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Purification and Properties of a Lipase from Thermophilic *Geobacillus stearothermophilus* Strain-5

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Abstract: The study was aimed to purify the extracellular lipase produced from a thermophilic *Geobacillus stearothermophilus* strain-5. The enzyme was purified using ultrafiltration followed by three column chromatographies: Q-Sepharose ion exchange chromatography, Sephadex G-100 gel filtration and adsorption on hydroxyl apatite to 22.6-fold with 8.8% recovery. The lipase had a molecular weight of about 61 kDa. The enzyme showed optimal activity at 55-60°C and pH 8.0 and retained about 87.5% of its original activity after exposure to 70°C for 15 min. Furthermore, 95-100% of the original activity was retained after incubation at pH 5-9 at 60°C for 30 min. The presence of 1 mM of the following metal ions: Fe^{2+}, Ca^{2+}, Mn^{2+} and Mg^{2+} enhance the lipase activity whereas Zn^{2+}, Hg^{2+} and Cu^{2+} inhibited it. The purified enzyme exhibited good tolerance to some organic solvents especially butanol and isopropanol. The presence of detergent such as SDS, Tween 20, Tween 80 and Triton X-100 had a slight effect on the lipolytic activity. The enzyme hydrolyzes both soluble and insoluble emulsified substrates; it showed highest affinity to tributyrin. The values of K_m and V_{max} of the lipase using p-nitrophenyl palmitate as calculated from the Linweaver-Burk plot were 0.588 mg mL^{-1} and 129.7 U mL^{-1}, respectively. The obtained enzyme showed stability to different pH values, temperatures and tolerance to some detergents and organic solvents.

Key words: *Geobacillus stearothermophilus*, characterization, purification, enzyme stability, thermostable lipase

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are a class of enzymes which catalyze the hydrolysis of long chain triglycerides. Lipases are ubiquitous in nature and are produced by various plants, animals and microorganisms. Microbial lipases are currently receiving much attention with the rapid development of enzyme technology (Hasan *et al*., 2006). Because many industrial processes operate best at high temperature, thermostable enzymes

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are gaining wide industrial and biotechnological interest as biocatalysts for the industrial applications (Haki and Rakshit, 2003). The advantages of application of thermostable enzymes as biocatalyst include increased substrate solubility, the diffusion rate, mass transfer effect due to reduced viscosity and reduced risk of contamination (Abdel-Fattah and Gaballa, 2008; Haki and Rakshit, 2003). However, only microbial thermostable lipases are commercially significant for their potential use in industries, such as organic syntheses (Li and Zhang, 2005). They have diverse applications in a wide variety of industries ranging from detergent; oleochemical; organic synthesis; dairy, fat and oil modification to pharmaceutical. The main reason for the steadily growing interest in lipases is because of their enantioselective, regioselective and chemoselective nature (Gupta *et al*., 2004). Recently, a number of thermostable lipases has been produced and purified mainly from thermophilic microorganisms isolated from different exotic ecological zones of the planet earth in order to be used for such applications (Abdel-Fattah and Gaballa, 2008; Dharmsthiti and Luchai, 1999; Kambourova *et al*., 2003; Rahman *et al*., 2007; Soliman *et al*., 2007).

Geobacillus srearothermophilus strain-5 is a thermophilic strain isolated from desert soil and identified using biochemical and molecular techniques. This isolate produce a thermostable lipase with optimal growth and lipolytic enzyme activity at 60°C and pH 7.0. An inducible nature of lipolytic enzyme synthesis using glycerol and glucose was demonstrated (Berekaa *et al*., 2009). In this study, we describe the purification and characterization of the purified enzyme produced from this strain.

MATERIALS AND METHODS

Microorganism and Production Conditions

The bacterial strain used in this study was isolated from desert soil sample and identified by 16S rRNA as *Geobacillus stearothermophilus* (accession number DQ923400). Production of lipase was carried out in 500 mL Erlenmeyer flasks containing 100 mL of the medium composed of (g L^{-1}): peptone, 10; yeast extract, 5; NaCl, 5 and $CaCl_2$, 0.02; pH 7.5. The production medium was inoculated and incubated at 60°C under shaking (150 rpm) for 24 h. The cell free supernatant was used for purification process. This study was carried out in 2007 at the University of Alexandria, Egypt.

Lipase Assay

Lipolytic activity was determined colorimetrically using two solutions (Vorderwuelbecke *et al*., 1992). Solution 1 contained 90 mg p-nitrophenyl palmitate (pNPP) dissolved in 30 mL 2-propanol. Solution 2 contained 2 g Triton X100 and 0.5 g gum arabic dissolved in 450 mL buffer (Tris/HCl, 50 mM, pH 8). The assay reagent was prepared by adding 1 mL solution 1 to 9 mL solution 2 dropwise to get an emulsion that remained stable for 2 h. The assay mixture contained 900 μL of the emulsion and 100 μL appropriately diluted enzyme solution. The liberated p-nitophenol was measured at 410 nm using spectrophotometer (Helios α/England). One unit of enzyme was defined as the amount of enzyme that releases 1 μmol p-nitrophenol from the substrate per min.

To determine the substrate specificity of the enzyme, lipase assay was performed using olive oil emulsion as a substrate. The emulsion was prepared by suspending 0.5 mL of olive oil into 5 mL of 2% Arabic gum in 0.1 M sodium phosphate buffer solution (pH 8.0). The mixture was homogenized for 3 min in a blender. After the addition of 100 μL of 1.1 M $CaCl_2$, the reaction mixture was incubated at 37°C for 1 min. The enzymatic reaction was initiated by the addition of 1 mL of the enzyme solution to 19 mL of the emulsion and the mixture was

incubated for 30 min at 60°C with agitation at 200 rpm. The enzymatic reaction was halted by the addition of 20 mL of acetone/ethanol mixture (1:1, v/v) and the liberated free fatty acids were titrated with a solution of 0.05 N NaOH. Phenolphthalein was used as indicator. One unit of lipase was defined as the amount of enzyme that liberates 1 μmol of free fatty acid min^{-1} (Kermasha *et al*., 1998).

Protein Determination

The protein content of cell free supernatant was determined according to Lowry method (Lowry *et al*., 1951).

Enzyme Purification

The extracellular lipase was first concentrated by ultrafiltration. The cell free supernatant was passed through a 10 kDa membrane and one-tenth of the retentate was collected. The concentrate was applied to Q-Sepharose column (2.5×7 cm) equilibrated with 20 mM Tris-HCl, pH 8.0. Elution was performed at a flow rate of 60 mL h^{-1} with a linear gradient of NaCl (0-1 M) in the same buffer. The active fractions that contained lipase were pooled and were further purified using sephadex G-100 column (45×1.6 cm) pre-quilibrated with 20 mM Tris-HCl, pH 8.0 at a flow rate of 45 mL h^{-1}. Protein fractions containing lipolytic activity were pooled and subjected to Hydroxyl Apatite (HA) chromatography on Bio-Gel HTP (BioRad). The column (10×1 cm) was pre-equilibrated with distilled water at a flow rate of 45 mL h^{-1} and the lipolytic enzyme was applied. Non binding material was eluted by washing the column with two bed volumes of distilled water. Elution was carried out by the application of a linear gradient of distilled water and 0.2 M potassium phosphate buffer pH 8.0. Active fractions were pooled and used for SDS-PAGE, zymogram and enzyme characterization.

Protein Analysis by SDS-PAGE and Zymogram Analysis

The proteins were analyzed by SDS-PAGE (10%) according to Laemmli (1970). The gel was stained with Coomassie Brilliant blue R-250. To confirm the lipolytic activity of this protein and to determine the exact molecular weight of the produced lipase, a zymogram was conducted as follow: an SDS gel was prepared as described above. After migration, part of gel was stained for protein detection with Coomassie Brilliant Blue R-250. The other part was washed by agitating during one hour in 250 mL of a renaturation buffer [20 mM Tris/HCl buffer, pH 8.0 containing 2.5% (w/v) of Triton X-100]. The renatured gel was put into a Petri dish containing 30 mL of a solidified 20 mM Tris/HCl buffer, pH 8.0 with 1% tributyrin (vol/vol). The Petri dish was incubated overnight at 50°C. The appearance of a clear halo indicated a lipolytic activity. Comparison of the two parts of the gel indicated the molecular mass of the enzyme (Nawani and Kaur, 2000).

Characterization of the Purified Lipase

The pH optimum of the purified enzyme was determined at 60°C using different pH values (5.0-11.0). The following buffers (50 mM) were used: potassium phosphate (pH 5.0-7.0), Tris-HCl (pH 7.0-9.0) and bicarbonate (pH 9.0-11.0). Reaction mixture (1 mL) contained (pNPP) as a substrate, in one of these buffers and 100 μL of appropriately diluted enzyme sample. The pH stability was determined by pre-incubation of lipase in different pH buffers (pH 5.0-11.0) at 60°C for 30 min. The residual enzymatic activity was determined using spectrophotometer (Helios α/England). The temperature optimum of the enzyme activity was evaluated at pH 8.0 using different temperatures. Thermal enzyme stability was determined by measuring the residual activity after 15 min of pre-incubation of the enzyme in Tris-HCl buffer (pH 8.0) at various temperatures.

The effect of metal ions on lipase activity was obtained by measuring the residual activity after pre-incubation of the enzyme with 1 mM of metal ion for 30 min at 60°C. The effect of some inhibitors and activators on the enzyme activity was determined after pre-incubation of the enzyme solution with each of these inhibitors and activators for 30 min at 60°C and then the residual activity was estimated. The effect of organic solvents was obtained by measuring the residual activity after pre-incubation of the enzyme solution with organic solvents for 30 min at 60°C. The final concentration of the organic solvents was 50%. Substrate specificity of the enzyme was determined at pH 8.0 and 60°C by titration method as described above using 1% emulsion of olive oil, tributyrin or Tween 80. The effect of substrate concentration (pNPP) on the reaction rate was assayed using spectrophotometric method (Vorderwuelbecke *et al*., 1992). Enzyme assays with 100 μL of purified enzyme were performed in assay buffer, pH 8.0 at 60°C with increasing concentrations of pNPP from 0.2 to 4.0 mg mL^{-1}. The Michaelis Menten constant (K_m) and the maximum velocity for the reaction (V_{max}) were calculated by Lineweaver-Burk plot using Hyper 32 program.

RESULTS

Purification of the Extracellular Lipolytic Enzyme

The extracellular lipolytic enzyme produced by the thermophilic *G. stearothermophilus* strain-5 was purified employing a 4 step procedure. The enzyme was found to be purified to almost 22.6 fold with 8.8% recovery as indicated in Table 1. The purity was confirmed by the presence of a single band on SDS-PAGE and its molecular weight was estimated to be 61 kDa (Fig. 1). To confirm the lipolytic activity of this protein bands, a zymogram was conducted

Table 1: Purification table of extracellular lipase from *G. stearothermophilus* strain-5

Purification step	Volume (mL)	Activity (U mL^{-1})	Protein (mg mL^{-1})	Specific activity (U mg^{-1})	Fold	Yield (%)
Crude	62.0	35.46	1.380	25.60	1.00	100.00
Ultrafiltrate	10.0	152.77	2.910	52.49	2.05	69.48
Q Sepharose	4.2	251.26	1.600	157.03	6.13	48.00
Sephadex G100	7.4	91.80	0.270	340.00	13.30	30.80
Hydroxyl apatite	4.2	44.60	0.077	579.22	22.60	8.80

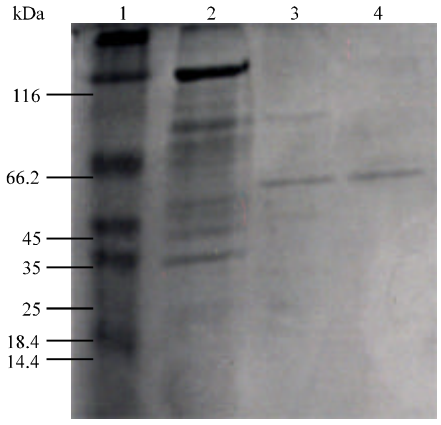

Fig. 1: Molecular weight determination of purified lipase through SDS-PAGE. ultrafiltrated (Lane 2), gel filtration (Lane 3), hydroxyl apatite purified lipase (Lane 4) and molecular weight markers (Lane 1)

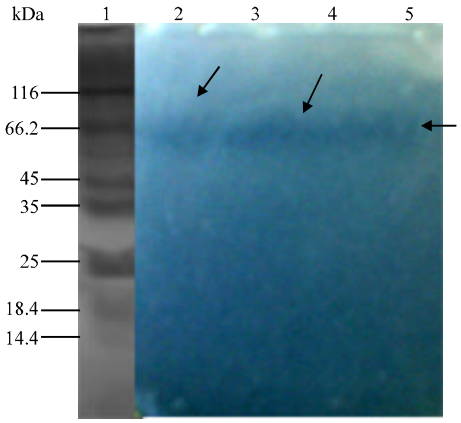

Fig. 2: Zymogram showing clear zone on agar/tributyrin plate. Enzyme was loaded onto SDS-PAGE and after the completion of the run the gel was then overlaid onto agar/tributyrin plate (lanes 2. 3, 4 and 5). Lane 1 represents molecular weight markers protein

using tributyrin as a substrate, as it is shown in Fig. 2, a unique clear zone appeared. Moreover, this specific precipitation zone is corresponding to a protein of molecular weight of about 61 kDa, which matched to the purified lipase described previously.

Characterization of the Purified Enzyme

The purified lipase exhibited maximum lipolytic activity at pH 8.0. Moreover, the activity was detectable over a wide range of pH between 5-10 (Fig. 3A). Lipase of *G. stearothermophilus* strain-5 retained 95-100% of its original activity after incubation at pH 5.0-9.0 at 60°C for 30 min (Fig. 3B). On the other hand, purified enzyme retained 58.22% and 27.27% of the original activity at pH 10 and 11, respectively.

The enzyme was most active in the temperature range 55-70°C with maximal activity at 55-60°C (Fig. 4A). Thermal stability of the enzyme was investigated for a period of 15 min (Fig. 4B). Purified enzyme retained 100% of its original activity at 30-60°C. On the other hand the enzyme retained about 87.5% of its original activity after 15 min at 70°C. However, the activity was strongly inhibited after the incubation of the enzyme at 90-100°C for 15 min.

The presence of Fe^{2+}, Ca^{2+}, Mn^{2+} or Mg^{2+} (1 mM) enhanced the activity of the purified enzyme after 30 min of incubation at 60°C and the residual activity was higher in the case of Ca^{2+} than with other ions (Table 2). However, no lipase activity was detected in the presence of 1mM Hg^{2+} or Cu^{2+}.

Lipase of *G. stearothermophilus* strain-5 exhibited good tolerance to some organic solvents, especially, butanol and isopropanol. Moreover, the presence of glycerol maintains the lipolytic activity after the incubation of the enzyme at 60°C for 30 min (Fig. 5). However, hexane, toluene, acetone and chloroform displayed a strong inhibitory effect on the lipolytic activity.

The enzyme retained about 59% of its original activity in the presence of 1 mM PMSF after 30 min of incubation at 60°C (Table 3). The purified enzyme retained about 47% of the original activity in the presence of 1 mM EDTA.

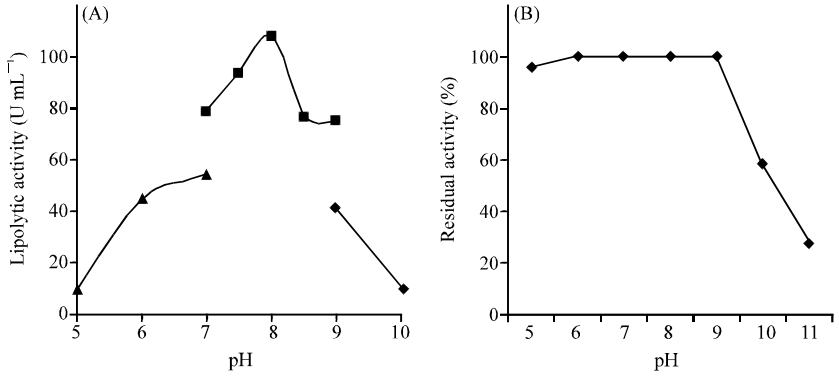

Fig. 3: Effect of pH on the activity and stability of the enzyme. Activity (A) was measured at different pH values and 60°C. pH stability (B) was established after pre-incubation of lipase solution at different pH values during 30 min at 60°C. Residual activity was expressed as percentage of original activity at pH 8.0, 60°C

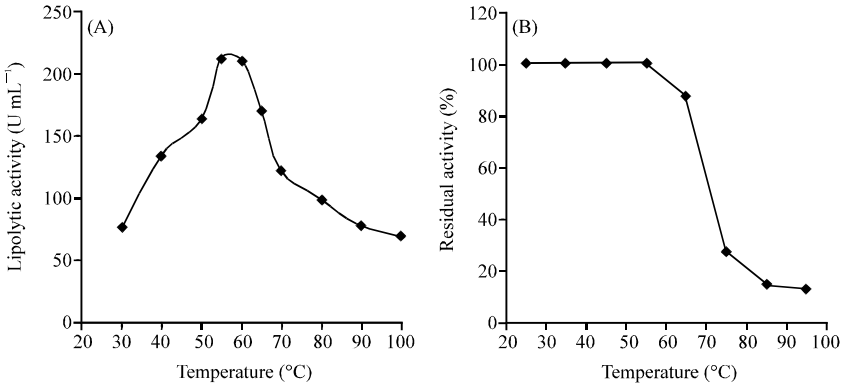

Fig. 4: Optimum temperature (A) was determined by measuring the activity at pH 8 and different temperature. Thermal stability (B) was determined by incubation of the enzyme for 15 min at different temperatures. Residual activity was expressed as percentage of original activity at pH 8.0, 60°C

Table 2: Effect of metal ions on the purified enzyme

Metal ions	Residual activity (%)
$CaCl_2$	155.00
$MgCl_2$	130.55
$HgCl_2$	0.00
$MnCl_2$	116.66
$ZnSO_4$	25.30
$CuSO_4$	0.00
$FeSO_4$	140.22

Result in Table 3 showed that the addition of 0.1 and 1% detergent had a slight effect on the enzyme activity. The enzyme retained 82.0, 82.53, 72.94 and 80.01% of its original activity for 30 min at 60°C in the presence of SDS, Tween 20, Tween 80 and Triton X-100, respectively.

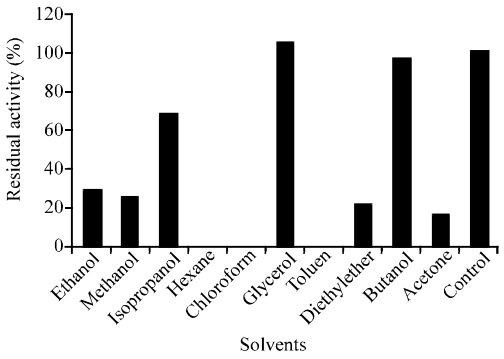

Fig. 5: Effect of organic solvents on the enzyme activity. The enzyme was incubated with different organic solvents for 30 min at 60°C. Activity was then determined at pH 8, 60°C

Table 3: Effect of EDTA, PMSF, and detergents on the purified lipase

Effector	Residual activity (%)
PMSF (1 mM)	59.00
EDTA (1 mM)	47.00
SDS (0.1%)	90.00
SDS (1%)	82.00
Tween 20 (0.1%)	89.41
Tween 20 (1%)	82.35
Tween 80 (0.1%)	96.74
Tween 80 (1%)	72.94
Triton X-100 (0.1%)	82.54
Triton X-100 (1%)	80.01

Table 4: Substrate specificity of purified lipase. Activity was measured by titration method

Substrate	Activity (U mL^{-1})
Tween 80	27.5
Tributyrin	175.0
Olive oil	42.5

The enzyme hydrolyzed both soluble and insoluble emulsified substrates (Table 4); it showed highest affinity towards tributyrin. The values of K_m and V_{max} of the purified lipase from *G. stearothermophilus* strain-5 using pNPP as calculated from the Linweaver-Burk plot were 0.588 mg mL^{-1} and 129.7 U mL^{-1}, respectively.

DISCUSSION

The aggregation may be the main cause of the slight reduction in the lipolytic activity during the purification steps of our enzyme. Generally, the yield in reported lipase purification procedures is relatively low (between 2 and 20%) (Nawani and Kaur, 2000; Lee *et al*., 2001; Sharma *et al*., 2002). Several reports attributed the low yield of purification to some aggregation-related problems (Dharmsthiti and Luchai, 1999; Kambourova *et al*., 2003). Reported molecular weights of microbial lipases vary considerably, ranging from 12 to 76 kDa (Castro-Ochoa *et al*., 2005). In particular, molecular weights of lipase from thermophilic bacteria range from 11 kDa in *B. thermoleovorans* CCR11 (Castro-Ochoa *et al*., 2005), to 69 kDa in *Bacillus* sp. THLO27 (Schmidt-Dannert *et al*., 1994). It seems that our enzyme belonged to the high molecular weight lipases.

The majority of lipolytic enzymes from thermophilic bacteria have neutral (Dharmsthiti and Luchai, 1999; Lee *et al*., 1999; Saeed *et al*., 2005), or alkaline pH optima (Castro-Ochoa *et al*., 2005; Sharma *et al*., 2002). However, several lipases are active over a broad pH range (Fucinos *et al*., 2005; Lee *et al*., 2001). Our enzyme showed stability in a broad range of pH, as most lipolytic enzymes from thermophilic bacteria (pH 5-pH 11) (Wang *et al*., 1995).

Optimum temperature of lipase activity from thermophilic bacteria has been reported to be generally in the range of 50-80°C (Dharmsthiti and Luchai, 1999; Sharma *et al*., 2002; Fucinos *et al*., 2005; Lee *et al*., 2001). The characteristics of our lipase make it belonged to thermostable enzyme because it showed an optimal activity at about 60°C.

Metal ions can enhance or inhibit the enzyme activity. Like our results, it was reported that Ca^{2+} ions increased lipase (Li and Zhang, 2005; Castro-Ochoa *et al*., 2005; Lee *et al*., 1999; Schmidt-Dannert *et al*., 1996) and esterase (Çolak *et al*., 2005) activities of several thermophilic bacteria. The influence of calcium ions was considered to be caused by their action on the release of fatty acids and on enzyme structure stabilization (Saeed *et al*., 2005). As reported above, Zn^{2+}, Cu^{2+}, Hg^{2+} and Co^{2+} have a strong effect on lipase activity of several thermophilic bacteria (Schmidt-Dannert *et al*., 1994; Fucinos *et al*., 2005; Sinchaikul *et al*., 2001). Gupta *et al*. (2004) reported that, lipases are generally stable in organic solvents, with few exceptions. Similarly, lipases from thermophilic *Bacillus* and *Bacillus thermoleovorans* ID-1 are stable in organic solvents (Lee *et al*., 2001; Kim *et al*., 2000). In our case, lipase exhibited good tolerance to butanol and isopropanol.

The enzyme under study retained about 59% of its original activity in the presence of 1 mM PMSF which suggests the presence of a serine residue at the catalytic triad of the active site. Nearly all lipases have a catalytic triad consisting of Ser-His-Asp/Glu, similar to that in serine protease (Tyndall *et al*., 2002). Serine inhibitors such as PMSF have a strong effect on the lipolytic activity from thermophilic bacteria (Ewis *et al*., 2004). The purified lipase lost about a half of its original activity in the presence of 1 mM EDTA. In accordance with this result, several reports indicated that EDTA had an inhibitory effect on lipases activity of thermophilic *Bacillus* sp. (Lee *et al*., 2001; Castro-Ochoa *et al*., 2005). However, no effect of EDTA on lipase activity of *Geobacillus* sp., *B. stearothermophilus* P1 and *G. stearothermophilus* was reported (Saeed *et al*., 2005; Sinchaikul *et al*., 2001; Ewis *et al*., 2004).

The response of reported thermophilic lipases to the presence of detergents is variable, except for Triton X-100 which has, in general, a stimulatory effect or has no effect on lipases activity (Castro-Ochoa *et al*., 2005; Li and Zhang, 2005; Kim *et al*., 2000). Comparable to our results, Tween 20 and SDS had a slight effect on lipase activity from *Geobacillus* TW1 (Li and Zhang, 2005) whereas a total loss of lipolytic activity of lipases from *B. thermoleovorans* CCR11 in the presence of Tween 80, Tween 20 and SDS was reported (Castro-Ochoa *et al*., 2005). Generally, a slight effect of detergent was shown on our enzyme. The ability to hydrolyze olive oil and pNPP specified the enzyme under study as a true lipase; in addition, it has some esterase activity as well. The same results were reported by Kambourova *et al*. (2003).

In conclusion, the obtained purified enzyme showed a high stability in broad range of pH values, high temperatures and in the presence of some detergents and organic solvents. These properties make it a good candidate for biotechnological applications.

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