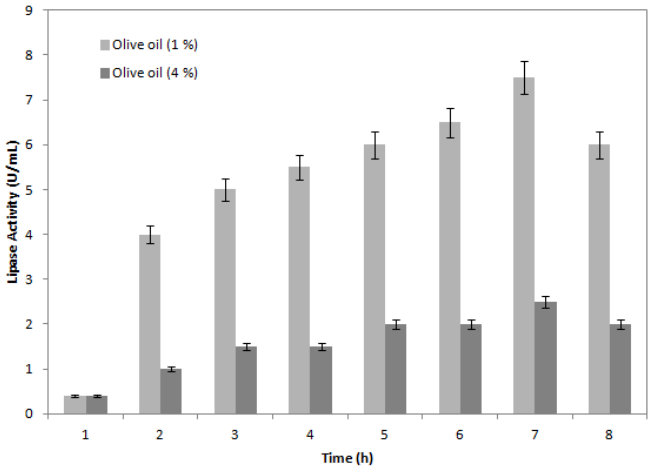

Figure 2. Effect of the concentration of olive oil on the lipolytic activity of the *Bacillus subtilis* lipase.

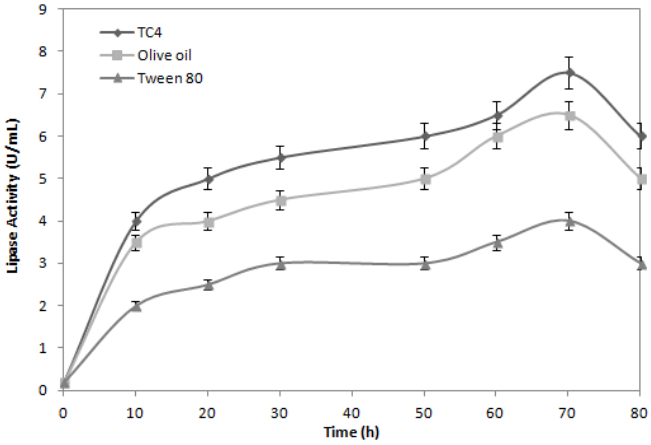

Figure 3. Effect of various substrates on the lipolytic activity of the *Bacillus subtilis* lipase.

Table 2. The composition of the standard range of oleic acid

	control tube	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
oleic acid (ml)	**0**	**0,1**	**0,05**	**0,025**	**0,0125**	**0,006**
phosphate buffer pH=7(ml)	**5**	**4,9**	**4,95**	**4,957**	**4,9875**	**4,994**
CaCl2 0 ,02M (20µl)	**20**	**20**	**20**	**20**	**20**	**20**
Culture filtrate (ml)	**1**	**1**	**1**	**1**	**1**	**1**

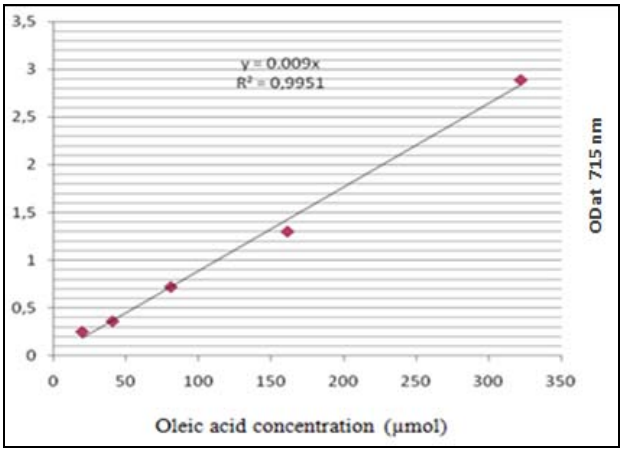

Figure 4. Standard range of oleic acid.

Effect of pH and Temperature on the Activity and stability of *B. subtilis* lipase

The activity of BSL was investigated at different pH using olive oil emulsion as substrate. Like some previously described bacillus lipases (Shah and Bhatt 2011), our results show that BSL remains active at a pH range from 6.0 to 10.0 (Figure 5A). In the pH stability study, the lipase is stable at abroad range of pH values between pH 5–10 after 24 h incubating (Figure 5B). The lipase activity was also determined at different temperatures under standard assay conditions (Figure 5C). The BSL activity increased significantly with increasing the temperature to reach its maximum value at 45 °C. The thermostability of BSL was also determined by measuring the residual activity after incubation of the enzyme at various temperatures (Figure 5D). The enzyme has significant activity at temperatures ranging from 47 °C to 60 °C which differs from the results observed in *Bacillus subtilis* by Fariha *et al.*, (2006); it preserves almost 92% of its activity and begins to lose its activity after 60 °C to lose up to 70% of its activity.

Ca^{2+} dependence

The metal cations, particularly Ca^{2+}, play an important role in influencing the structure and function of the enzymes.

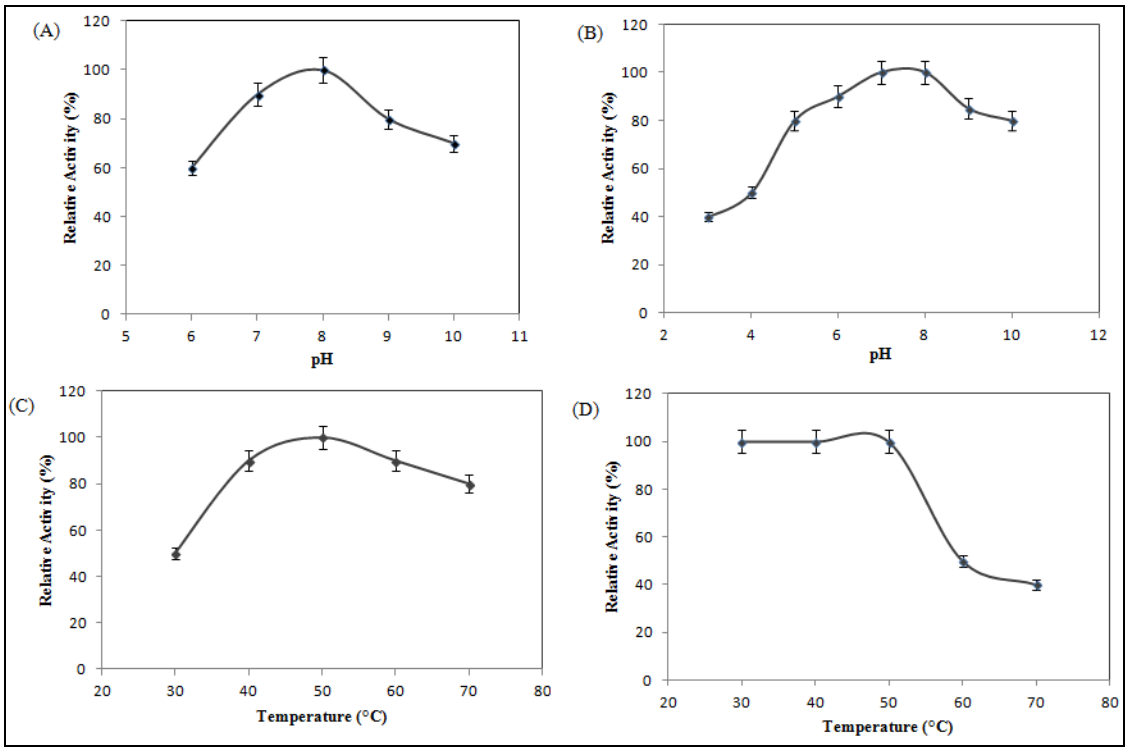

Figure 5. Effect of pH on enzyme activity (A) and stability (B) of BSL. Optimal pH was determined with tributyrin at 45 °C under the standard conditions. Stability was analyzed after preincubating the pure enzyme for 24 h in different buffer solutions at various pH ranging from 3 to 10. Temperature effect of on BSL activity (C) and stability (D). For temperature stability the pure enzyme was preincubated at different temperatures for 30 min and the remaining activity was measured under the standard conditions.

Different concentrations of $CaCl_2$ were used to determine its effects on the lipase activity (Figure 6). The lipase activity is increased in the presence of 40 mM concentration of $CaCl_2$, but at low concentrations the activity drop. The main role of Ca^{2+} seems to eliminate the released fatty acids, but the catalytic effect of calcium on a lipase has been explained by the removal of free fatty acids from the interface. In a system free of calcium, the lipase cannot be adsorbed to the water-fatty acid interface, and therefore no lipolytic activity occurs. It is possible that the calcium ions created offset electrostatic repulsion between the enzyme and the substrate. (Shastry and Raghavendra Rao, 1971).

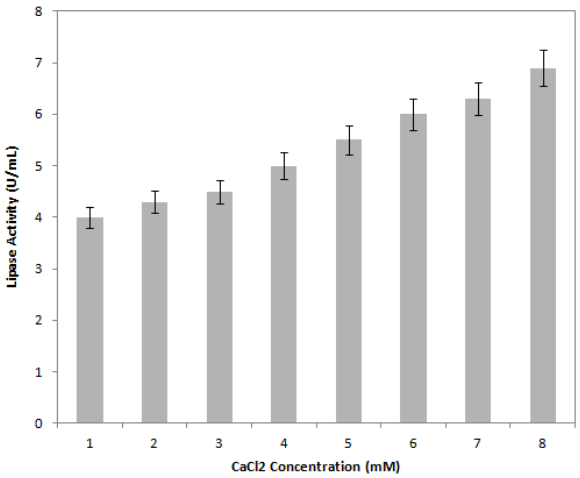

Figure 6. Effect of different concentrations of $CaCl_2$ on lipase activity

Conclusion

This study was carried out to identify the strains producing bacterial lipolytic traditional tanning led to the

enzymes. The bacterial screening made from a sample of the identification of a bacterial strain capable to hydrolyze all the different lipid substrates added to the fermentation medium. Among the substrates tested olive oil appears to be a good inducer for the lipase production when used as only carbon source in the culture medium. Furthermore, the production of lipase is to be associated with bacterial growth and its secretion begins when the bacterial cell is in exponential growth phase. However, the results obtained showed that the enzymatic production period may vary from several hours to several days depending on the bacterial species and also to the environmental conditions from which the bacterium is isolated.

According to our preliminary results, these studies must be continued and deepened by targeting qualitative and quantitative production of lipases followed by a characterization of them. It would also be important to study the effect of some physicochemical parameters (various carbon and nitrogen sources, metal ion …) on lipase production by the isolated bacterial strain.

References

Ben Bacha A., Gargouri Y., Ben Ali Y., Miled N., Reinbolt J., Mejdoub H. (2005). Purification and biochemical characterization of ostrich pancreatic lipase. Enz. Microb. Technol. **37**: 309-317.

Bhardwaj K., Raju A., Rajasekharan R. (2001). Identification, Purification, and Characterization of a Thermally Stable Lipase from Rice Bran. A New Member of the (Phospho) Lipase Family. Plant Physiol. **127**: 1728-1738.

Cardenas F., Alvarez E., Castro Alvarez M.S., Sanchez- Montero J.M., Valmaseda M., Elson S.W., Sinisterra J.V. (2001). Screening and catalytic activity in organic synthesis of novel fungal and yeast lipases. J. Mol. Catal. B: Enzym. **14**: 111-123.

Fariha H., Aamer A.S., Abdul H. (2006). Influence of culture conditions on lipase production by Bacillus sp. FH5. Ann Microbiol. **56(3)**: 247-252.

Hills M.J., Kiewitt I., Mukherjee K.D. (1990). Lipase from *Brassica napus* L. discriminates against *cis*-4 and *cis*-6 unsaturated fatty acids and secondary and tertiary alcohols. Biochim. Biophy. Acta **1042**: 237-240.

Immanuel G., Esakkiraj P., Jebadhas A., Iyapparaj P., Palavesam A. (2008). Investigation of Lipase Production by Milk Isolate *Serratia rubidaea*. Food Technol. Biotechnol. **46**: 60 65.

Kwon D.Y., Rhee J.S. (1986). A simple and rapid colorimetric method for determination of free fatty acid for lipase assay. J. Am. Oil Chem. Soc. **63**: 89-92.

Mosbah H., Sayari A., Mejdoub H., Dhouib H., Gargouri Y. (2005). Biochemical and molecular characterization of *Staphylococcus xylosus* lipase. Biochem. Biophy. Acta **1723**: 282-291.

Parola P., Roux V., Camicas J.L., Baradji I., Brouqui P., Raoult D. (2000). Detection of ehrlichiae in African ticks by PCR. Trans R Soc Trop Med Hyg. **94**:707-708.

Rosenstein R., Götz F. (2000). Staphylococcal lipases: biochemical and molecular characterization. Biochimie **82**: 1005-1014.

Sanz L.C., Olias J.M. (1990). Characterization of lupin seed lipase. Food Chem. **37**: 221- 228.

Sayari A., Agrebi N., Jaoua S., Gargouri Y. (2001). Biochemical and molecular

characterization of *Staphylococcus Simulans* lipase. Biochimie **83**: 1-9.

Shah K.R., Bhatt S.A. (2011). Purification and characterization of lipase from Bacillus subtilis Pa2. J. Biochem Tech **3(3)**: 292-295.

Shastry B.S., Raghavendra Rao M.R. (1971). Studies on rice bran lipase. Indian J. Biochem. Biophys. **8**: 327-332.

Sidhu P., Scharma R., Soni S.K., Gupta J.K. (1998). Production of extra cellular alkaline lipase by a new thermophilic *Bacillus sp*. Folia microbial. **43**: 51-54.

Steiner J.M., Williams D.A. (2002). Purification of classical pancreatic lipase from Dog. Pancreas. Biochimie **84**: 1243-1251.

Sztajer H., Maliszewska I., Wierczorek J. (1988). Production of exogenous lipases by bacteria, fungi and actinomycetes. Enz. Microb. Technol. **10**: 492-497.

Verger R. (1980). Enzyme kinetic of lipolysis. Methods Enzymol. **64**: 341-392.

Verger R. (1984). Pancreatic lipases, in: Borgström B., Brockman H.L. (Eds.), Elsevier, Amsterdam, 83-149.

Verger R., de Hass G.H. (1973). Enzyme reactions in a membrane model. 1: A new technique to study enzyme reactions in monolayers. Chem. Phys. Lipids **10**: 127-136.

Verger R., de Hass G.H. (1976) Interfacial enzyme kinetic of lipolysis. Annuel Review Biophys. Bioeng. **5**: 77-117.

Zouari N., Miled N., Cherif S., Mejdoub H., Gargouri Y. (2005). Purification and characterization of a novel lipase from the digestive glands of a primitive animal: The scorpion. Biochim. Biophy. Acta **1726**: 67-74.