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Enzymatic activity of a novel halotolerant lipase from *Haloarcula hispanica* 2TK2

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A strain of *Haloarcula hispanica* isolated from Tuzkoy salt mine, Turkey exhibited extracellular lipolytic activity. Important parameters such as carbon sources and salt concentration for lipase production were investigated. Optimal conditions for the enzyme production from *Haloarcula hispanica* 2TK2 were determined. It was observed that the lipolytic activity of *Haloarcula hispanica* was stimulated by some of the carbon sources. The high lipase acitivity values were obtained in the presence of 2% (v/v) walnut oil (6.16 U/ml), 1% (v/v) fish oil (5.07 U/ml), 1% (v/v) olive oil (4.52 U/ml) and 1% (w/v) stearic acid (4.88 U/ml) at 4M NaCl concentration. Lipase was partially purified by ammonium sulfate precipitation and ultrafiltration. Optimal temperature and pH values were determined as 45°C and 8.0, respectively. Lipase activity decreased with the increasing salt concentration, but 85% activity of the enzyme was maintained at 5M NaCl concentration. The enzyme preserved 41% of its relative activity at 90°C. The partially purified lipase maintained its activity in the presence of surfactants such as Triton X-100 and SDS. Therefore, the lipase which is an extremozyme may have potential applications especially in detergent industry.

Keywords: *Haloarcula hispanica*, extremely halophilic archaea, extremozyme, lipase, lipolytic activity.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) belong to the hydrolase family of enzymes. They hydrolyze triacylglycerides into fatty acids and glycerol on an oil-water interface. Additionally they can catalyze esterification, interesterification, and transesterification of fatty acids[1, 2]. Therefore, lipases can be used in various industrial processes such as pharmaceutical, detergents, cosmetic, food, textile, hide and leather industries, pulp and paper industries, biodegradation of plastics, wastewater treatment, production of optically active compounds and production of biodiesel[1–3].

Industrial lipases are mainly produced from microorganisms that secrete extracellular lipase[4]. Microbial lipases are usually produced by submerged fermentation and their activities are influenced by the type and concentration of carbon/nitrogen sources, pH and temperature[5]. Lipases can also be obtained from extremophiles. Extremophilic organisms have drawn much scientific interest and have important biotechnological potential because of their molecular adaptation and their ability to synthesize stable and active macromolecules in extreme environmental conditions[6]. Industrial processes generally occur under harsh conditions such as extreme temperatures, pH and salt concentrations or presence of organic solvents. For instance, lipases used in detergents should have stability and high activity over a wide range of pH and temperature, as well as in the presence of surfactants and metal ions. The exploration of new lipases will enhance the variety of lipolytic enzymes and thus assisting the selection of appropriate biocatalysts for challenging reactions[7]. Therefore, lipases derived from extremophiles, offer alternative solutions to important industrial processes.

Halophilic microorganisms are extremophilic organisms that have the ability to adapt to high salt concentrations. Halophilic proteins carry out their activities without losing resolution and preserving the 3D conformation in extreme conditions such as high temperature, high salt concentration, the presence of organic solvents and surfactants which cause denaturation of other proteins[8]. Therefore, the importance of characterization studies on stable and active lipolytic enzymes in extreme saline environments are recently increasing. *Haloarcula hispanica* which is an *archaea* thrive at high salt concentration and high temperatures[9, 10]. Extremozymes obtained from halophilic *archaea* adapt to high salt concentrations and maintain their stabilities and functions in extreme environments[11, 12]. Therefore, extremozymes are suitable for using in the processes carried out under extreme conditions[13]. Hence, *Haloarcula hispanica* used in this study may have important application potential, especially in industrial processes carried out under harsh conditions[14, 15].

In the present study, an extreme halophilic *archaea Haloarcula hispanica* 2TK2 strain isolated from Tuzkoy Salt Mine of Turkey, was examined for its lipase activity. The parameters influencing the lipase production such as temperature and pH were optimized. Lipase produced by the halophilic *archaea* strain was partially purified. Activity of partially purified lipase in extreme conditions and its stability in different salt concentrations and resistance to surfactants were investigated. Stability of the enzyme was investigated in the presence of certain detergent ingredients, and its suitability in these conditions were examined. This study is important because of the lack of study in the literature related to the production of lipase from extremely halophilic *Haloarcula hispanica* 2TK2 strain. The lipase produced by this strain may have an important potential in detergent industry.

MATERIAL AND METHODS

Microorganism and culture conditions

Haloarcula hispanica 2TK2 strain was obtained from culture collection of Biology Department, Marmara Univer-

sity. The cells were grown aerobically at 40°C in a Brown medium containing 25% NaCl, 2% $MgSO_4 \cdot 7H_2O$, 0.5% yeast extract, 0.3% $C_6H_5Na_3O_7 \cdot 2H_2O$, and 0.2% KCl (w/v)[16, 17]. The pH was adjusted to 7.5 before autoclaving. Bacterial growth was monitored by measuring optical density at 600 nm spectrophotometrically (Scinco S-3100, Seoul, Korea). All cultures were harvested in the late stationary phase and stored at –20°C.

Optimization of medium components

First, different compositions of Brown media were prepared in order to investigate optimal salt concentration and carbon source (Table 1). Salt concentration and carbon source of the medium was modified to obtain the highest activity of lipase. Effect of salinity was firstly analyzed using 3, 4 and 5 M NaCl in the culture medium containing 1% (v/v) olive oil in order to find the optimum salt concentration for the production of *Haloarcula hispanica* 2TK2 extracellular lipase. The test medium containing 1% olive oil (v/v) and test microorganism was incubated at 120 rpm 40°C for 60 days. The effect of carbon sources in the culture medium was investigated using several oils or fatty acid esters such as olive (*Olea europaea* L.) oil (1%, v/v), sesame (*Sesamum indicum* L.) oil (1%, v/v), marigold (*Calendula officinalis* L.) oil (1%, v/v), bitter melon (*Momordica charantia* L.) oil (1%, v/v), polyanthus (*Primula polyantha* L.) oil (1%, v/v), fish oil (1%, v/v), walnut (*Juglans regia* L.) oil (1% – 4%, v/v), tributyrin (1%, v/v), stearic acid (1%, w/v) and oleic acid (1%, v/v). Then, the effect of carbon sources was examined after the optimum salt concentration for *Haloarcula hispanica* 2TK2 was determined. Incubation conditions of the microorganism were 4 M NaCl at 40°C and pH 7.5 (Table 1).

Table 1. Investigated carbon sources in Brown media for lipase production

Medium	Carbon source
1	1% Olive oil
2	1% Sesame oil
3	1% Marigold oil
4	1% Bitter melon oil
5	1% Polyanthus oil
6	1% Fish oil
7	1% Walnut oil + 1% Tributyrin
8	1% Walnut oil + 1% Oleic acid
9	1% Walnut oil + 1% Stearic acid
10	1% Walnut oil
11	2% Walnut oil
12	3% Walnut oil
13	4% Walnut oil

Enzyme extraction and partial purification

Lipase production was carried out by inoculating 1 ml of fresh culture into 25 ml of the medium in a 100 ml flask. The test medium was incubated at 40°C on an orbital shaker (Lab Companion SI-300R, JeioTech, Seoul, Korea) at 120 rpm for 30 days. The culture was centrifuged at 15 000 rpm for 10 min at 4°C. Then, the cell free supernatant was precipitated using 40% ammonium sulfate. After precipitation, samples were centrifuged at 4800 rpm for 10 min at 4°C and supernatants were collected and Amicon stirred cell (10 kDa nitrocellulose membrane, Merck Millipore, Darmstadt, Germany) was used for ultrafiltration. Partially purified enzyme solution was washed with distilled water and phosphate buffer twice. Partially purified extracellular lipase was concentrated with ultrafiltration process by decreasing the total volume from 200–250 ml to 25–40 ml and stored.

The protein content of the enzyme extract was determined by the method of Bradford and was expressed as μg of total proteins per ml of the crude enzyme solution.

Lipase activity assay

Titrimetric method

A volume of 0.4 ml of enzyme extract and 0.5 ml of olive oil were added to 2.5 ml of phosphate buffer (100 mM, pH 7.2) and incubated at 37°C, 200 rpm for 30 minutes. The reaction was stopped with the addition of 2.5 ml ether/ethanol mixture (1:1 v/v). Obtained mixture was titrated with 0.08 N NaOH in the presence of phenolphthalein as indicator. All experiments were performed in triplicate. Lipase activity was calculated from NaOH consumption. One lipase unit was defined as the enzyme amount that causes the release of 1 μmol of fatty acids per minute, under the assay conditions. Enzyme activity was expressed as units per ml of the crude enzyme solution.

Spectrophotometric method

Lipase activity was also measured using p-nitrophenyl palmitate (pNPP) as substrate. The activity was determined by the addition of 0.4 ml of the crude enzyme solution to 2.5 ml of 1 M Tris-HCl buffer (pH 7.2) and 2.5 ml of 1.5 mM pNPP. The hydrolysis reaction was carried out in water-bath at 37°C while shaking at 150 rpm for 15 minutes. Reaction tubes were transferred to a boiling water bath and incubated for 3 minutes to stop the hydrolysis reaction. Absorbance of p-nitrophenol released from the hydrolysis reaction of pNPP was measured at 404 nm. One unit of lipase activity is defined as the amount of enzyme which releases 1 μmol of p-nitrophenol per minute under the assay conditions. Enzyme activity was expressed as units per ml of the crude enzyme solution.

Effects of temperature, pH and salt concentration on lipase activity

Optimum temperature, pH and salt concentration values for lipase (*H. hispanica* 2TK2) activity were determined. Hydrolysis reaction temperatures studied were 30, 40, 45, 50, 60, 70, 80 and 90°C. Since pNPP is degraded at high temperatures lipase activity at different temperatures was determined by titrimetric method as previously described. In order to determine the optimum pH for lipase activity pH values between 4–10 in different buffer solutions (acetate buffer at pH 4–5.6, phosphate buffer at pH 6–8, Tris-HCl buffer at pH 9, carbonate buffer at pH 9.2 and 10) were studied at 45°C. Because phenolphthalein is a pH dependent indicator the results were obtained spectrophotometrically at 404 nm as described previously. Maximum lipolytic activity was also studied in different salt concentrations ranging from 1 to 5 M NaCl in phosphate buffer. The results were obtained titrimetrically. All experiments were performed in triplicate.

Effects of surfactants

Partially purified lipase was incubated in the presence of Triton X-100 (1–70%, v/v) or SDS (1, 2.5, 5 and 10%, w/v). After incubation for 15 min at 45°C, 200 rpm and pH 8.0 the residual enzyme activity was measured using pNPP as substrate. Enzyme activity in the absence of surfactants was marked as the control. The results were given in means of relative activity. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Determination of lipase activity in the culture medium

Lipase production was induced by the presence of olive oil in the culture medium. Media containing different concentrations of NaCl (3, 4, 5 M) were used for bacterial growth. The highest level of activity in the culture medium was reached after 56 days of cultivation in Brown medium containing 4 M NaCl (Fig. 1). It has been known that *Bacillus* spp. is the most commonly used microorganism as a lipase producer. The highest lipase activity of this strain was obtained at between 48–72 h[19–21]. Although the incubation period of *Haloarcula hispanica* 2TK2 is much longer than the other lipase producing microorganisms, the test strain is much more stable than the other lipase producers. Halophilic microorganisms have the ability to adapt to high salt concentrations by balancing the osmotic pressure of their cytoplasm. Hypersaline media leads to protein aggregation by forming electrostatic interactions between the bonds of protein, but the halophilic proteins maintain their three-dimensional structure and conserve their activities in such environments[19]. Several lipolytic enzymes have been reported which are active and stable in extreme conditions such as hypersalinity[20, 21]. In our study, the lipase activity has continuously increased during the incubation period and the maximum activity (4.73 U/ml) was recorded at the 56th day in 4 M NaCl. The lipase activity of the cultures containing 3 M and 5 M NaCl were recorded as 3.85 U/ml and 4.34 U/ml, respectively which are higher than the results reported by Ozcan et al. (2009)[18].

Salt concentrations above 4M was determined not to have a significant effect on the lipase production, furthermore has an adverse effect on the lipolytic activity and cell growth. Oren (2000)[10] stated that the members of *Halobacteriales* need at least 1.5 M NaCl for growth and most of the strains have the optimum growth at 3.5–4.5 M NaCl concentration. This result is also comparable to the findings of Oren[10].

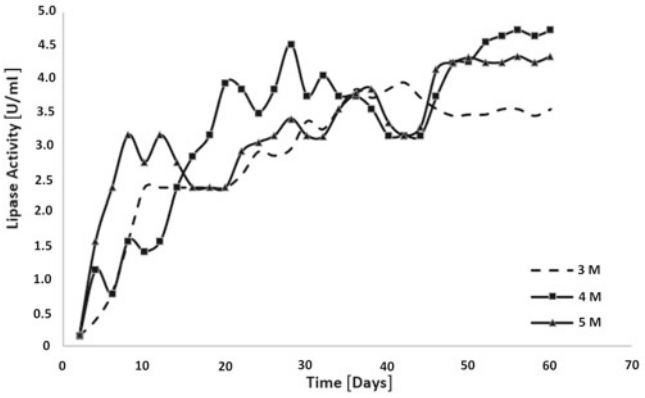

Figure 1. Lipase activity in means of salt concentration. Incubation performed in 500 ml flasks at 100 rpm 40°C for 60 days in presence of 1% olive oil. Results obtained by titrimetric method. All experiments performed in triplicate

Effect of carbon source in the culture medium

Lipase production was induced by the presence of different oil in the culture medium. Olive oil, sesame oil, marigold oil, bitter melon oil, polyanthus oil, fish oil and walnut oil were used as carbon source in the culture medium. All test media was analyzed for 60 days of incubation and the results were measured both by titrimetric and spectrophotometric methods. The results showed that maximum extracellular lipolytic activity decreased in the presence of oils in the following order: walnut oil > fish oil > olive oil > sesame oil > momordica oil > polyanthus oil = calendula oil (Fig. 2). The highest lipase activity has been reached in the culture media containing walnut oil and fish oil (5.43 and 5.07 U/ml) respectively, followed by olive oil (4.52 U/ml). Lipase from *Bacillus* spp. was produced by Sugihara (1991) in culture medium containing 1% olive oil[22]. It was reported that palm oil was the best substrate for the production of the extracellular lipase of *Rhodotorula glutinis*[23]. Marigold oil is a volatile oil and this oil has negative effect on cell growth. Hence, lipase production was inhibited. Likewise, polyanthus oil does not trigger the production of lipase, but it was found that the cell growth continued. In addition to the aforementioned carbon sources, tributyrin, stearic acid and oleic acid were used as carbon source to examine the effect of fatty acids on the lipase activity. All experiments were carried out at Brown media containing 4M NaCl. The lipase activities of media containing tributyrin, stearic acid and oleic acid were respectively detected as 4.88 U/ml, 4.88 U/ml and 3.62 U/ml. It was found that tributyrin and stearic acid have slightly increased lipase activity while oleic acid caused a decrease (Fig. 2). Our results are in agreement with the studies reported before[24, 25].

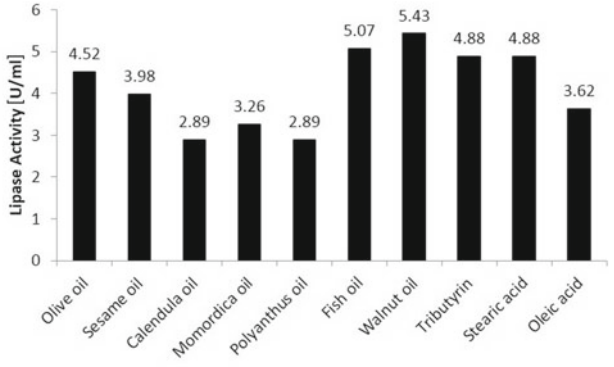

Figure 2. Lipase activity in means of carbon source. Several oils and fatty acids were used as carbon source of the growth medium in 500 ml flasks at 100 rpm 40°C for 60 days in presence of 4 M NaCl. Results obtained by titrimetric method. All experiments performed in triplicate

Effect of the carbon source concentration in the medium

To examine the effect of different concentrations of oil on lipase activity, 4 M NaCl and 1–4% (v/v) of walnut oil were used. The highest lipase activity was attained

in the medium containing 2% walnut oil (6.16 U/ml), but higher concentrations of oil (3–4% walnut oil) did not trigger lipase production (Fig. 3). Lipase activity decreased after 2% walnut oil concentration. It was reported that more than a certain amount of substrate causes an inhibition effect[26]. Finally, the optimum lipase production parameters were found to be 4 M NaCl and 2% walnut oil or tributyrin or stearic acid. Pérez et al. (2011)[27] reported a novel halophilic lipase produced by *Marinobacter lipolyticus* SM19 that exhibited high activity with short-medium length acyl chain substrates, although it also hydrolyzes long chain oils such as olive oil (1–2%) and fish oil (5%)[27].

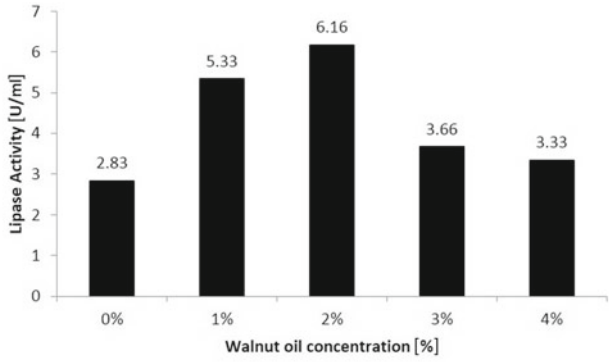

Figure 3. Lipase activity in means of carbon source concentration. Several concentrations of walnut oil were used in the growth medium in 500 ml flasks at 100 rpm 40°C for 60 days in presence of 4 M NaCl. Results obtained by titrimetric method. All experiments performed in triplicate

Partial purification of extracellular lipase

The extracellular lipase from *H. hispanica* 2TK2 was produced and partially purified as described previously. Ammonium sulfate precipitation and ultrafiltration were applied on the supernatant for the partial purification of the lipase. Optimum saturation of ammonium sulfate precipitation was 40% (w/v). The lipase activity was increased approximately 5 fold after partial purification (Table 2).

Table 2. Protein concentration after partial purification and lipase activity values before and after partial purification

Culture media	Activity [U/ml]		Protein concentration [mg/ml]	Specific activity [U/mg]
	before partial purification	after partial purification		
1	0.0127	0.119	0.379	0.31
5	0.00827	0.061	0.891	0.19
10	0.015	0.099	1.126	0.25
11	0.015	0.117	1.227	0.26
12	0.0173	0.175	1.546	0.32

Effect of temperature, pH and NaCl concentration on enzyme activity

The partially purified lipase was incubated at various temperatures and its activity was found to be maximum at 45°C. Lipase activity decreased at the temperature values above 45°C, however it did not lose its activity completely even at 90°C. 55, 44 and 41% of the lipase activity were preserved at 70, 80 and 90°C, respectively (Fig. 4A).

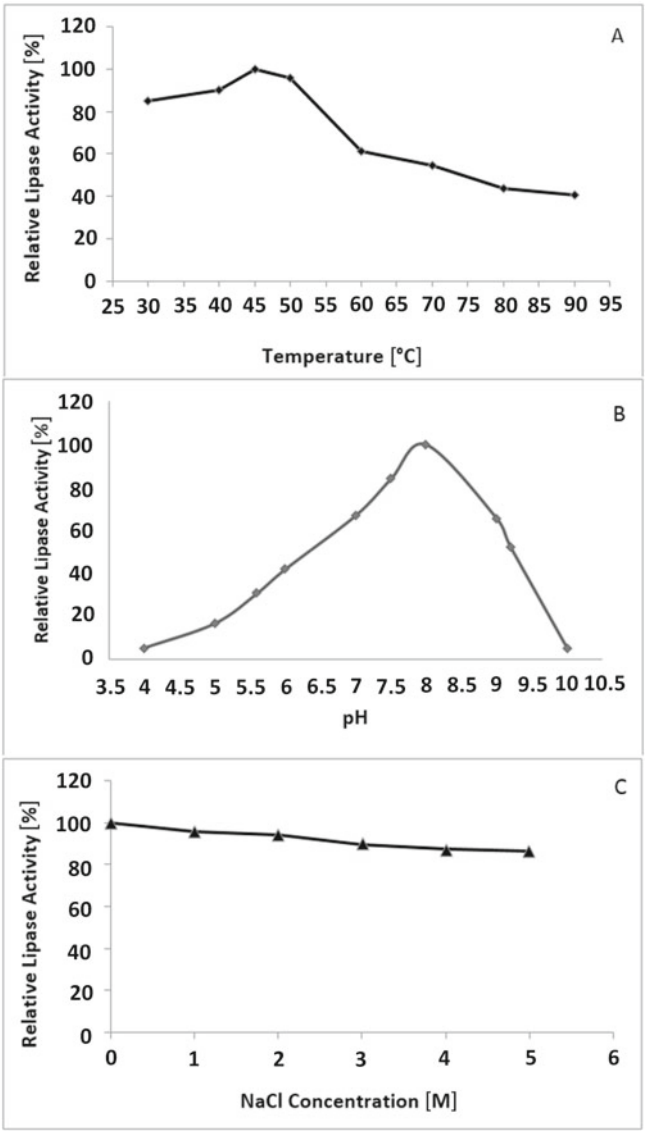

Figure 4. Effect of (A) temperature, (B) pH and (C) NaCl on 2TK2 lipase activity after 30 min of incubation. Relative lipase activity was measured using olive oil as substrate. Results in (A) and (C) were obtained by titrimetric method while (B) was spectrophotometric. All experiments performed in triplicate

Therefore *H. hispanica* 2TK2 lipase could be assumed as a thermostable lipase. Bhatnagar et al. (2005)[28] screened for lipolytic activity of *Natronococcus* spp. and results showed that optimum activity was attained at 40°C, 4 M NaCl and pH 7.5. However the enzyme lost all its activity at 50°C[28]. A thermostable lipase of the mesophilic fungus *Penicillium simplicissimum* was inactivated at 70°C[29], but 2TK2 lipase tends to be more stable at high temperatures than the lipases mentioned in the literature. It was reported that an organic solvent-tolerant lipase by *Haloarcula* sp. G41 strain displayed optimal activity at 70°C[30]. Still most of the known lipases by halophilic *archaea* have been reported to show maximal activities between 45 and 65°C[18, 20, 32]. Thus, the present lipase from *H. hispanica* 2TK2 had excellent thermostability which was observed over temperature ranging from 30 to 90°C.

In order to determine the pH value in which the enzyme activity is maximum, assays were carried out in buffer solutions with different pH values at 45°C, which was determined as the optimum temperature. The highest lipase activity was obtained at pH 8.0 and the 2TK2 lipase

was found to be active between pH 7.5–9.2 (Fig. 4B), retaining more than 50% of its activity. These results are similar to the results of a halotolerant *Staphylococcus* spp. extracellular lipase which was characterized by Daoud *et al.* (2013)[19]. Extracellular lipase of this strain showed optimal activity at pH 8.0[19]. Likewise, Li and Yu (2014)[30] reported that *Haloarcula* sp. G41 lipase was active at the same pH which is similar with our results[30].

The effect of NaCl concentration on the 2TK2 lipase activity was investigated over a concentration range of 1–5 M. The results were obtained at 45°C and pH 8.0. It was shown that the lipase preserved nearly 85% of its activity even at high salt concentrations such as 5 M (Fig. 4C). Results indicated that *Haloarcula hispanica* 2TK2 lipase was stable in a wide range of salt concentrations unlike most of the halophilic lipases[31, 32]. The lipase preserved its activity even at low salt concentrations. This feature is interest of biotechnological and industrial processes which are performed at high salt concentrations.

Effect of surfactants on enzyme stability

The effects of Triton X-100 and SDS were investigated on the partially purified lipase activity. Addition of 1% and 5% Triton X-100 (v/v) caused an increase of about 28% and 9% increase in lipase activity, respectively. The addition of 10% Triton X-100 (v/v) decreased lipase activity sharply (Fig. 5A). However, in the presence of 20% Triton X-100 (v/v) the enzyme preserved about 50% of its activity. Lipase activity decreased as Triton X-100 percentage increased. In the presence of 70% Triton X-100 (v/v), which is highly concentrated, lipase preserved about 16% of its activity. It was found that 1% SDS (w/v) in the reaction environment caused an increase of 32% in the lipolytic activity while 2.5% SDS (w/v) caused 9% increase. Results showed that the lipase retained 43% of its activity in the presence of 5% SDS (w/v) while in the presence of 10% SDS (w/v) lipolytic activity could not be observed (Fig. 5B). It has been reported that surfactants have strong inhibitory effects on lipases[33, 34]. Takac and Sengel (2010)[35] reported that Triton X-100 reduced lipolytic activity of *Debaryomyces hansenii* by 50% in the enzyme production medium containing 1.0% soybean oil[35]. These data showed that *H. hispanica* 2TK2 lipase is more stable than those in the literature in the presence of Triton X-100 and SDS. Thus, the present lipase from *Haloarcula* sp. 2TK2 can be rated among the higher thermoactive lipases.

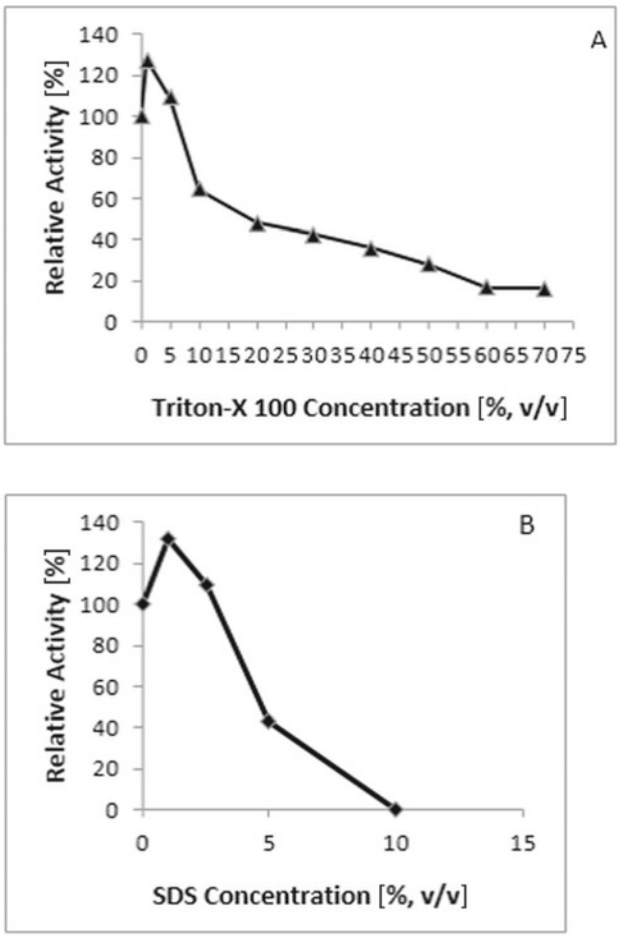

Figure 5. Effect of (A) Triton X-100 and (B) SDS on 2TK2 lipase activity. The enzymatic activity was measured on pNPP as substrate. All experiments performed in triplicate

CONCLUSIONS

Most of the industrial processes take place under harsh conditions. Hence it is very important to obtain enzymes that can maintain activity under unfavorable conditions such as extreme temperatures, salinity and pH, or the presence of organic solvents or surfactants. Therefore, lipases derived from extremophiles, offer alternative solutions to important industrial processes. The presented enzyme has several advantages such as being resistant to high surfactant levels, active at a wide range of pH, at high temperatures and NaCl concentrations.

When all results are considered, it can be said that the partially purified lipase by *Haloarcula hispanica* 2TK2 is thermostable and halophilic extremozyme, and has superior properties compared to other microbial lipases. It has a great biotechnological potential not only because of its thermostable properties, but also due to the low production cost. Thus it has diverse industrial applications ranging from using in detergent and paper manufacturing to the production of structured lipids, biodiesel, and biosurfactants. Also, the use of lipases in leather and hide industry to dissolve and remove fat is under rapid development and lipases are now an integrated part of leather processing in many parts of the world. Our group will focus on the purification of the enzyme in future. Determination of the molecular weight and isoelectric point of the lipase and its immobilization will be further studied.

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