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ORIGINAL PAPER

Characterization of extracellular esterase and lipase activities from five halophilic archaeal strains

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Abstract A total of 118 halophilic archaeal collection of strains were screened for lipolytic activity and 18 of them were found positive on Rhodamine agar plates. The selected five isolates were further characterized to determine their optimum esterase and lipase activities at various ranges of salt, temperature and pH. The esterase and lipase activities were determined by the hydrolysis of pNPB and pNPP, respectively. The maximum hydrolytic activities were found in the supernatants of the isolates grown at complex medium with 25% NaCl and 1% gum Arabic. The highest esterase activity was obtained at pH 8–8.5, temperature 60–65°C and NaCl 3–4.5 M. The same parameters for the highest lipase activities were found to be pH 8, temperature 45–65°C and NaCl 3.5–4 M. These results indicate the presence of salt-dependent and temperature-tolerant lipolytic enzymes from halophilic archaeal strains. Kinetic parameters were determined according to Lineweaver–Burk plot. The KM and V_{max} values were lower for pNPP hydrolysis than those for pNPB hydrolysis. The results point that the isolates have higher esterase activity comparing to lipase activity.

Keywords Halophilic archaea · Esterase · Lipase · Characterization

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Introduction

The extreme environments found on the planet are inhabited by microorganisms, which belong to the Archaeal and Bacterial domains of life [24]. Extreme environments comprise sites of extreme temperature, pH, pressure and salinity. Microorganisms that are able to grow in the presence of high salinity are determined in all three domains of life: Archaea, Bacteria and Eukarya [14]. The halophilic archaeal strains that require at least 12% (2 M) NaCl for growth have a number of useful applications in biotechnological processes and potential new applications are being investigated. For instance, they produce bacteriorhodopsin (used in information processing and ATP generation), novel extracellular polysaccharides, exoenzymes (amylase, cellulase, xylanase, lipase and protease) and poly-β-hydroxyalkanoate (used in biodegradable plastic production), and a protein from *Halobacterium salinarum* has significance in cancer research [3, 17, 23, 30].

The growing demand for more effective biocatalysts has been satisfied either by improving the properties of existing proteins or by producing new enzymes. The majority of the industrial enzymes known to date have been extracted mostly from bacteria and fungi [7, 9]. Until now, only a few archaeal enzymes have been found to be useful in industrial applications [10, 30]. Based on the unique stability of archaeal enzymes at high temperature and salt concentration and at extremes of pH, they are expected to be a very powerful tool in industrial biotransformation processes that run at harsh conditions. Particularly, halophilic archaea are the most likely source of such enzymes, because not only are their enzymes salt-tolerant, but many are also thermostable. Concerning to production of hydrolytic enzymes, moderately halophilic bacteria were reported not to be very promising, apart from production of DNase enzyme by

Gram-positive halophilic bacteria [26]. The potential usefulness of halophilic archaeal enzymes in biotechnological processes is still an open question. Interest in lipolytic microorganisms has increased in recent years due to the vast industrial applications of the enzyme [21]. Lipases as a class of hydrolytic enzymes have experienced the greatest market increase during the last few years with their wide applications ranging from detergents to food industry [8, 27, 28]. Esterases (EC 3.1.1.3) and lipases (EC 3.1.1.1) hydrolyze triglycerides to fatty acids and glycerol, and under certain conditions catalyze the reverse reaction [28]. Esterases catalyze the hydrolysis and formation of short-chain fatty acid esters, while lipases act mostly on long-chain acylglycerols [8]. Some lipases are also able to catalyze transesterification and enantioselective hydrolysis reactions [16]. The term "lipolytic activity" used in this report refers to activities of lipases and esterases.

The extremophiles are promising organisms for the production of enzymes, which are able to function under high temperature and salt concentration and under extremes of pH. Recently, it was published that Lakes of Turkey are rich sources of halophilic archaeal microorganisms [19] and some of these strains were characterized in a phylogenetic manner [20]. A screening method performed for the isolation of extracellular enzyme producers will provide the possibility to use different halophiles as a source of extremophilic enzymes in biotechnological processes. In this study, we describe a screening method for determination of extracellular lipolytic activity, which is produced by 118 halophilic archaeal strains isolated from different hypersaline environments in Turkey. The optimum pH, temperature and salt concentration of the extracellular esterase and lipase activities and kinetic parameters were also determined for further characterization.

Materials and methods

Archaeal strains, growth conditions and screening for lipolytic activity

A total of 118 halophilic archaeal collection of strains isolated from Salt Lake (Ankara), Aci Lake (Denizli) and Tuzla Lake (Kayseri) located in Turkey were routinely cultured in Sehgal–Gibbons (SG) medium, which contains the following (g/l): NaCl, 250; $MgSO_4 \cdot 7H_2O$, 20; KCl, 2; sodium citrate (trisodium salt), 3; casamino acids, 7.5; yeast extract, 1; $FeSO_4 \cdot 7H_2O$, 0.0023 [19]. Agar plates contained 2% (w/v) agar. All cultures were incubated at 37°C and liquid cultures were incubated in an orbital shaker (175 rpm).

The halophilic archaeal isolates were screened for lipolytic activity on Rhodamine B agar plates at 37°C. Sterilized SG medium was supplemented with 2.5% olive oil (w/v) and 0.001% (w/v) Rhodamine B solution. The cultures were grown at 37°C for 12 days. Afterwards, the screening of lipolytic activity of the strains was monitored under UV light at 350 nm as orange halos appearing around the colonies [1, 12]. The growth of the selected five isolates was determined by the increase in optical density at 600 nm, and lipolytic activity was routinely assayed during 7 days of culture period.

Esterase and lipase activity assays

The selected isolates were inoculated in 250-ml Erlenmeyer flasks containing 50 ml SG medium supplemented with either 1% gum Arabic or 1% olive oil or 1% gum Arabic + 1% olive oil. Cells were harvested by centrifugation at 15,000 rpm for 20 min at 4°C, and the supernatant was used as crude enzyme source. Lipase and esterase activities were determined by using pNPP (*p*-nitrophenylpalmitate) and pNPB (*p*-nitrophenylbutyrate) as substrates, respectively [2, 13]. Both substrates were dissolved in 2-propanol to give a final concentration of 1 mM in the reaction mixture. pNPP solutions were mixed with Tris–HCl buffer containing gum Arabic and Triton-X100 as emulsifying agents, which were not added for the pNPB assay. Following the addition of 1.5 ml of 0.25 M Na_2CO_3 solution to stop the reaction, the amount of pNP released at the end of the reaction time (5 min) was measured at 410 nm against a blank. One enzyme unit was defined as the amount of enzyme that liberates 1 μmol of pNP per min and per 1 ml of supernatant.

The effect of salt concentration, pH and temperature

The effect of salt concentrations on esterase and lipase activities were measured at 40°C in 50 mM Tris–HCl, pH 8, containing different final NaCl concentrations ranging from 2 to 5 M. The effect of pHs on esterase and lipase activities at 40°C and 4 M NaCl concentration were determined in 50 mM acetic acid/acetate buffer for pH range 5–6, and in 50 mM Tris–HCl buffer for pH range 6.5–9. The molar extinction coefficient values were different for varied pHs; therefore, the standard curves for pNP for each pH values were constituted. The dependence of lipolytic activity to temperature was determined in 50 mM Tris–HCl buffer at pH 8 and 4 M NaCl concentration by applying temperatures in the range of 30–70°C.

Kinetic studies

The effect of substrate (pNPP and pNPB) concentrations (0.067–1 mM) on the reaction rates of the crude lipolytic enzymes were assayed by using standard enzyme assay.

The Michaelis–Menten constant (K_m) and the maximum velocity for the reaction (V_{max}) with pNPP and pNPB as substrates were calculated by Lineweaver–Burk plot.

Results and discussion

Screening of isolates and lipolytic activity

The 118 strains were screened for their potential lipolytic activity and only 18 of them were positive on Rhodamine agar plates. The best five strains, which were named as A43, B53, E7, A138 and B49, were selected according to the size of halos formed in the specific plate assay. The screening results indicated that most of the halophilic isolates did not express any lipolytic activity. A study carried out by moderately halophilic bacteria gave similar results, and they reported that, from a total of 9,848 colonies isolated on the screening media, only 207 lipase producers were detected [26].

In this study, lipolytic activity positive isolates were tested for their potential to produce esterase and lipase activity on SG broth medium containing 1% gum Arabic or 1% olive oil or 1% olive oil + 1% gum Arabic by monitoring pNPB and pNPP hydrolysis, respectively. As a result, higher lipase production was found to be on SG broth with 1% gum Arabic. In the current study, addition of the gum Arabic to the medium led to high growth of the isolates and presumably this affected the enzyme production. Several surfactants, such as gum Arabic, have often been proposed as a means to increase extracellular lipase production of certain microorganisms, due to their potential ability to increase cell wall permeability and/or to release cell bound enzymes [5].

When the lipase activities of the strains in the culture broth were compared with the lipase activities of the strains on the agar media by comparing the size of halos, there was no correlation. The level of maximum growth rates was reached at the fifth day for each of growth periods, and then the cultures entered into stationary phase. The maximum lipolytic activity of the each strain was also detected in the fifth day, after which the rates of pNPP and pNPB hydrolyzing activities were decreased. This decrease may be due to the production of protease by the strains at the same time as explained before [29].

Effect of pH on pNPB- and pNPP-hydrolyzing activities

The influence of pH on the pNPB-hydrolyzing activity (Fig. 1) and pNPP-hydrolyzing activity (Fig. 2) of the isolates was determined. B53, A138 and B49 had the maximum lipolytic activity at pH 8 for both pNPB and pNPP substrates. However, in the case of A43 and E7, the maximum activities were detected at pH 8.5 for pNPB-hydrolyzing activity and at pH 8 for pNPP-hydrolyzing activity, respectively. It was observed that while pH was strongly effective on the pNPB-hydrolyzing activities of E7 and pNPP-hydrolyzing activities of B49 and A138, the hydrolytic activities of other isolates were not affected greatly with pH differences.

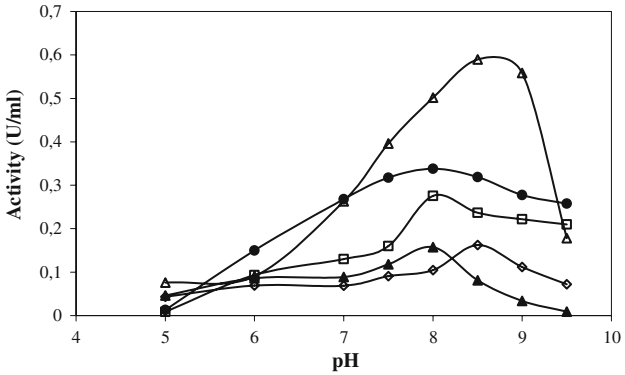

Fig. 1 The effect of the pH on the esterase activity of the isolates determined by pNPB hydrolysis. Isolates were represented as follows: (*filled circle*) 49, (*open square*) 53, (*open diamond*) 43, (*open triangle*) 7, (*filled triangle*) 138

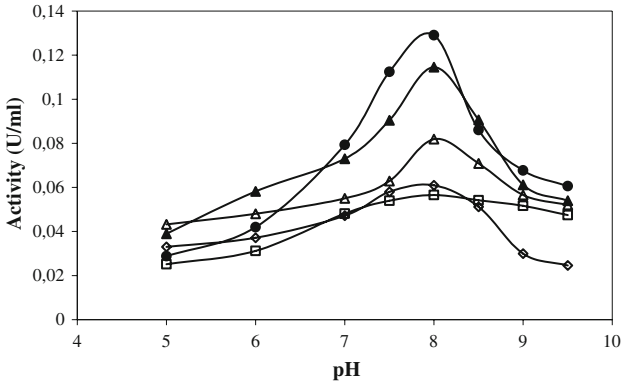

Fig. 2 The effect of the pH on the lipase activity of the isolates determined by pNPP hydrolysis. Isolates were represented as follows: (*filled circle*) 49, (*open square*) 53, (*open diamond*) 43, (*open triangle*) 7, (*filled triangle*) 138

In our knowledge, there have been only a few studies on the production of lipolytic enzymes by this class of organisms. Bhatnagar et al. [1] investigated the lipolytic activity of *Natronococcus* TC6 strain by growing them at medium with different pH. They found the highest activity on the medium with pH 8 for pNPB-hydrolysis and with pH 7.5 for pNPP-hydrolysis. However, in that study, the effect of pH was investigated as a component of the media other than reaction mixture like we did in the present study. In another study with the same strain, the maximum activity for pNPP-hydrolysis was reported to be at pH 7 [2].

Effect of salt concentration on pNPB- and pNPP-hydrolyzing activities

The effect of salt concentrations on the extracellular hydrolytic activity of the strains for the hydrolysis of pNPB (Fig. 3) and pNPP (Fig. 4) was determined in the salt concentrations range between 2 and 5 M NaCl. Although supernatant of the strains showed their maximum esterase and lipase activities between 3 and 4.5 M NaCl, it was obvious that the highest activities of the isolates on pNPP hydrolysis have a more narrow range comparing to pNPB hydrolysis (Figs. 3, 4). In the current study, we determined that the lipase activities of the strains increased until 4 M NaCl and then dropped markedly. In a similar study, Boutaiba et al. [2] investigated the effect of salt concentrations of extracellular lipolytic activity of *Natronococcus* sp. and found that the activity increased with salt concentration up to 4 M NaCl and then decreased. In a biotechnological view, enzymes excreted into the media, such as the lipase studied here, also must work at salt concentrations at which other enzymes would lose their properties. These properties make them valuable because halophilic enzymes are not only able to deal with high ionic strength in their environment but also need it to maintain function and structure [6]. In addition, it is well established that hydrolytic enzymes of halophilic archaea are salt-dependent enzymes. For instance, the best activity of α-amylase is at 3 M NaCl [22], protease is at 4.3 M NaCl [11], extracellular serine-protease is at 4 M NaCl [25], and of xysilosidase is at 2.8 M NaCl [31].

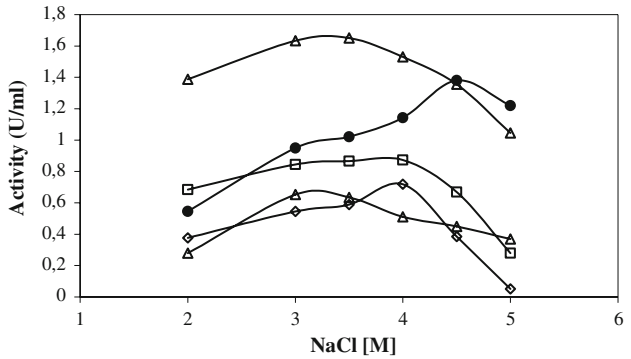

Fig. 3 The effect of the NaCl concentration on the esterase activity of the isolates was determined by pNPB hydrolysis. Isolates were represented as follows: (*filled circle*) 49, (*open square*) 53, (*open diamond*) 43, (*open triangle*) 7, (*filled triangle*) 138

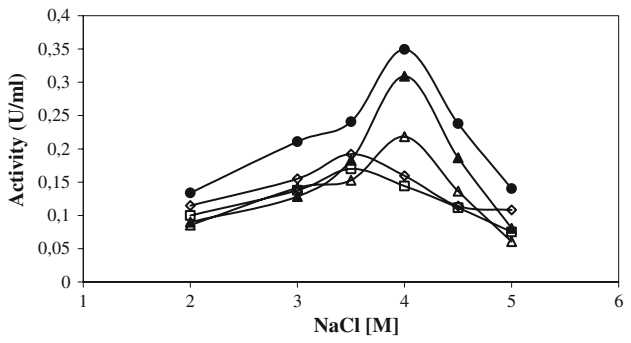

Fig. 4 The effect of the NaCl concentration on the lipase activity of the isolates determined by pNPP hydrolysis. Isolates were represented as follows: (*filled circle*) 49, (*open square*) 53, (*open diamond*) 43, (*open triangle*) 7, (*filled triangle*) 138

Effect of temperature on pNPB- and pNPP-hydrolyzing activity

The effect of temperature ranging from 30 to 70°C on the pNPB (Fig. 5) and pNPP-hydrolyzing activities (Fig. 6) of the strains were investigated at 4 M NaCl and at pH 8. The maximum esterase activities occurred at 65°C for pNPB hydrolysis for all strains except B49, for which it was observed at 60°C (Fig. 5). In the case of pNPP hydrolysis, the maximum lipase activities were observed at 45°C for A43, 55°C for B53 and E7, 60°C for B49 and 65°C for A138 (Fig. 6). Maximum pNPB-hydrolyzing activities usually were reached at higher temperatures when compared with the temperatures at which pNPP hydrolyzing occurred. However, A138 and B49 showed the maximum pNPP and pNPB hydrolysis at the same temperature. In this study, the maximum activities generally exhibited at 55°C or higher temperatures; therefore, lipolytic activity of our halophilic strains can be defined as not only salt-dependent but also as thermo-tolerant. We also noted that lipolytic activity of our strains is sort of thermo-dependent until about 60°C, after which activities dropped (Figs. 5, 6).

Kinetic parameters

The kinetic parameters determined in this study were obtained from crude enzymes other than purified enzymes.

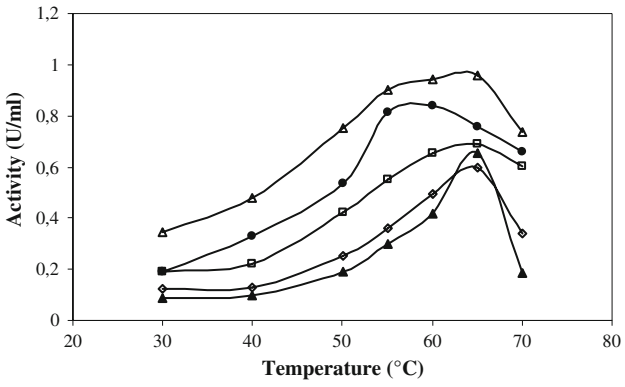

Fig. 5 The effect of the temperature on the esterase activity of the isolates determined by pNPB hydrolysis. Isolates were represented as follows: (*filled circle*) 49, (*open square*) 53, (*open diamond*) 43, (*open triangle*) 7, (*filled triangle*) 138

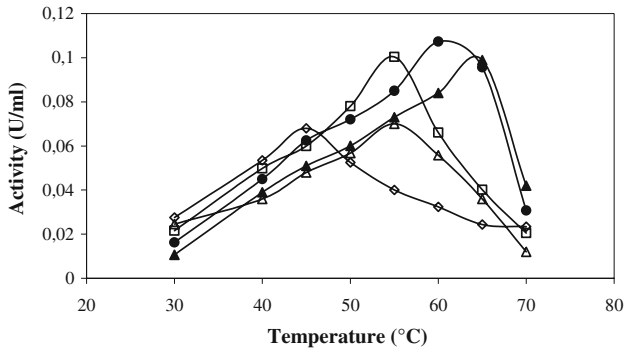

Fig. 6 The effect of temperature on the lipase activity of the isolates determined by pNPP hydrolysis. Isolates were represented as follows: (*filled circle*) 49, (*open square*) 53, (*open diamond*) 43, (*open triangle*) 7, (*filled triangle*) 138

K_M and V_{max} values were calculated from Lineweaver–Burk plot constructed by using activity values depending on substrate concentrations for esterase activity by pNPB and for lipase activity by pNPP. The activation energies (E_a) were determined from the temperature dependence of the activity according to Arrhenius equation. Results are shown in Tables 1 and 2 for pNPB- and pNPP-hydrolyzing activities, respectively. It was found that, the K_M and V_{max} values were lower for pNPP hydrolysis than those for pNPB hydrolysis. The values of K_M, which were getting higher while the affinity of enzyme to substrate was getting lower, vary considerably from one enzyme to another or for a particular enzyme with different substrates [4]. Therefore, to compare enzymes of the isolates for their catalytic efficiencies, the V_{max}/K_M ratios are more useful. As seen in the results, the V_{max}/K_M ratios are higher at esterase activities for all strains; therefore, it can be said that in the current study in terms of lipolytic activity the isolates have higher esterase activity. In parallel to our results, various studies comprising the lipolytic activity of halophilic organisms revealed that esterase activity is usually higher than lipase activity [15, 26]. However, Boutaiba et al. [2] reported the highest lipolytic activity against pNPP (include 16-C fatty acid) when they tested other substrates containing C2 to C18 long-chain fatty acids.

Table 1 Kinetic parameters of extracellular esterase activity of halophilic archaeal isolates for pNPB hydrolysis

Isolate	V_{max} (U ml^{-1})	K_M (mM)	V_{max}/K_M	E_a (kJ mol^{-1} K^{-1})
43	1.597	0.437	3.654	46,805
53	0.936	0.296	3.162	36,800
7	2.458	0.463	5.309	24,434
138	0.768	0.119	6.454	54,516
49	2.211	1.363	1.622	38,124

Table 2 Kinetic parameters of extracellular lipase activity of halophilic archaeal isolates for pNPP hydrolysis

Isolate	V_{max} (U ml^{-1})	K_M (mM)	V_{max}/K_M	E_a (kJ mol^{-1} K^{-1})
43	0.092	0.071	1.296	45,547
53	0.090	0.167	0.539	49,934
7	0.070	0.052	1.355	35,036
138	0.053	0.064	0.828	49,597
49	0.078	0.089	0.876	48,294

Halophilic microorganisms that are able to live in saline environments offer a multitude of actual or potential applications in various fields of biotechnology. Enzymes of halophilic archaeas can provide distinct advantages over their classical counterparts in the development of new bioconversion processes, potentially offering resistance to high salt conditions or high temperatures and enabling the use of organic solvents at low water activity [14, 18].

The current study was carried out with the purpose of defining lipolytic enzymes in halophilic archaea isolates, which are adapted to live at extreme salt environments. Following the discovering of the lipolytic enzymes, they were further characterized to determine their tolerance with pH, temperature and salt alterations, and finally, their kinetic parameters were calculated. As a result, we have shown that the lipolytic enzymes from our halophilic isolates are also active under high temperature (until 60–65°C) and pH conditions (until pH 8–8.5). In addition, the isolates were found to have higher esterase activity comparing to lipase activity on the basis of kinetic parameters. However, it would be more informative if lipolytic enzymes are purified from the isolates and then characterized, which is the next step of the current work.

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