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EXPERIMENTAL ARTICLES

Phylogenetic Analysis and Characterization of Lipolytic Activity of Halophilic Archaeal Isolates[1]

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Abstract—Five isolates designated as B45, D83A, A206A, A85 and E49 found to possess lipolytic activities were taxonomically classified on the basis of their phylogenetic, phenotypic and chemotaxonomic characteristics. The isolates were determined to be gram-negative, catalase and oxidase positive, hydrolyzing Tween 80 and 60 but not starch, need 3.5–4 M NaCl for optimal growth and lack of anaerobic growth with arginine or DMSO. All isolates had the highest lipolytic activity at pH 8.5. Lipase and esterase activities increased with salt concentration up to 3–4.5 M NaCl, and decreased at 5 M NaCl. Esterase and lipase showed their maximal activities at 50–55°C and 60–65°C, respectively. The phylogenetic tree constructed by the neighbor-joining method indicated that the strain B45 and A85 were closely related to the members of genera *Halovivax* and *Natrinema*, respectively. The closest relative of the strain A206A and D83A were found to be *Haloterrigena saccharevitans*. The strain E49 displayed a more distant relationship to known strains.

Keywords: halophilic archaea, lipase, esterase, phylogeny.

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Such industries as food processing, washing, biosynthetic processes and environmental bioremediation require biocatalysts that are stable in harsh environments. Recently, the extremophilic microorganisms became popular sources of these enzymes, as they remained catalytically active at the extremes of temperature, salinity, pH and pressure [1–3]. In biotechnology, it is still a challenge to identify the most suitable enzymes and best reaction conditions for an efficient application. There are two main strategies to obtain obtaining enzymes with desired properties [4], namely the genetic engineering of currently known enzymes and the search for new activities in previously uncharacterized microorganisms [5, 6]. Within the second approach, the search for enzymes in extremophiles seems to be particularly promising since the enzymes of these organisms have particular mechanisms of increased stability in adverse environments, which can potentially also increase their stability in the harsh environments in which they are to be applied in biotechnology [7–10].

Lipases (carboxyl ester hydrolases; E.C. 3.1.1.3) are ubiquitous in nature, produced by animals, plants, and fungi, as well as bacteria [11]. Recently halophilic archaea attract more attention as the sources of lipolytic enzymes. For example, a halophilic archaeon *Haloarcula marismortui* whose genome contains genes encoding for putative esterase and lipase [12], and these genomic predictions have been recently verified [13]. It was reported that *Haloarcula marismortui* displayed esterase and lipase activity intracellularly and extracellularly. While lipase was accumulated mainly extracellularly, esterase was generally accommodated intracellularly [13]. The esterase and lipase genes of *Pyrococcus furiosus*, a hyperthermophilic archaeon, were cloned in *E. coli* and functional properties were determined. The archaeal enzyme was reported to be the most thermostable and thermoactive esterase known to date [14, 15].

Hypersaline environments are commonly present in Turkey. Several studies have been carried out to isolate and characterize halophilic archaeal strains from various saline parts of Turkey [16–18]. In a recent study, 118 archaeal strains from Turkey were screened for their lipolytic activity and it was found that lipolytic enzymes were active under high temperature (up to 65°C) and pH conditions (up to pH 8.5) and the kinetic parameters indicated that the isolates retained higher esterase activity than lipase activity [19]. The phylogenetic studies revealed that the halophilic archaeal isolates from Turkey clustered closely to genera *Halorubrum*, *Haloarcula*, *Natrinema*, *Halobacterium* and *Natronococcus* [20, 18]. However, there has been little effort for the identification of these isolates.

In this study, 5 isolates that were selected because of their high lipolytic activity from a collection of

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archaeal strains were further characterized in terms of their lipolytic activity under various temperature, salt and pH conditions. Furthermore, these isolates were taxonomically characterized based on their phylogenetic, phenotypic and chemotaxonomic characteristics.

MATERIALS AND METHODS

Archaeal isolates, media and culture conditions. Halophilic archaeal isolates were cultured in SG medium, containing (g L^{-1}): NaCl, 250; $MgSO_4 \cdot 7H_2O$, 20; trisodium citrate, 3; KCl, 2; casamino acids, 7.5; yeast extract, 1; $FeSO_4 \cdot 7H_2O$, 0.0023. The medium was adjusted to pH 7.3 prior to sterilization. The cultures were incubated at 37°C on an orbital shaking incubator at 175 rpm. The isolates were cultured on SG agar plates containing 2.5% olive oil and 0.001% (w/v) Rhodamine B solution to observe lipolytic activities which were monitored under UV light at 350 nm as orange halos appearing around the colonies. The isolates designated as the strain E49, A206A, D83A, A85 and B45 were found to be the best producer of extracellular lipolytic activities and they were selected for further studies. The growth of the selected isolates was determined by the increase in optical density at 600 nm, and the lipolytic activities were routinely assayed during 7 days of culture period.

Identification of isolates. Phenotypic characterization was carried out in accordance with the recommended minimal standards for the description of new taxa in the order *Halobacteriales* [21]. Cell motility and morphology of exponentially growing liquid cultures were examined using an Olympus BX51 microscope equipped with phase-contrast optics. Colony morphology was observed on agar medium after incubation at 37°C for 10 days. Gram staining was carried out as described by Dussault [22]. Anaerobic growth was tested in the presence of 5 g L^{-1} DMSO or L-arginine in filled stoppered tubes by measuring culture turbidity at 600 nm [23]. Cytochrome oxidase, catalase, nitrate reduction, indole and H_2S production, hydrolysis of gelatin, casein, starch and Tween 20, 40, 60 and 80 were determined as explained before [21, 20]. The optimum salt concentrations for growth were determined in media containing 0.5–5 M NaCl by turbidity measurement. Acid production was carried out in unbuffered medium with glucose, sucrose, fructose, arabinose, galactose, xylose and maltose as substrates. Antibiotic susceptibility was tested according to the methods described by Stan-Lotter et al. [24].

Polar lipids were extracted with methanol/chloroform as described by Oren and Litchfield [25]. The lipids were separated by thin-layer chromatography with single development on silica plates (Kieselgel 60 F_{254}; Merck) gel in a chloroform/methanol/acetic acid/water (85 : 22.5 : 10 : 4, v/v) solvent system. Glycolipids/phospholipids were visualized by spraying the plates with 0.5% α-naphthol in 50% methanol, and then with 5% sulfuric acid in ethanol followed by heating at 150°C for 5 min.

DNA was extracted by using phenol/chloroform extraction followed by ethanol precipitation, as explained by Ozcan et al. [20]. The gene encoding 16S rRNA was amplified by PCR with the forward primer 5'-ATTCCGGTTGATCCTGCCGGAGGTC-3' (positions 1–25) and the reverse primer 5'-AGGAGGTGATCCAGCCGCCAGATTCC-3' (positions 1448–1472) according to the *Halobacterium cutirubrum* NCIMB 763 (GenBank Accession no. AB073366). The PCR product was sequenced by using an ABI BigDye3.1 sequencing kit (Applied Biosystems) and an automated DNA sequencer (model ABI3100; Applied Biosystems). Phylogenetic analyses were conducted using the neighbor-joining method (MEGA version 4.1) [26].

The phylogenic relationship of isolates were determined by comparing the sequencing data with those of *Natrinema*, *Haloterrigena*, *Natronococcus* and *Halovivax* genera available in GenBank database of the National Center for Biotechnology Information. The 16S rDNA sequences of halophilic archaeal isolates were deposited in the GenBank database under the following accession numbers: strain B45 FJ686121, strain A85 DQ309079, strain D83A FJ686122, strain A206A FJ686119 and strain E49 FJ686123.

Enzymatic assays. Esterase and lipase assays were carried out as described before [19]. Lipase and esterase activities were determined by spectrophotometric assay using pNPP (*p*-nitrophenylpalmitate) and pNPB (*p*-nitrophenylbutyrate) as substrates, respectively. The final concentration of substrates in reaction mixture was 1 mM. Triton X-100 (0.4%) and gum arabic (0.1%) were added for solubilization of pNPP. The reaction was carried out at 40°C for 5 min and the absorbance was measured at 410 nm. One enzyme unit was defined as the amount of enzyme that liberates 1 μmol of pNP per min.

Effect of pH, temperature, and NaCl concentration on enzyme activity. Optimum pH was determined in reaction mixtures containing 50 mM sodium acetate buffer for pH range 5–6 and in 50 mM Tris–HCl for pH range 6.5–9. The assays were performed with 4 M NaCl at 40°C. The molar extinction coefficients of pNP were different for varied pH values; therefore, the standard curves for each pH value were constituted. Optimum temperature was determined in 50 mM Tris–HCl at pH 8 with 4 M NaCl. The assays were carried out at temperatures ranging from 30 to 70°C. The effect of NaCl concentrations ranging from 2 to 5 M NaCl on enzyme activity were measured at 40°C, pH 8.

K_m and V_{max} values of the esterase and lipase were determined by measurement of enzymes activity with various concentrations of *p*-nitrophenyl butyrate and *p*-nitrophenyl palmitate substrate ranging from

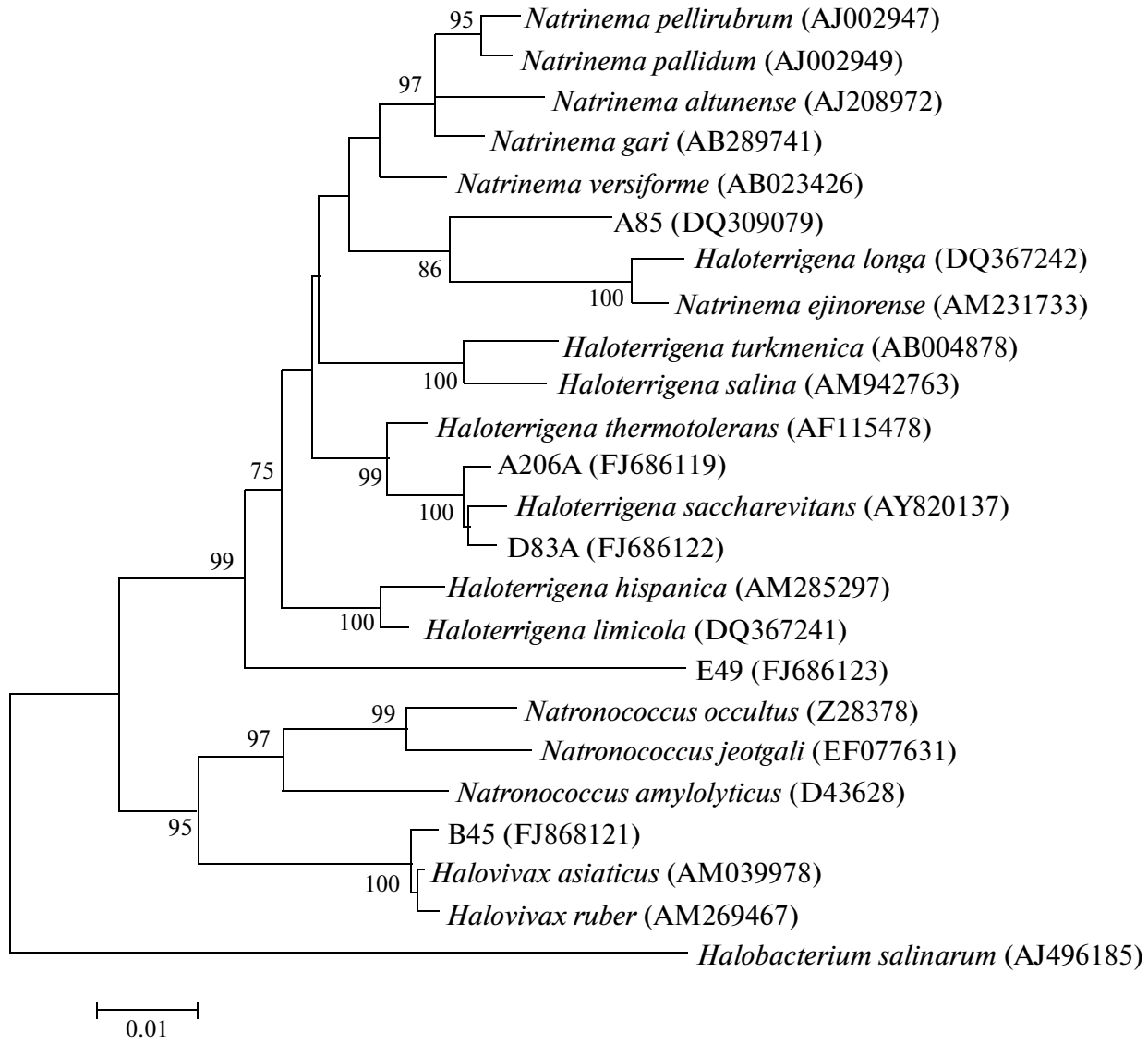

Fig. 1. Neighbor-Joining tree showing the position of isolates A85, A206A, B45, D83A and E49 to members of the *Natrinema*, *Haloterrigena*, *Natronococcus* and *Halovivax* species based on 16S rRNA gene sequences. Bootstrap values greater than 70% are indicated. Bar, 1% sequence divergence.

0.067–1 mM. Kinetic constants were calculated using Lineweaver-Burke plot.

RESULTS AND DISCUSSION

A large collection of halophilic archaea strains obtained from various saltern areas of Turkey was screened for their lipolytic activity on agar plates containing olive oil and Rhodamine B. Among them five strains that produced significant lipolytic activity were selected for the characterization of their extracellular esterase and lipase activities and the evaluation of their taxonomic position based on phylogenetic, chemotaxonomic and phenotypic characteristics.

Phylogenetic and phenotypic characteristics of lipolytic archaea. Comparative sequence analyses of the 16S rRNA gene of the five strains and their closest phylogenetic relatives were performed. The phylogenetic tree (Fig. 1) constructed by the neighbor-joining method indicated that the isolates B45 and A85 were parts of clusters within the genera *Halovivax* and *Natrinema*, respectively. The remaining isolates (D83A, A206A and E49) were clustered within *Haloterrigena* genus. Phenotypic characteristic of the isolates is shown in Table 1. All isolates were gram-negative, catalase and oxidase positive, hydrolyzing Tween 80 and 60 but not starch, needed 3.5–4 M NaCl for optimal growth and lacked the ability for anaerobic growth with arginine or DMSO (Table 1).

The 16S rRNA gene analysis indicated that the closest phylogenetic relatives of B45 are *Halovivax ruber* and *Halovivax asiaticus* which displayed a simi-

Table 1. Features of the halophilic archaeal strains

Characteristic	E49	A85*	D83A	A206A	B45
Colony morphology	Pink-red, Opaque	Red, Translucent	Pale-light red, Opaque	Pale red, Opaque	Pale-white pink, Opaque
Cell morphology	Rod	Rod	Rod	Rod	Rod to pleomorphic
Motility	+	+	+	+	+
Gram reaction	–	–	–	–	–
NaCl optimum (M)	3.5–4	3.5–4	3.5–4	3.5–4	3.5–4
Reduction of nitrate to nitrite	+	+	+	+	+
Gas formation from nitrate	–	–	–	–	–
Anaerobic growth in:					
L-arginine	–	–	–	–	–
DMSO	–	–	–	–	–
Indole formation	+	+	+	+	–
Hydrolysis of:					
Starch	–	–	–	–	–
Gelatin	+	–	+	+	+
Casein	+ (weak)	–	+ (weak)	+ (weak)	+
Tween 20	+	+	+	+	+
Tween 40	+	+	+	+	+
Tween 60	+	+	+	+	+
Tween 80	+	+	+	+	+
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
H_2S formation	+	–	+	+	+
Sensitivity to antibiotics†	Rif, B, Nv	Rif, B, SXT, Nv	Rif, B, SXT, Nv	Rif, B, SXT, Nv	Rif
Acid from sugar	–	Glucose, xylose, arabinose	–	–	–
Polar lipids‡	PG, PGP-Me, S_2-DGD, S-DGD one glycolipid	PG, PGP-Me, PGS, four glycolipid	PG, PGP-Me and S_2-DGD, S-DGD, one glycolipid	PG, PGP-Me and S_2-DGD, S-DGD, one glycolipid	PG, PGP-Me, S-DGD, three glycolipid

* Some phenotypic data from Ozcan et al. (2007).

† Rif—rifampicin, B—bacitracin, Nv—novobiocin, SXT—trimethoprim-sulphamethoxazole.

‡ PG—phosphatidylglycerol, PGP-Me—phosphatidylglycerol phosphate methyl ester, PGS—phosphatidylglycerol sulfate, S_2-DGD: mannose-2,6-disulfate (1⟶2)-glucose glycerol diether, S-DGD—sulfated diglycosyl diether.

larity level of 99.3 and 99.5%, respectively. Cells of strain B45 were motile and predominately rod-shaped in young cultures but became pleomorphic, from rods to triangles or squares, when further incubated. Comparison of phenotypic properties has revealed some differences among *Halovivax* species and B45 isolates. While these two species of the *Halovivax* genus are non-motile and unable to reduce nitrate, B45 is motile and able to reduce nitrate. Although the colonies of *Halovivax asiaticus* and B45 are pale white pink, *Halovivax ruber* has red colonies. On the other hand, although *Halovivax ruber* and B45 are sensitive to Rifampicin and unable to produce acid from D-xylose, *Halovivax asiaticus* is resistant to Rifampicin and able to produce acid (Table 1) [27, 28]. Strain B45 has a polar lipid content including phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me) and a pattern of glycolipids (GL) (Fig. 2) which is similar to that of *Halovivax* species retained [27, 28].

Phylogenetic position and some phenotypic properties of the isolate A85 have been previously reported by Ozcan et al. [20]. In this study, 16S rDNA sequence analysis of the strain A85 which also has high lipolytic activity was repeated and its sequence was updated on GenBank. The phylogenetic analysis of 16S rRNA

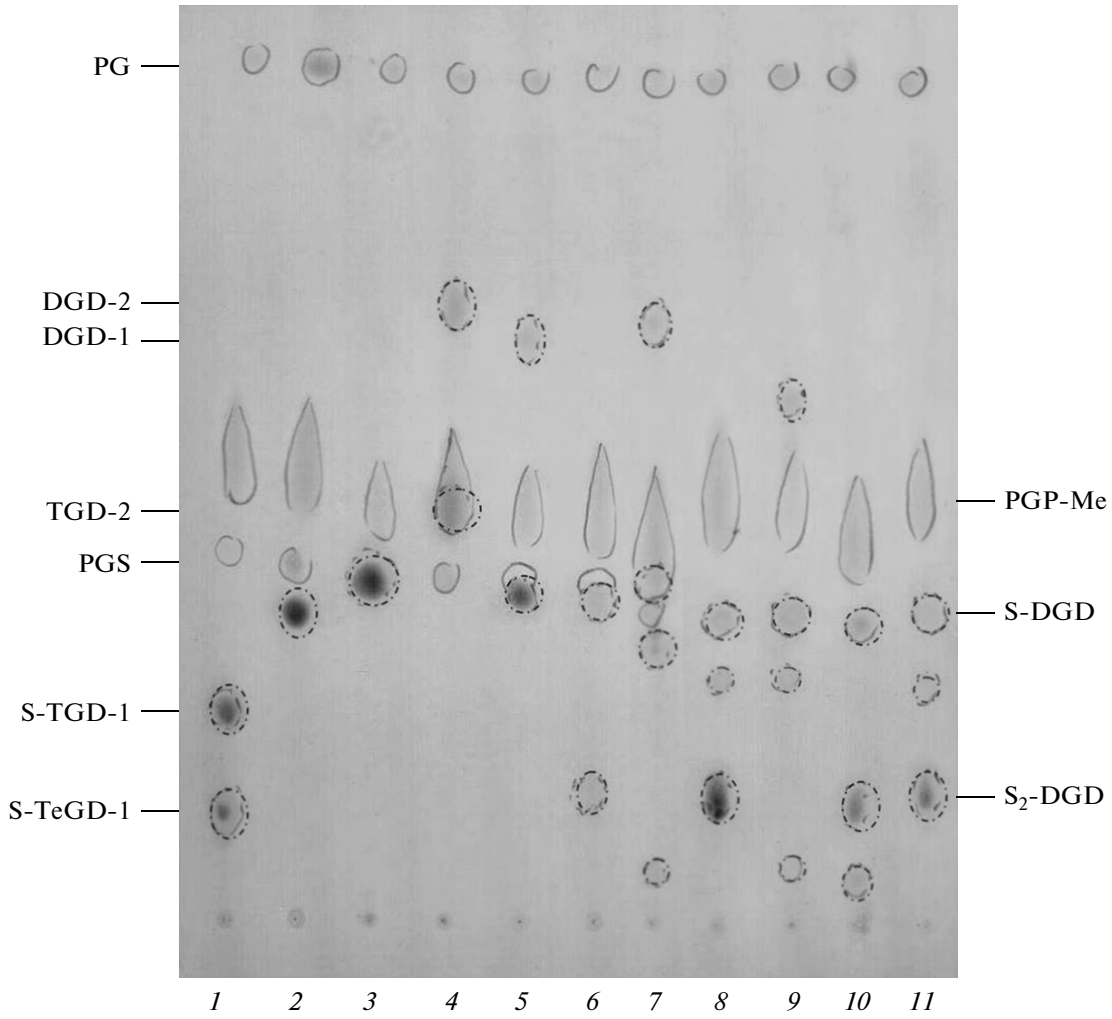

Fig. 2. Thin-layer chromatogram on a Merck silica gel 60 F_{254} glass-backed plate of total polar lipids from strains A85 (lane *7*), A206A (lane *8*), B45 (lane *9*), E49 (lane *10*), D83 (lane *11*), and reference strains *Halobacterium salinarum* ATCC 33170 (lane *1*), *Halorubrum saccharovorum* ATCC 29252T (lane *2*), *Haloferax mediterranei* (lane *3*), *Haloarcula vallismortis* CGMCC 1.2048T (lane *4*), *Halomicrobium mukohatei* (lane *5*) and *Natrialba asiatica* (lane *6*).

gene performed by neighbor-joining method [29], showed the position of strain A85 as a branch in the *Natrinema* clade (Fig. 1). Although the sequence similarity rate between A85 and *Natrinema versiforme*^{T} was revealed as 98.5% previously [20], in the current study the similarity rate was found lower, 97.6%. The characteristics that differentiate the strain A85 from *Natrinema versiforme* are the motility and rod-shaped form of cells, acid production from glucose, arabinose and xylose, hydrolysis of Tweens 80, 60 and 40, unable to form hydrogen sulphide and gas from nitrate and susceptibility to trimethoprim-sulphamethoxazole (Table 1). On the other hand, the high rate of similarity of 16S rDNA sequences and polar lipid content (PG, PGP-Me, PGS-phospho glycerol sulfate and several GL) between A85 and *Natrinema* genus have revealed that A85 is a member of genus *Natrinema* [30]. A85 has shown second high similarity rate (96.1%) to *Haloterrigena longa*. A85, however, differs from *Haloterrigena longa* by its ability to produce acid from some sugars mentioned above, bacitracin sensitivity, the ability to hydrolyze Tween 80 (Table 1) and lack of mannose-2,6-disulfate (1⟶2)-glucose glycerol diether (S_2-DGD) (Fig. 2) which is the major polar lipid of most *Haloterrigena* species [31–33].

The strains A206A, D83A, and E49 are closely related to the members of genus *Haloterrigena* on the basis of common phenotypic features (Table 1) and phylogenetic relationship (Fig. 1). The species of *Haloterrigena* possess mannose-2,6-disulfate (1⟶2)-glucose glycerol diether (S_2-DGD), but lack phosphatidylglycerol sulfate (or contain levels below the limit of detection) [31, 34]. The strains A206A, D83A, and E49 found to have S_2-DGD, and this data support the results of phenotypic and phylogenetic studies. The strains A206A and D83A are similar to each other and *Haloterrigena saccharevitans* with the level

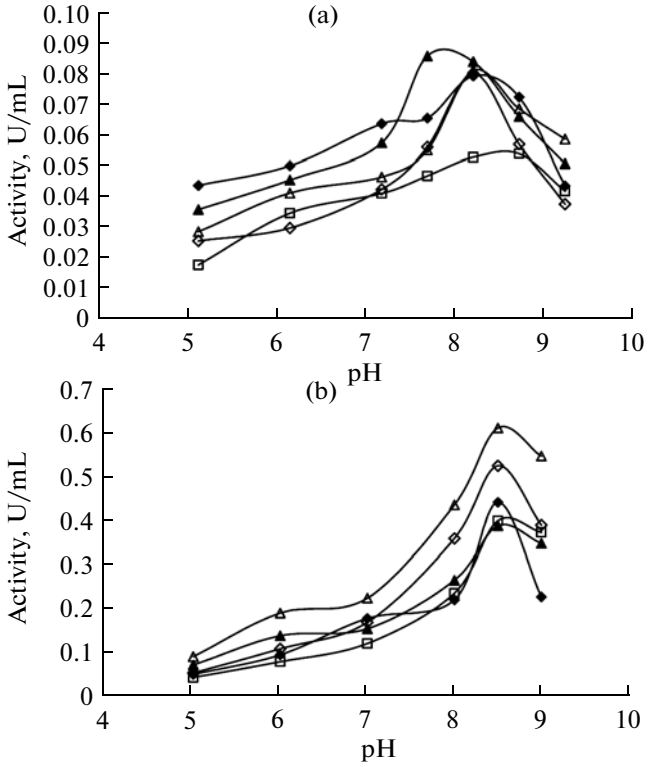

Fig. 3. Effect of pH on the activities of esterase and lipase of E49, B45, D83A, A85 and A206A strains monitored by hydrolysis of *p*-nitrophenyl butyrate (pNPB) (a) and *p*-nitrophenyl palmitate (pNPP) (b). (□—E49; ◇—B45; △—D83A; ▲—A85; ◆—A206A).

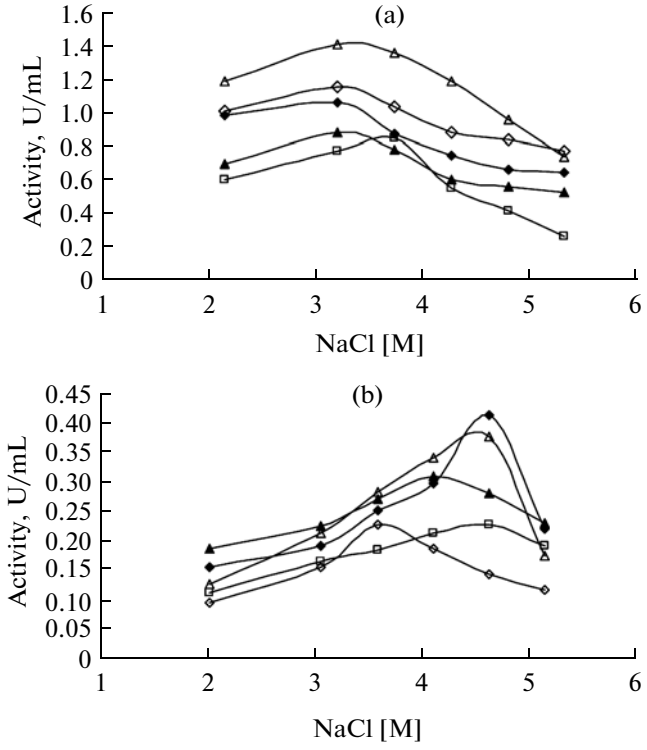

Fig. 4. Effect of the NaCl concentration on the esterase (a) and lipase (b) activity. pNPB and pNPP was used as substrates for esterase and lipase activity, respectively. Assays were carried out at the range of 2–5 M NaCl concentrations in 50 mM Tris–HCl, at pH 8 and 40°C. □—E49; ◇—B45; △—D83A; ▲—A85; ◆—A206A).

of similarity above 99%. A206A and D83 could be distinguished from *Haloterrigena saccharevitans* by the ability to hydrolyse gelatin and casein (weakly), indol formation from tryptone, sulfated diglcosyl diether (S-DGD) and one GL content, and their resistance to tetracycline (Table 1) [35].

Apparently, *Haloterrigena thermotolerans* is the closest relative of strain E49, having 16S rRNA sequence similarity value of 94%. The phenotypic characteristics of *Haloterrigena thermotolerans* are different from E49 in terms of indole production, weak hydrolysis of gelatin, nonmotile cell and susceptibility to trimethoprim-sulphamethoxazole [34]. The phylogenetic tree in Fig. 1 shows the relationship between strain E49 and the representatives of the family *Halobacteriaceae*.

Detection of esterase and lipase activities. High level pNPB- and pNPP-hydrolyzing activities were detected within the pH range 7–8.5. Esterase activity was highest at pH 7.5 for strain A85, pH 8 for strains B45, D83A and A206A and pH 8.5 for strain E49 (Fig. 3a). All strains were shown to possess the highest lipase activity at pH 8.5 (Fig. 3b). It was determined that there was a prominent rise in enzyme activities of A85, B45, D83A and A206A at pH 7–7.5 and above, while E49 was not affected greatly with pH differences.

The effect of NaCl concentrations on the esterase and lipase activities of the isolates is presented in Fig. 4. The esterase and lipase activities of the strains were monitored in the range of 2 to 5 M salt concentrations. It was observed that the isolates have produced maximal lipase activity, but not esterase activity, at higher NaCl concentration. Both enzyme activities increased with salt concentrations up to 3–4.5 M NaCl, and then started to decrease at 5 M NaCl. The temperature profile of esterase and lipase activities of the strains is illustrated in Fig. 5. The optimum temperatures for the esterase and lipase activities were found as 60–65°C and 50–55°C, respectively. The kinetic parameters of esterase and lipase activities of the strains were determined by Lineweaver–Burk plot (Table 2). In general, the strains were found to have higher esterase efficiency comparing to lipase efficiency on the basis of V_{max} values. For the pNPB and pNPP hydrolysis it was found that strains A85 and A206A had the highest substrate affinity, respectively.

In the current study, we have characterized the lipolytic activities of 5 halophilic archaeal isolates that were screened from a large collection obtained from various saline areas of Turkey.

Halophilic enzymes could be used in industrial processes containing high salt concentrations and

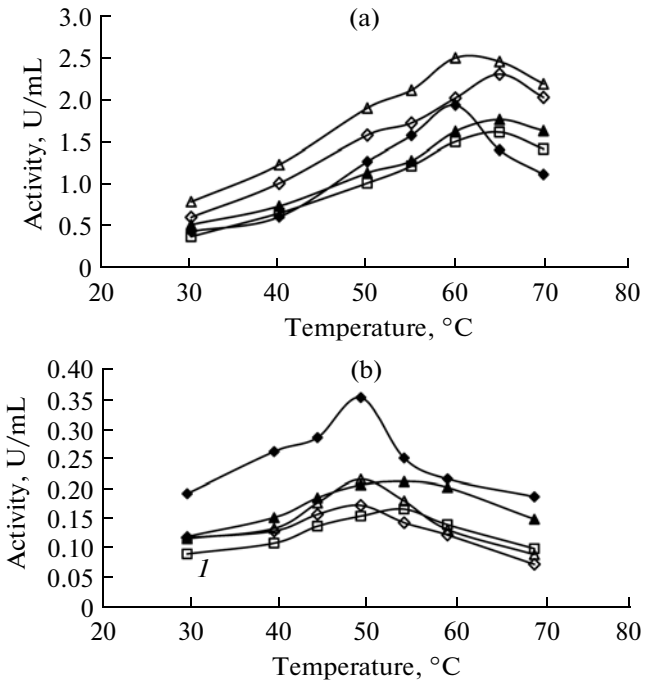

Fig. 5. Effect of temperature on the activities of esterase and lipase of E49, B45, D83A, A85 and A206A strains monitored by hydrolysis of *p*-nitrophenyl butyrate (pNPB) (a) and *p*-nitrophenyl palmitate (pNPP) (b). Assays were carried out in 50 mM Tris–HCl, at pH 8 and 4 M NaCl at different temperatures. (□—E49; ◇—B45; △—D83A; ▲—A85; ◆—A206A).

hydrophobic organic solvents [36]. In this study, the isolates were grown in a range of 2–5 M NaCl. All isolates produced higher lipolytic activity in the range of 3–4.5 M NaCl. It was found that the lipolytic activity dropped at 5 M NaCl. A crude preparation of extremely halophilic archaeon, *Natronococcus* sp. strain TC6, reported to have lipolytic activity optimally at 4 M NaCl, with no activity in the absence of salt. With 4 M NaCl, activity was maximal at 50°C and pH 7 [37]. The enzymes from many halophilic archaea have been shown to be not only salt dependent but many are also thermostable [9, 38]. The lipolytic activities of our isolates have their temperature optima between 50–65°C. Therefore, these enzymes can be classified as moderately thermoactive lipolytic enzymes. Similar results were reported for different halophilic archaeal strains previously [9, 13, 39].

Table 2. Kinetic parameters of extracellular esterase and lipase activity of the halophilic archaeal strains for pNPB and pNPP hydrolysis

	Strain	V_{max} (U/mL)	K_m (mM)
pNPB	B45	1.408	0.933
hydrolysis	E49	0.891	0.727
	D83A	1.926	1.142
	A85	0.949	0.588
	A206A	2.558	3.724
pNPP	B45	0.571	3.067
hydrolysis	E49	0.054	0.115
	D83A	0.080	0.184
	A85	0.062	0.057
	A206A	0.056	0.017

The isolates B45, E49, D83A, A85 and A206A displayed a high level pNPB- and pNPP-hydrolyzing activities within the pH range 7–8.5. These results are in agreement with previous studies which also announced neutral pH optimum for lipolytic activity of *Natronococcus* strain TC6 and *Haloferax mediterranei* [19, 37].

It is generally accepted that strains belong to different species if the similarity value based on 16S rDNA sequences is lower than 97%. If the similarity value is lower than 95%, the strains are considered to belong to different genera [21, 40]. In the current study the phylogenetic results imply that the strain B45 is closely related to the members of genus *Halovivax* (a similarity level above 99%). Phylogenetic position and phenotypic properties showed the position of strain A85 as a member of genus *Natrinema* with the maximum similarity level 97.6%. A206A, D83A and *Haloterrigena saccharevitans* found to share sequence similarity above 99%. Data from sequence similarity rate, polar lipid pattern, phenotypic properties and whole cell protein profiles (data not shown here) clearly implied that these organisms are very close relatives. On the other hand, strain E49 is distantly related to the isolates and the other known genera. *Haloterrigena thermotolerans* was found to be the closest relative of strain E49, having a sequence similarity value of 94%. Thus, the strain E49 may represent a new species within *Haloterrigena* or a new genus in *Halobacteriaceae* family.

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