Determination of Hydrolytic Enzyme Capabilities of *Halophilic Archaea* Isolated from Hides and Skins and Their Phenotypic and Phylogenetic Identification

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Abstract

This research aims to isolate extremely halophilic archaea from salted hides, to determine the capacities of their hydrolytic enzymes, and to identify them by using phenotypic and molecular methods. Domestic and imported salted hide and skin samples obtained from eight different sources were used as the research material. 186 extremely halophilic microorganisms were isolated from salted raw hides and skins. Some biochemical, antibiotic sensitivity, pH, NaCl, temperature tolerance and quantitative and qualitative hydrolytic enzyme tests were performed on these isolates. In our study, taking into account the phenotypic findings of the research, 34 of 186 isolates were selected. These isolates were identified by 16S rRNA sequence analysis and 15 different strains of extreme halophilic archaea were identified. 13 strains of these were identified for first time from salted raw hide and skin in our study including *Natrialba aegyptia, Halococcus thailandensis, Halococcus dombrowskii, Halovivax asiaticus*, *Halovivax sp.* E107, Haloarchaeon, *Natronococcus sp.*, *Halorubrum sp.*, *Halomicrobium zhouii, Natronococcus jeotgali*, *Haloterrigena thermotolerans*, *Natrinema versiforme*, *Halobacterium noricense*. At the same time detecting *Natrialba aegyptia* in 6 of 8 hide samples showed that this strain is widely found in hide and skin samples. Research results are expected to contribute to other studies and solving microbial problems in leather industry.

Introduction

The main constituent of the raw hide is protein, mainly collagen (33% w/w), and remainder is moisture and fat. During storage of raw hide, collagen's excessive proteolysis by lysosomal autolysis or proteolytic bacterial enzymes can lead to the disintegration of the structure of collagen fibers.[1] Biodeterioration is among the major causes of impairment of aesthetic, functional and other properties of leather and other biopolymers or organic materials and the products made from them. Due to the fact that prevention of biological degradation is very important in conservation and processing of leather, great effort is being made for decontamination of these processes from microorganisms and for the development of more effective methods.[2,3,4]

In Europe, North America and other regions with temperate climates, conservation varieties are the protection of raw hide, such as drying, salt and brine, and the most common method traditionally used is salt curing.[5] Halophiles which require high salt concentration to grow, and develop different strategies to survive in halophilic environments, are a large group and have both prokaryotic and eukaryotic representatives. These microorganisms are located in salt lakes, salt production facilities, salted fish and on the surface of hides and skins.[6] Halophiles are divided into 4 groups according to their need for optimum amount of NaCl to grow in.

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Non-halophiles prefer less than 1% NaCl, while slightly halophiles favor 1 to 3%, moderately halophiles 3 to 15%, and extremely halophiles more than 15% NaCl.[7] In addition to these groups, there are halotolerant organisms developing in the presence or absence of NaCl.[6] The family *Halobacteriaceae* in the domain *Archaea,* thoroughly consisting of halophiles, is the only family in order *Halobacteriales*. The ones, which are the most salt-tolerant, and the most in need of salt in all microorganisms are included in this family.[8]

Isolation and molecular identification of these microorganisms that cause financial losses in the leather production are of great importance because microbial control methods can be developed intended for these particular microorganisms by determining hydrolytic enzyme activities, especially the proteolytic and lipolytic ones exhibited by these microorganisms. In recent years, some studies have been carried out on the basis of 16S rRNA gene sequences for the identification of microorganisms found in leather industry. In a study on raw buffalo hide with 16S rRNA gene sequence analysis method, the identification of 17 different species of belonging to 11 bacteria genera (*Bacillus, Acinetobacter, Aeromonas, Escherichia, Proteus, Myroides, brevibacterium, Vagococcus, Staphylococcus, Mycoplana*, and *Weeksell*) was reported.[9] By using comparative partial 16S rRNA gene sequence analysis of the isolates from salted hide, Yılmaz[10] identified different Bacteria and Archaea strains, namely *Salimicrobium album, Salimicrobium halophilum, Halomonas eurihalina, Salimicrobium luteum, Halomonas koreensis, Halomonas elongata, Halomonas halmophila, Halomonas alimentaria, Marinococcus halophilus, Halorubrum saccharovorum, Halorubrum tebenquichense, Halorubrum lacusprofundi, Chromohalobacter salexigens, Oceanobacillus picturae, Thalassobacillus devorans, Alkalibacillus salilacus, Natrinema pallidum, Natrinema gari*. Moreover, this researcher suggests that salted hides and skins contain various moderate halophilic bacteria species and halophilic bacteria counts are controlled in well-conserved hides.

On the other hand, microorganisms are used as enzyme sources for their wide biochemical diversity and their appropriateness for genetic practices.[11] Hydrolytic enzymes such as protease, amylase, amidase, esterase and lipase constitute a large part of the industrial enzymes. Recently, lipases (triacylglycerol acylhydrolase EC 3.1.1.3) have emerged as key enzymes increasingly growing in biotechnology due to their wide range of use in industrial practices such as food technology, detergent production, chemical industry, and biomedical sciences thanks to their versatile features.[12,13] Little is known about proteolysis in Archaea although it has been studied in bacteria and eukaryotes.[14] Haloarchaea and their enzymes are active and stable in high saline environments,[15] therefore they have a great potential as biocatalysts in application procedures requiring low water activity such as high salinity or organic solvent processes.[14] It was observed that most of the moderate or extreme halophilic microorganisms such as *Salinivibrio* sp. and *Natronococcus* sp. produce lipases which are stable at high temperatures.[16] Boutaiba *et al*.[17] pre-characterized lipase activity of *Natronococcus* sp. which is an extreme halophilic archaea.

Leather processing is performed with pre-tanning which includes a series of operations such as soaking, dehairing, bating, degreasing, pickling[18] tanning and post-tanning steps. Microbial enzymes are used in many of these steps and leather waste treatment.[11] The conservation of rawhide and skin to prevent the development of many of the microorganisms is not sufficient for complete protection. Halophilic microorganisms that especially cause red spot on salted raw hides/skins can damage the hide/skin with their proteolytic and lipolytic properties. To prevent these problems, a wide variety of antimicrobial agents are assessed[19] along with their synergistic effects[20] in leather industry and currently studies are being conducted for the detection of more effective, and eco-friendly new agents[21] efficient in controlling microorganisms.

As a result of the literature review, it was found that studies with salted rawhide and skin were carried out mostly on halophilic bacteria and some of their enzymes; but not research on exiguous halophilic archaea. For this reason, the present study is intended to isolate extremely halophilic archaea from salted rawhide and skin, to determine their hydrolytic enzyme capacities, and to identify these isolates by means of phenotypic analyses and phylogenetically by 16S rRNA sequence analysis.

Experimental

Eight salted hides and skins (4 local and 4 foreign), which were processed in Turkey, were used in this study. The 1st sample was obtained from Nigde, the 2nd from Aksaray, the 3rd from Kayseri, the 4th from Gaziantep, the 5th from Iraq, the 6th from Turkmenistan, the 7th from Kazakhstan, and the 8th from Armenia. Isolates from these samples were separately enumerated. SW 25 medium was used for culture isolation, activation and phenotypic tests. This medium was composed of 833.4 ml/l SW 30 solution, 5 g/l yeast extract, 20 g/l agar. SW 30 solution contains 234 g/l NaCl, 39 g/l $MgCl_2$, 61 g/l $MgSO_4$, 1 g/l $CaCl_2$, 6 g/l KC1, 0.25 g/l $NaHCO_3$, 0.7 g/l NaBr. Media were adjusted to pH 7.5 with 1N NaOH.[22]

Microorganisms were isolated via dilution plate and spreading culture techniques.[23] After incubation at 37°C for 14 days, microorganisms were selected by using binocular loop according to colony and color differences.[24] Chosen colonies were inoculated via duplicate streak plate technique and stored in their respective plates at +4°C. Longer storage was conducted in 20% glycerol at -20°C. For the identification of phenotypic characteristics of the isolates, most of diagnostic

tests required by Oren et al.[25] were used. The incubations were carried out at 37°C for 7 days. Dussault[26] Gram staining was performed for the identification of Gram reactions and cell morphology of the isolates. Of biochemical tests, oxidase,[27,28] catalase, indole,[29] and nitrate-to-gas and nitrate-to-nitrite reduction tests[28,30] were utilized.

In the identification of the growth conditions of the isolates, they were inoculated in spots in the plates containing 0 M, 0.4 M, 0.7 M, 1 M, 1.3 M, 1.5 M, 1.7 M, 2 M, 2.3 M, 2.5 M, 2.7 M, 3 M, 3.3 M, 4 M NaCl and 0.5% yeast extract to determine their salt tolerances.[31] SW-25 agar plates where isolates were inoculated in spots were left for incubation at 20, 27, 37, 40 and 50°C to determine the level of their heat resistance.[24] For pH tolerance, growth rates of the isolates inoculated in the SW 25 agar plates adjusted to pH 3, 4, 5, 6, 7, 8, 9, 10 were examined.[24]

In order to determine the lowest level of need for yeast extract, SW-25 agar plate with 0%, 0.01%, 0.1% and 0.5% yeast extract was used.[32] To monitor the growth in a single carbon source, SW-25 agar containing 0.01% yeast extract was used. D-Galactose, Lactose, D-Fructose, Maltose, Mannitol, Sucrose, Trehaloz, Glucose carbon sources were sterilized outside the petri with a 0.22 µm syringe filter and added to the extent that the last volume would be 1%.[33] SW-25 agar plates containing 0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 3.2% $MgCl_2$ were used to determine the lowest level of need for Mg^{+2} (by adding 3.4% Na_2SO_4 instead of $MgSO_4$).[34] For the antibiotic sensitivity test, disc diffusion method was used and antibiotic discs with 6 mm diameters were used for Ampicillin 10 μg (A 10), Bacitracin 10 μg (B 10), Erytromycin 15 μg (E 15), Novobiosin 30 μg (NV 30), Penicillin G 10 U (P 10), Streptomycin 10 μg (S 10), Tetracycline 30 μg (T 30), Vankomycin 30 μg (VAN 30), Chloramphenicol 30 μg (C 30), and Cefotaxime 30 μg (CTX 30).[33,35,36] Caseinase,[37] gelatinase,[38,39] amylase,[40,41] cellulase,[42] esterase,[43,44,45] and lipase tests[44,46] were performed for the qualitative screening of extracellular hydrolytic enzymes; and for DNase test, DNase agar plate prepared with SW 25 solution was used by adding 0.005% methyl green.[47]

Caseinase, keratinase, collagenase, esterase and lipase enzyme activities of some of the isolates exhibiting qualitative enzyme activity were quantitatively measured. Selected isolates were cultured in 150 rpm shaking incubator at 37°C in 250 ml Erlenmeyer flasks containing 50 ml SW-25 liquid media. Samples were taken daily. 1% inductor was added in the media. Kazein was used as inductor for protease, collagenase and keratinase activities, olive oil for lipase activities and tween 80 for esterase activity. Daily samples were centrifuged at 10.000xg for 10 minutes and their supernatants used as enzyme sources. Growth curves were created for each isolate to compare the enzyme activities.[40] The modified version of the procedure by Karadzic *et al*.[48] was used with 0.6% Hammersten Kazein (50 mM Tris-HCl, 2M NaCl, pH:7.5) substrate solution. The modified version of the procedure by Cheng *et al*.[49] was utilized for the identification of collagenase and keratinase activities. 0.4% keratin-azure (50 mM Tris-HCl, 2 M NaCl, pH 7.5) substrate solution was used for keratinase and 0.5% azocoll for collagenase. One unit (U/ml) of enzyme activity was determined to be the required enzyme amount for a 0.01 unit of absorbance increase per minute under the above mentioned reaction conditions. By modifying the method by Comacho *et al*.,[50] esterase and lipase activities were investigated by spectrophotometrically measuring p-Nitrophenyl butyrate (pNPB) hydrolysis for esterase activity and p-Nitrophenyl laurate (pNPL) hydrolysis for lipase activity. The enzyme amount producing 1 μmol p-Nitrophenol per minute under aforementioned reaction conditions was identified as the 1 unit (U/ml) enzyme activity.

The genomic DNA isolation of the isolates was conducted by using the modified version of the method of Dyall-Smith[51] and Geneon® bacterial DNA isolation kit. For Genomic DNA isolation, 1.5 ml of the culture samples ($OD_{600} \cong 1$) grown in SW-25 liquid medium was transferred to sterile microcentrifuge tubes and centrifuged at +4°C at 10.000xg for 10 minutes. After removing the supernatants of the samples, 400 µl of sterile cold ultra pure water was added in the tubes and shaken for a short time to disintegrate the cells. Samples were centrifuged at +4°C' at 10.000xg for 3 minutes to remove supernatants and genomic DNA isolation was conducted in line with the instructions of the commercial kit. 0.8% agarose gel (80 V, 100 mA, 1 hour) was prepared with 1X TAE for control and Syber® Green I and 6X gel loading dye was used to load the samples onto the gel.

In the Polymerase Chain Reaction of 16S rRNA genes of the obtained genomic DNAs, Arc7f (5'- ttc ygg ttg atc cyg cc-3') was used as forward primer and Arc1384r (5'- cgg tgt gtg caa gga gca-3') as reverse primer.[52] In the configuration of Polymerase Chain Reaction (PCR), Geneon® Maximo Taq DNA Polymerase kit was used. This PCR program consisted of 30 repetitive cycles with a strand separation step at 94°C for 45s, an annealing step at 53°C of 45s, and an elongation step for 1m at 72°C. PCR products were purified with PCR cleanup kit (Geneon®) and sequence analyses were conducted by Bioeksen. *Haloferax* sp. HSC4 was used as a control. Chromatograms of the sequence analyses were viewed and edited via Chromas®. Forward and reverse sequences were aligned on the NCBI's (National Center of Biotechnology Information) website, and then 16S rRNA gene sequences were obtained by determining the shared parts. These sequences were analyzed by using nucleotide BLAST (Basic Local Alignment Search Tool) at http://www.ncbi.nlm.nih.gov and compared with existing16S rRNA sequences in the GenBank database.

Results

In this study, a total of 186 extremely halophilic microorganisms were isolated, to be more specific, 59 isolates (number 101- 166) from sample 1, 29 (number 201-229) from 2, 17 (number 301-317) from 3, 19 (number 401-419) from 4, 20 (number 501-520) from 5, 10 (number 601-610) from 6, 16 (number 701-717) from 7, 16 (number 801-817) from 8. The isolate colonies were observed to be light red, red, dark red, cream or transparent. Of 186 isolates, 82 were identified to be cocci, 72 to be bacilli, and 32 to be pleomorphic as a result of microscopic examination. Gram-staining results revealed that all of the isolates were gram negative [Gr(-)]. It was determined that 73% of the obtained isolates were oxidase negative, while 68% were catalase positive. Moreover, 15 isolates producing indole from tryptophan, 10 isolates producing gas from nitrate, and 115 isolates reducing nitrate to nitrite were identified. All of the isolates displayed optimal growth at 37 and 40°C. It was observed that 35% of the isolates were able to grow in a pH range 6-9, 32% in 5-9, 19% in 6-10 and 12% in 5-10. Minimum NaCl amount in which the isolates were able to grow was found to be 1.5 M for 118 isolates, 2 M for 39 isolates, 1 M for 23 isolates, 2.5 M for 4 isolates and 0.4 M for 2 isolates.

It was also discovered that the minimum $MgCl_2$ amount needed by all of the isolates to grow in was 0.05%, while they needed at least 0.1% yeast extract. It was revealed that all were able to grow in glucose, used as the sole carbon source. Besides, it was determined that 63% of the isolates were able to use lactose, and 26%; sucrose, 25%; galactose, 15%; fructose 8%; maltose, 7%; mannitol and 5%; trehalose as the sole carbon source (last volume 1%). In the antibiotic sensitivity test, 8 mm and smaller zone diameters were considered resistant.[53] It was found that all of the isolates were susceptible to bacitracin and novobiocin. Also, it was observed that 50 isolates (27%) were sensitive to tetracycline, 16 isolates (9%) to chloramphenicol, 14 isolates (8%) to erythromycin, 7 isolates (4%) to ampicillin, 5 isolates (3%) to penicillin G, 2 isolates to vancomycin, 1 isolate to streptomycin and 1 isolate to cefotaxime antibiotics.

Qualitative Findings Concerning Hydrolytic Enzyme Activities

In the qualitative enzyme analyses in the present study, it was observed that all of the isolates had positive DNase activity. Moreover, no isolate was found out to have cellulose activity. It was discovered that out of 186 isolates, 23 isolates (12%) had caseinase, 106 isolates (57%) had gelatinase and 21 isolates (11%) had amylase activity. 65 isolates (35%) were found to be esterase positive and 33 (18%) to be lipase positive.

Quantitative Findings Concerning Hydrolytic Enzyme Activities

24 isolates with considerable hydrolytic enzyme activity were selected by making use of qualitative enzyme analyses. Out of these isolates, 21 proteolytic isolates' (102, 103, 107, 111, 112, 114, 116, 123, 125, 126, 129, 149, 150, 151, 153, 165, 221, 505, 511, 514 and 519) protease (caseinase) activities were quantitatively measured (Fig.1). Keratinase and collagenase activities of 10 isolates (107, 114, 116, 125, 149, 150, 151, 153, 505 and 514), which exhibited a high level of protease (caseinase) activity were quantitatively determined (Fig.2). Of these, esterase and lipase activities were also quantitatively measured (Fig.3) for 10 isolates (107, 129, 140, 144, 149, 150, 151, 505, 514 and 516).

Molecular Identification

According to the findings obtained from 34 isolates identified in this study, 15 isolates were found to phylogenetically resemble different species. 16S rRNA sequence analysis showed that the findings elicited from the comparison between these 34 isolates and 16S rRNA gene sequences available in the gene bank database and record numbers retrieved from the gene bank are presented in Table I.

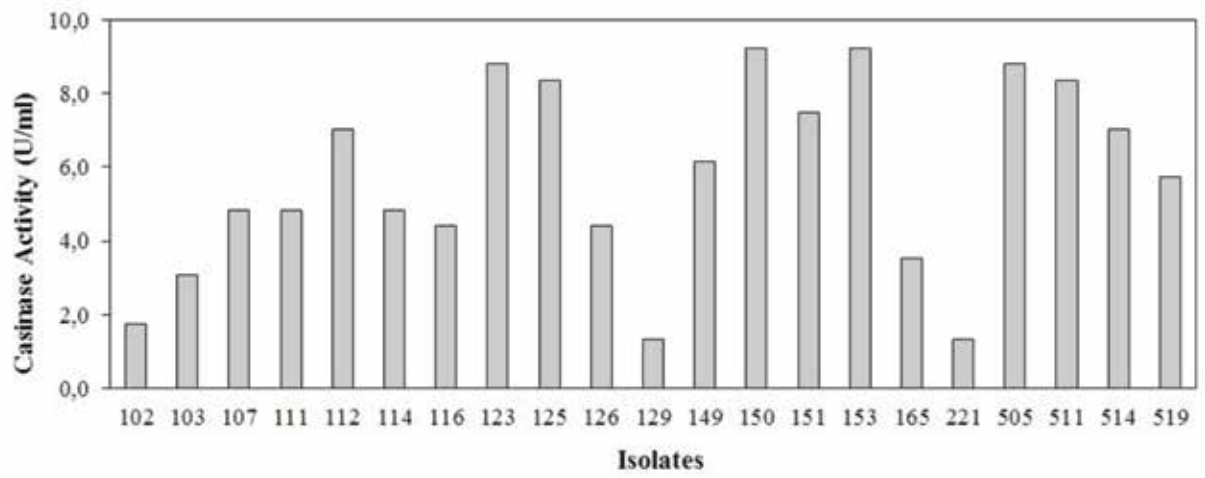

Figure 1. Caseinase activity.

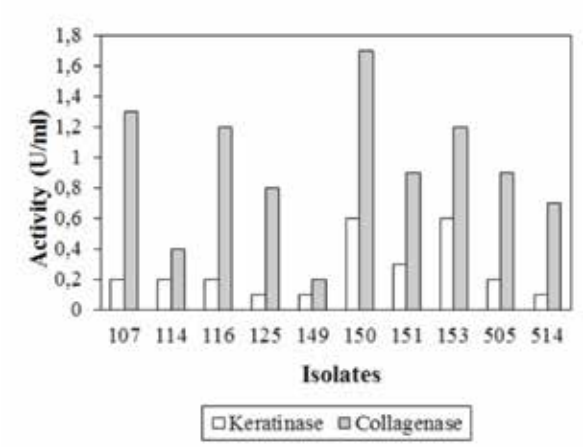

Figure 2. Keratinase and Collagenase activity.

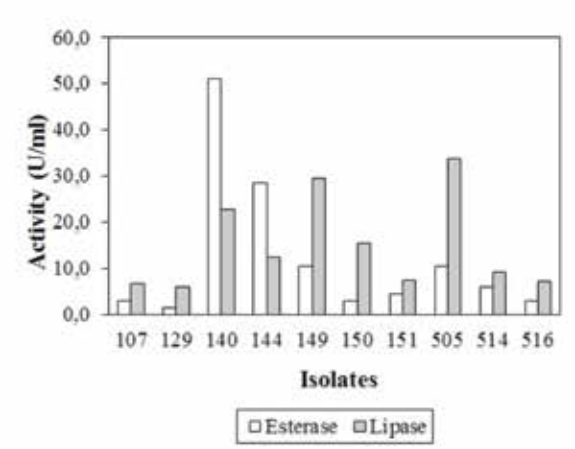

Figure 3. Esterase and Lipase Activity.

TABLE I
Description of Archaeal 16S rRNA gene from salted hides.

Isolates	Accession number in this study	Closest described relative and accession number[a]	% similarity[a]
103	JX481739	*Natronococcus sp*. F30AI (DQ309081.1)	99
107	JX481740	*Natrialba aegyptia* 40 (NR_028176.1)	99
112	JX481741	*Halovivax asiaticus* (AB477227.1)	99
114	JX481742	*Natrialba aegyptia* 40 (NR_028176.1)	99
116	JX481743	*Halovivax* sp. E107 (FJ686124.1)	99
123	JX481744	*Halococcus morrhuae* (X00662.1)	99
125	JX481745	*Halococcus thailandensis* (AB549236.1)	100
129	JX481746	Uncultured haloarchaeon clone YA32 (EF533956.1)	99
140	JX481747	*Halorubrum* sp. CH3 (FJ686129.1)	99
142	JX481748	*Natrialba aegyptia* 40 (NR_028176.1)	97
144	JX481749	*Halomicrobium zhouii* (HQ215547.1)	94
149	JX481750	*Natrialba aegyptia* 40 (NR_028176.1)	99
150	JX481751	*Natrinema pallidum* R-fish (AF367370.2)	99
151	JX481752	*Natrinema pallidum* R-fish (AF367370.2)	99
153	JX481753	*Natrinema pallidum* R-fish (AF367370.2)	100
213	JX481754	*Natrialba aegyptia* 40 (NR_028176.1)	99
221	JX481755	*Halococcus thailandensis* (AB549236.1)	100

Table I continued on the following page.

Table I continued.

226	JX481756	*Natronococcus jeotgali* (AB477232.1)	99
305	JX481757	*Natrialba aegyptia* 40 (NR_028176.1)	99
312	JX481758	*Halococcus thailandensis* (AB549236.1)	99
313	JX481759	*Natrialba aegyptia* 40 (NR_028176.1)	99
410	JX481760	*Halococcus dombrowskii* H4 (NR_028186.1)	99
415	JX481761	*Haloterrigena thermotolerans* Z4 (EU557270.1)	99
505	JX481762	*Natrinema pallidum* R-fish (AF367370.2)	99
511	JX481763	*Halococcus morhuae* (X00662.1)	99
514	JX481764	*Halococcus thailandensis* (AB549236.1)	99
516	JX481765	*Natrialba aegyptia* 40 (NR_028176.1)	92
517	JX481766	*Halovivax asiaticus* (AB477227.1)	99
519	JX481767	*Halococcus dombrowskii* H4 (NR_028186.1)	99
603	JX481768	*Natrialba aegyptia* 40 (NR_028176.1)	99
701	JX481769	*Natrialba aegyptia* 40 (NR_028176.1)	99
708	JX481770	*Natrinema versiforme* XF10 (NR_028150.1)	99
714	JX481771	*Halobacterium noricense* A1 (NR_028187.1)	99
805	JX481772	*Halococcus thailandensis* (AB549236.1)	99

[a]Based on FASTA under nucleotid collection database

Discussion

All of the 186 isolates exhibited Gram negative reaction, which is in accordance with the typical properties of Archaea domain. According to the results of NaCl tolerance, all the isolates were able to grow in 3.3 and 4 M NaCl, but not in non-NaCl medium. In consideration of the classification created by Kushner[7] according to salinity concentration, this finding indicates that the isolates were extremely halophilic.

In the test of the sole carbon source utilization of the isolates, the concentration of the yeast extract added in the media was determined to be 0.01%. The lowest $MgCl_2$ for all of the isolates was found to be 0.05% (5 mM). Montalvo-Rodriguez et al.[33] calculated this percentage as 0.005%. It was detected that the isolates were able to grow at pH 7-8 optimum. This finding overlaps with the physiological properties of species identified by molecular methods. The antibiotic sensitivity tests revealed that all of the isolates were susceptible to the antibiotics bacitracin and novobiocin, and 97% of them to penicillin G, which indicates that the isolates might be Archaea. Accordingly, Holmes and Dyall-Smith[54] express that DNA gyrase inhibitor of most halophilic archaea species are sensitive to bacitracin and novobiocin. Çetinkuş[55] identified the isolated halophilic microorganisms as Archaea or Bacteria via 16S rRNA analysis and found that the isolates were susceptible to bacitracin and novobiocin, and resistant to penicillin G. Likewise, the present study discovered with antibiotic tests and molecular identification that the 34 isolates were Archaea.

Discussion of Findings Concerning Measurement of Hydrolytic Enzyme Activity

The review of the literature on leather microbiology suggests that studies on enzyme activity mostly focus on bacteria and halophilic bacteria. Besides, there is research on extremely

halophilic archaea isolated from saline soil samples, saltpans, salt lakes, salt fermented food and their enzymes. The literature review also revealed that most of the existing research is related to the characterization of the optimum activity conditions of an enzyme isolated from an isolate or an already identified species. The current study is intended to quantitatively determine the protease, keratinase, collagenase, lipase and esterase enzyme activities in the conditions where isolates qualitatively chosen out of isolates exhibit enzyme activity. Measurements of the caseinase, keratinase and collagenase enzyme activity were performed in 2M NaCl and at 37°C and pH 7.5 reaction conditions. In these reaction conditions, the highest level of activity was realized by number 150, whose caseinase, keratinase and collagenase enzyme activities were measured as 9.2 U/ml, 0.6 U/ml and 1.7 U/ml, respectively.

In a study which investigates the esterase and lipase activities of 5 halophilic archaeal strains, maximum esterase activity was found to occur in reaction conditions of 3–4.5 M NaCl, pH 8-8.5 and 60-65°C , and maximum lipase activity in 3.5-4 M NaCl and at pH 8 and 45-60°C. Moreover, it was also detected that values of esterase activity were higher than those of lipase activity.[56] Another study showed that optimum conditions of *Halobacteria* strain were 3.5 M NaCl, pH 8, 40°C for esterase and 4 M NaCl, pH 7.5 40°C for lipase. It is also suggested that esterase activity occurs faster than the lipase activity.[44] In a study where esterase and lipase are produced and characterized from *Haloarcula marismortui*, it was found out that extracellular esterase and lipase activities were 0.06 U/l and 0.26 U/l, respectively.[50] In the present study, esterase and lipase activities were measured in 2 M NaCl, at 37°C and pH 7.5. In this conditions, the highest esterase activity (51 U/ml) was exhibited by number 140, while the highest lipase activity (33.8 U/ml) was realized by the isolate number 505. It was also observed that isolates 140 and 144 had high esterase activity, whereas isolates 140, 149 and 505, too, exhibited high lipase activity.

Discussion of the Findings Concerning Molecular Identification

Fifteen different species, 8 genera and 1 haloarcheon were identified as a result of 16S rRNA sequence analysis of 34 isolates chosen in consideration of many phenotypic properties of 186 isolates. Relatedly, it was discovered that 10 strains were phylogenetically similar to *Natrialba aegyptia* 40, 5 strains to *Halococcus thailandensis*, 4 strains to *Natrinema pallidum* R-fish, 2 strains to *Halococcus dombrowskii* H4, 2 strains to *Halovivax asiaticus*, 2 strains to *Halococcus morrhuae* and 1 strain to each of *Halovivax sp.* E107, uncultured haloarchaeon clone YA32, *Natronococcus sp.* F30AI, *Halorubrum* sp. CH3, *Halomicrobium zhouii*, *Natronococcus jeotgali*, *Haloterrigena thermotolerans* Z4, *Natrinema versiforme* XF10, *Halobacterium noricense* A1. These results revealed that molecularly identified isolates of the current study were extremely halophilic archaea. In previously studies, all of the resembling species isolated in this study were identified and isolated from such saline media as saline soil, saltpans, salt lakes, salt-fermented food.

Natrinema pallidum[10,57] and *Halococcus morhuae*[2] species, isolated from hides in previous studies, were also detected in the present study. However, this study is the first in Turkey to identify 13 different extremely halophilic archaea species, namely *Natrialba aegyptia* 40, *Halococcus thailandensis*, *Halococcus dombrowskii* H4, *Halovivax asiaticus*, *Halovivax* sp. E107, uncultured haloarchaeon clone YA32, *Natronococcus sp.* F30AI, *Halorubrum* sp. CH3, *Halomicrobium zhouii*, *Natronococcus jeotgali*, *Haloterrigena thermotolerans* Z4, *Natrinema versiforme* XF10, *Halobacterium noricense* A1 by isolating them from salted hides. *Natrialba aegyptia* 40 was discovered in 6, and *Halococcus thailandensis* in 5 of 8 hide samples. Thereby, these extremely halophilic archaea were found to be common species in the hide samples on hand.

According to the data obtained from http://www.ncbi.nlm.nih.gov/, *Natronococcus sp.* F30AI, *Halovivax* sp. E107, *Halorubrum* sp. CH3 are extremely halophilic archaea species which were identified by Özcan *et al.*[56,58] after being isolated from various saline regions in Turkey and isolated from local hide samples for the first time by this study. It can be suggested that the source of these archaea is salt used for conservation.

Isolate 140 was identified as *Halorubrum* sp. CH3 and it was found out that this isolate exhibited higher level of esterase and lipase activity. Moreover, isolates 150, 151, 153 and 505 were identified as *Natrinema pallidum* R-fish strain and they were observed to exhibit considerable hydrolytic enzyme activities like caseinase, keratinase, collagenase and lipase.

The 16S rRNA sequence analysis revealed that isolates 107, 114, 142, 149, 213, 305, 313, 516, 603, and 701 were *Natrialba aegyptia* 40; and isolates 125, 221, 312, 514, and 805 exhibited the characteristics of *Halococcus thailandensis*, but some similar isolates were found out to differ in terms of enzyme activities. It was considered that such species might belong to a different subspecies.

Conclusion

Previous studies on leather microbiology majorly focus on bacteria, fungi and their proteolytic and lipolytic enzymes. This study showed that the identification of halophilic archaea with such hydrolytic enzyme activities as caseinase, keratinase, collagenase, esterase and lipase in salted raw hide and skin is very important. It was considered that characterization of some of the archaea identified in the current study can substantially contribute to the industry. Plus, the study also found out that it is necessary to use antimicrobial agents in tanning processes to control the growth of these archaea

whose hydrolytic activities were detected. Furthermore, a halophilic archaea, which had never been cultured before, was cultured in this study. The examination and identification of the halophilic archaea isolates on hand via more advanced methods are assumed to contribute to the halophilic archaeal diversity in leather industry and microbial diversity.

In this study, extremely halophilic archaea were isolated and identified in the samples from salt-conserved raw hides and skins by means of culture-based methods. Identification of microorganisms in such hides via non-culture-based molecular methods may remarkably contribute to knowledge of microbial diversity in salt-conserved hides and skins ready for processing that contain considerable microbial load. The researchers are of the opinion that use of these archaea exhibiting remarkable enzyme activity in various industrial areas may yield significant results.

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