Research Article

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Highly active and stable protease production by an extreme halophilic archaeon Haloarcula sp. TG1 isolated from Lake Tuz, Turkey

Tuz Gölü, Türkiye’den izole edilen ekstrem halofilik arke Haloarcula sp. TG1’in yüksek derecede aktif ve kararlı proteaz üretimi

Abstract

Objective: Isolation of halophilic microorganisms from Çankırı salt mine and Lake Tuz in Turkey to explore versatile protease producers for industry and characterization of protease enzyme from the best protease producer among the isolated strains.

Methods: Extreme halophiles were isolated from salt samples of Çankırı salt mine and Lake Tuz. Their protease activities were determined. The isolate with the highest protease activity was characterized. Its protease activity was evaluated in different NaCl concentrations, temperature and pH ranges, and in the presence of different inhibitors and metals. Thermostability and pH stability were also determined.

Results: The highest protease producer strain was identified as Haloarcula sp. on the basis of 16S rRNA analysis. The isolate namely, Haloarcula sp. TG1, was found to be 99% identical to Haloarcula salaria strain HST01-2R.

The TG1 protease was found to possess very high activity and stability over a broad pH and temperature ranges. Its maximum activity was recorded at pH: 4.0, 50°C and 4 M NaCl. Among inhibitors tested, dimethyl sulfoxide (DMSO) and ethanol caused the highest decrease (ca. 25%) in its activity.

Conclusion: Due to the high activity and stability over a wide range of extreme conditions, Haloarcula sp. TG1 protease reported here is a promising candidate in biotechnology.

Keywords: Haloarcula sp. TG1; 16S rRNA; protease activity; Lake Tuz; salt mine.

Özet

Amaç: Türkiye’deki Çankırı kaya tuzu mağarası ve Tuz Gölü’nden halofilik mikroorganizmaların izole edilmesi, endüstriyel anlamda çok yönlü proteaz üreticilerinin keşfedilmesi ve izole edilen suşlar arasında en iyi proteaz üreticisine ait proteaz enziminin karakterizasyonu.

Metod: Çankırı kaya tuzu mağarası ve Tuz Gölü’nden ekstrem halofiller izole edilmiş ve proteaz aktiviteleri belirlenmiştir. En iyi proteaz aktiviti ile izole edilmiş, sıcaklık ve pH aralığı ile farklı inhibitör ve metallerin varlığında proteaz aktivitesi değerlendirilmiştir. Ayrıca, enzimin termostabilitesi ve pH stabiliteleri tespit edilmiştir.

Bulgular: En yüksek proteaz üreticisi suş 16S rRNA analizi ile Haloarcula sp. olarak tanımlanmıştır. Haloarcula sp. TG1 suşu Haloarcula salaria HST01-2R’e %99 özdeş bulunmuştur. TG1 proteazının oldukça yüksek ve kararlı enzim
activitesine sahip olduğu belirtilmiştir. Maksimum akti- vite pH: 4.0, 50°C ve 4 M NaCl koşullarında elde edilmiştir. Test edilen inhibitörler içerisinde, aktiviteden fazla düşüşe (%25) DMSO ve etanol neden olmuştur.

**Sonuç:** Çok geniş aralıklı ekstrem koşullarda yüksek aktivite ve kararlılığının dolayısıyla çalışılmada rapor edilen *Haloarcula* sp. TG1 proteazı endüstriyel anlamda üst verici bir adaydır.

**Anahtar kelimeler:** *Haloarcula* sp. TG1; 16S rRNA; proteaz aktivitesi; Tuz Gölü; tuz mağarası.

**Introduction**

Hypersaline environments harbor diverse microbial populations from mostly halophilic organisms belonging to three domains of life [1]. Halophilic organisms are categorized as slight halophiles (optimum growth occurs at 0.2–0.5 M NaCl), moderately halophiles (0.5–2.5 M NaCl) and extremely halophiles (growth over 2.0 M NaCl until to saturation) [2] on the basis of their salt requirements for growth. Although some extremely halophiles are found in Eubacteria domain, major representatives of this group are the members of domain Archaea [3]. Therefore, archaeal species are the subject of many researches because of capability for living in extreme environments with high salt concentrations. Haloarchaea thrive in hypersaline environments such as salt lakes or salt rock deposits having in excess of 2 M NaCl, and the cellular integrity depends on high molar salt ratio. In general, the haloarchaea are named as extremophiles because of being very well adapted to live even in saturated amounts of sodium chloride. There are 48 different genera of haloarchaea containing 177 species and studies to explore new species are ongoing [4]. Up to now, complete genomes of 10 haloarchaea have been published (http://halo4.umbi.umd.edu). Among them, those of *Haloarcula marismortui* and *Haloarcula hispanica* were released in 2004 [5] and 2011 [6], respectively. The survival of haloarchaea under harsh conditions allow them to produce a vast repertoire of metabolites. Hydrolytic enzymes such as proteases, lipases, cellulases and amylases produced by haloarchae are among the invaluable candidates for industrial approaches as because they are more stable and functional in extreme conditions such as high temperature, NaCl and pH [7, 8]. Proteases are one of the most important hydrolytic enzymes that exist in the first rank of worldwide enzyme market. The protease enzyme exerts its effect on protein degradation. The proteases produced by extremophile haloarchaea in particular, attract researchers’ attention due to its broad activity range in extreme conditions. Halophilic proteases are widely used in washing detergent, food, baking, dairy, tannin and leather industries, in aspartame production, in pharmaceutical industry and in the manufacture of soy products [9, 10]. The characterization and high titer production of such kind of novel enzymes are of great biotechnological importance. Therefore, studies still are going on discovery of novel halophilic hydrolytic enzymes with optimum activity and stability [7, 10, 11].

Turkey possesses extremely saline environments harboring diverse halophilic microorganisms. Different phylogenetic studies have been conducted to explore this biodiversity [12–17]. However, there is limited study on characterization of industrially important enzyme production by haloarchaea isolated from saline environments in Turkey [18]. Çankırı salt mine and Lake Tuz are among the most important sources of commercial salt in Turkey. Çankırı salt mine is an ancient 5000 years old cave and one of the most important rock salt deposit of the country. On the other hand, Lake Tuz, a second largest lake of Turkey with a geologically tectonic origin, is one of the greatest salty lakes in the world and is distinguished with attractive flora and fauna. Therefore, in 2000, the lake and its surrounding region was announced as Special Environment Protection Area by UNESCO [19]. It has a salt ratio of 32.4% [20]. In this study, we aimed at isolation and identification of halophilic microorganisms from Çankırı salt mine and Lake Tuz in Turkey to explore versatile protease producers in industrial manner. Next, activity and stability of the protease enzyme from *Haloarcula* sp. TG1, the best protease producer among the isolated strains, were determined for the first time.

**Materials and methods**

**Sampling site and isolation of halophilic microorganisms**

The salt samples were obtained from Çankırı salt mine (40°32′39.1″N 33°45′34.7″E) and Lake Tuz (39°04′13.1″N 33°24′29.5″E) in Turkey. The Çankırı and Lake Tuz Basins are located in northern part of central plateau and in the dry central plateau of Turkey, respectively. The mineral composition of Lake Tuz is consisted of mainly Na and Cl, with few quantities of SO₄, Mg, Ca and K [20]. The halophilic microorganisms were isolated using slightly modified procedure described by Nagaoka et al. [21] and Enache et al. [1]. One gram of samples were inoculated into 10 mL of Sehgal–Gibbons (SG) medium in the presence of penicillin (6 μg/mL). All cultures were incubated...
at 37°C for 2 weeks in an orbital shaker with 200 rpm. The liquid cultures were then used to inoculate SG agar plates in order to obtain pure cultures of halophiles, and were incubated at 37°C for 1 week. SG medium includes the following components in g/L: NaCl, 250; MgSO4·7H2O, 20; KCl, 2; sodium citrate (trisodium salt), 3; casamino acids, 7.5; yeast extract, 1; FeSO4·7H2O, 0.0023; agar, 15 g, pH: 7.55 [22].

**Protease activity of isolated strains**

A slightly modified procedure of Pathak and Sardar [23] was used to determine protease activity of the isolates. One week cultures (OD600 ~1.9) were centrifuged and the supernatants were used for the assay. One percent of casein dissolved in 50 mM phosphate buffer (pH 7.5) was used as substrate. One milliliter of supernatant was mixed with 1 mL of substrate and incubated at 30°C for 30 min by vortexing. The mixture was incubated at room temperature for 15 min and then centrifuged. The absorbance of supernatants was measured at 280 nm. Tyrosine (0–50 mg/L) was used as the standard. One unit protease activity was defined as the amount of enzyme that is required for release of 1 μg tyrosine per minute [24].

**Phylogenetic analysis**

Genomic DNA from the isolate with the highest protease activity was extracted using QIAmp genomic DNA kit (Qiagen) according to manufacturer’s instructions. Universal primers, Arch F (5′-TCCGGGTATCCTGCCGA-3′) [25] and AIIR (5′-GGTTACCTTGTTACGACTT-3′) [26], were used to amplify 16S rRNA gene sequence. Polymerase chain reaction (PCR) reaction solution included the following components: 1× Taq DNA polymerase buffer (ThermoScientific), 1.5 mM MgCl2, 0.2 μM forward and reverse primers, 200 μM dNTP mix, 50 ng template DNA and 1 U Taq DNA polymerase. The reaction conditions were started with an initial denaturation step (5 min at 94°C), were proceeded with 35 cycles of amplification (45 s at 95°C, 30 s at 55°C, 1 min 40 s at 72°C) and a final extension step (10 min at 72°C). PCR amplicon was run on 1% agarose gel. Then, it was purified by using PCR purification kit (Qiagen) and sequenced at BGI (Europe). The analysis of 16S rRNA gene sequence was carried out using the Basic Local Alignment Search Tool (BLAST) software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). The 16S rRNA gene sequence of the selected isolate was aligned using CLUSTALW [27] and the phylogenetic tree was constructed with MEGA6 using a Neighbor Joining algorithm and Kimura two-parameter corrections [28]. 16S rRNA sequence of Halobacillus mangrovi strain MS10 (accession number DQ888316.1) was used as the outgroup.

**Cultural and phenotypic characterization of Haloarcula sp. TG1**

Pure culture of extreme halophile Haloarcula sp. TG1 was observed under microscope and its gram staining property was determined. Colony morphology, color and shape were observed by growing on SG agar plate. The salt range that the isolate needs for growth was defined adding 1–5.5 M NaCl to the SG medium and incubation at 37°C for 1 week and their growth were monitored spectrophotometrically at OD600. In addition, different pH (5.0–10.0) and temperature (20, 28, 37 and 50°C) requirements of the isolate were also tested.

**Biochemical characterization of Haloarcula sp. TG1**

Extracellular enzyme activities (lipase, amylase and protease) of Haloarcula sp. TG1 were tested [29]. Besides, it was inoculated to sulfate indole motility (SIM), urea agar, triple sugar iron agar (TSIA) and nitrate broth media to determine its biochemical activity.

**Effect of temperature, pH and salt concentration on the Haloarcula sp. TG1 protease activity**

Optimum conditions for protease activity were determined by analysing different temperatures, pH values and salt concentrations. Thus, the enzyme activity was assayed at 25, 30, 35, 40, 50, 60, 70, 80, and 90°C. Optimum pH value was tested at 3.0, 4.0, 5.0, 6.0, 7.0, 7.5, 8.0, 9.0 and 10.0 at optimum temperature, 50°C. Maximum protease activity was determined in the presence of 1, 2, 3, 4 and 5 M NaCl under optimized temperature at 50°C and pH value of 4.0. All experiments were performed as triplicate and repeated two times.

**Effect of some inhibitors on Haloarcula sp. TG1 protease activity**

To determine the effect of inhibitors on the protease activity, different metal ions (2 mM MgCl2, ZnSO4, KCl, CaCl2, MgCl2), detergents (5 M urea, 0.1% SDS, 0.1%
β-mercaptoethanol, 0.1% tween 80, 0.1% dimethyl sulfoxide (DMSO), 10 mM EDTA] and an organic solvent (25% ethanol) [24, 30] were added to the phosphate buffer and the supernatants was preincubated at 25°C for an hour. The residual activity was assayed at optimized conditions. The protease activity in the absence of any metal ion, solvent or detergent was defined as 100% and the other values were calculated as relative to it [30]. All experiments were done in triplicate and repeated two times.

**Results**

**Identification of the extreme halophilic archaea**

The extreme halophilic archaea were isolated using two different salt samples from Çankırı salt mine and Lake Tuz in Turkey. In SG agar plates, the pink colored colonies with smooth and round appearance were obtained (Figure S1). A total of 15 colonies were cultured in SG for determination of protease activity (data not shown). The isolate that exerted the highest protease activity among the isolated ones was selected for further studies. The isolate was used for 16S rRNA gene sequence analysis, and was identified as *Haloarcula* sp. with the GenBank accession number of KU051670.1. This isolate namely, *Haloarcula* sp. TG1, was found to be 99% identical to *Haloarcula salaria* strain HST01-2R (FJ429318.1). The phylogenetic tree based on 16S rRNA sequence of *Haloarcula* sp. TG1 is shown in Figure 1. Microscopic observation of *Haloarcula* sp. TG1 showed that

**Thermostability and pH stability of the *Haloarcula* sp. TG1 protease**

The culture supernatants were preincubated for an hour in tested temperatures and pH values at 25°C one by one. Then, the protease activity was assayed at optimized conditions. The enzyme activity in the control was defined as 100% and the others were calculated relative to it.

![Figure 1: Phylogenetic relationship of the haloarchaeal isolate based on 16S rRNA gene sequence. The tree was constructed using Neighbour-Joining method with 1000 replicates of bootstrap test. 16S rRNA gene sequence of *Halobacillus mangrovi* strain MS10 (accession number DQ888316.1) was used as the outgroup.](image-url)
Table 1: Comparison of phenotypic and biochemical characteristics of Haloarcula sp. TG1 and Haloarcula salaria strain HST01-2R [37].

| Characteristics                | Haloarcula sp. TG1 | Haloarcula salaria strain HST01-2R [37] |
|--------------------------------|--------------------|---------------------------------------|
| Source                         | Lake Tuz           | Fish sauce                            |
| Gram staining                  | Negative           | Negative                               |
| Cell shape                     | Pleomorphic rod    | Pleomorphic rod                        |
| Motility                       | Non motile         | Non motile                            |
| Pigmentation                   | Pink red           | Red                                   |
| NaCl range (M)                 | 2.2–5.2            | 2.6–5.1                               |
| Optimum NaCl (M)               | 3.4–4.3            | 3.4–4.3                               |
| pH range                       | 6.5–9              | 6–8                                   |
| Optimum pH                     | 6.5–9              | 7                                     |
| Temperature range (°C)         | 15–50              | 15–45                                 |
| Optimum temperature (°C)       | 37–50              | 37                                    |
| Starch hydrolysis              | +                  | +                                     |
| Tween 80 hydrolysis            | +                  | +                                     |
| Nitrate reduction              | +                  | +                                     |
| H₂S production                 | –                  | ND                                    |
| Indole utility                 | –                  | ND                                    |
| Urease activity                | –                  | ND                                    |
| Acid from glucose/sucrose/galactose | –/–/–               | –/ND/–                                |
| Utilization of peptone         | +                  | ND                                    |

ND, not determined.

it has pleomorphic rod shaped cells with gram negative characteristics (Figure S2). The growth temperature and the pH ranges were 15–50°C and 6.5–9.0, respectively. The phenotypic and biochemical characterization data showed that Haloarcula sp. TG1 isolate can grow in the presence of 2.2–5.2 M NaCl; is negative for H₂S production, tryptophan degradation, and non motile determined using SIM medium; is positive for amylase, lipase, and nitrate reductase enzymes but negative for urease; is negative for utilization of glucose, sucrose, and lactose but positive for peptone utilization determined using TSIA medium (Table 1).

Characterization of the protease activity of Haloarcula sp. TG1

As the highest protease activity was provided by Haloarcula sp. TG1, its protease activity was characterized in terms of optimum temperature and pH, thermal and pH stability as well as the effects of NaCl and some enzyme inhibitors.

The protease activity assayed at different incubation temperatures (25, 30, 35, 40, 50, 60, 70, 80, and 90°C) and pH values (3.0–10.0). Thus, the optimum temperature and pH for the TG1 protease activity were found to be 50°C (Figure 2) and pH 4.0 (Figure 3), respectively. Although the enzyme was identified as an acid protease, the activity was high at pH 8.0, as well.

To determine the thermal stability, TG1 supernatant was incubated between 25 and 90°C for an hour prior to protease activity measurement. The highest activity was found at 25°C incubation while more than 92% of the activity was preserved at 90°C incubation (Figure 4). Next, the pH stability was defined via incubation of the culture supernatant at 25°C and pH values of 3.0–10.0 for an hour. The highest stability was observed both at pH 8.0 and 9.0 while ca. 99% activity was remained at pH 3.0, and more than 97% of the protease activity was protected at all experimented pH values (Figure 5).
In order to determine optimum NaCl concentration for protease activity in *Haloarcula* sp. TG1 supernatants, the protease activity was measured in the absence and presence of (1–5 M) NaCl. The activity was elevated by the increasing salt concentrations, reached maximum at 4 M NaCl, and decreased at 5 M NaCl, therefore indicating the NaCl requirement of the enzyme for maximum activity (Figure 6). The effect of some metals and inhibitors on the enzyme activity was also evaluated using MgCl₂, CaCl₂, ZnSO₄, MnCl₂, KCl, Tween-80, DMSO, EDTA, ethanol, β-mercaptoethanol, urea, and SDS (Figure 7). Interestingly, none of the substances used do not lead to an increase in enzyme activity. The highest inhibition (ca. 25%) in TG1 protease activity was recorded with the use of DMSO and ethanol. Minimum activity inhibition was found to be 5% in presence of SDS.

Discussion

The saline environments such as natural brines, salt mines, salt lakes or hypersaline seas are attractive locations for isolation of extremely halophilic microorganisms [31]. Turkey, with its geological past, has many resources for the isolation of halophiles. There are some predictions for long term maintenance of these microorganisms in ancient salt habitats, such as dormancy or growth interspersed with relatively short periods of dormancy [32]. There are different studies regarding with halarchaea isolation and characterization from natural habitats of Turkey. For instance, in 2006, 95 archaeal strains belonging to the family *Halobacteriaceae* were isolated using samples collected from six different locations [15].

![Figure 4](image4.png)

*Figure 4: Thermostability of the protease enzyme in a temperature range between 25 and 90°C.*

![Figure 6](image6.png)

*Figure 6: The effect of NaCl concentration on the protease activity of Haloarcula sp. TG1.*

![Figure 5](image5.png)

*Figure 5: pH stability of the protease enzyme.*

![Figure 7](image7.png)

*Figure 7: The effect of different inhibitors on the protease activity of Haloarcula sp. TG1.*
Birbir et al. [33] also isolated 27 archaeal strains from Lake Tuz and its salterns. Both study performed detailed phylogenetic analyses and biochemical characterization. However, they did not find any archaeal strain that has 16S rRNA gene sequence similarity to *H. salaria* and protease activity of all isolates was evaluated by qualitative observation of casein hydrolysis. A detailed report regarding with characterization of extracellular esterase and lipase activities of five halophilic archaeal strains isolated from other salt lakes located in Turkey were performed in only one study conducted by Özcan et al. [18].

There are different reports on halophilic microorganisms identified from some other locations in the world. Makhdoumi-Kakhki et al. [34] reported *Halorubrum*, *Haloarcula*, *Salinibacter*, *Salicola*, *Rhodovibrio*, *Halorhabdus*, *Haloquadratum*, *Halanaerobium*, *Halocella*, *Halorhodospira* and *Cyanobacteria* from Aran-Bidgol Salt Lake, the largest hypersaline playa in Iran. Ghozlan et al. [35] isolated moderately halophilic bacteria, *Pseudoalteromonas*, *Flavobacterium*, *Chromohalobacter*, *Halomonas*, *Salegentibacter*, *Halobacillus*, *Salinicoccus*, *Staphylococcus* and *Tetragenococcus* in hypersaline habitats in Alexandria, Egypt. Moreover, in 2014, many archaea from the Capuchin Catacombs of Palermo, Italy, belonging to different genera (*Bacillus*, *Virgibacillus*, *Arthrobacter*, *Oceanobacillus*, *Halobacillus*, *Idiomarina*, *Chromohalobacter*, *Nesterenkonia*, *Staphylococcus equorum* and *Halomonas*) were isolated [36].

Lake Tuz and its surrounding salterns are main salt sources especially for food and leather industries in Turkey and they are used in crude form in such industrial applications [33]. Therefore, it is expected to observe haloarchaea from salted products such as fish, tomato paste and vegetables. Hence, it is quite logical the finding of 99% sequence similarity of *Haloarcula* sp. TG1 16S rRNA gene to that in *H. salaria* strain HST01-2R isolated from salt in a fish sauce sample from Thailand [37]. Besides, phenotypic and biochemical characterization results of *Haloarcula* sp. TG1 were found to be quite similar to *H. salaria* strain HST01-2R characteristics as tabulated in Table 1.

The extreme environments that the halophilic bacteria survive attract the attention to their stable hydrolytic enzymes such as amylases, lipases and proteases with biotechnological importance [10]. Enache et al. [38] reported a variety of enzymes belonging to *Haloarcula*, *Halobacterium*, *Halofex*, and *Halobrunum* isolated from hypersaline environments in Romania hydrolyzing starch, casein, Tween 80 and carboxymethylcellulose. Sehar and Hameed [39] characterized the production of an alkaline protease from a moderately halophilic *Bacillus* sp. isolated in Khewra salt range (Pakistan). Optimum biomass production and proteolytic activity was found at 37°C, pH 8.0 with 8% salinity and 150 rpm agitation, using the soybean meal as a nitrogen source, and the rice bran as a carbon source. Besides, the Ca2+ and Mg2+ ions were boosted the protease activity while Cu2+ and Zn2+ ions caused a significant reduction. However, there are limited number of studies on the characterization of proteases from halophilic archaea. For instance, Manikandan et al. [24] investigated the protease of *Halofex lucentensis* VKMM 007 isolated from brine samples in Tamil Nadu, India. The protease was found to be stable in the temperature range of 20–70°C, NaCl concentrations of 0.85–5.13 M and pH range of 5.0–9.0 being optimum at 60°C, 4.3 M NaCl and pH 8.0. The Ca2+, K+, Mg2+, Na+, and Fe2+ ions promoted the enzyme activity. In a recent study, Pathak and Sardar [23] characterized the protease of *Halofex APP15* isolated from a marine solar saltern in Mumbai, India. The enzyme was found to be active at the NaCl concentrations of 1 M–5 M, pH range of 6.0–9.0, temperature range of 30–60°C with optimum activity at 4 M NaCl, pH 8.0 and 50°C. The crude protease was shown to be highly active in the presence of CaCl2, KCl, MgCl2 and NaCl while CuCl2, HgCl2, MnCl2, ZnCl2, and BaCl2 were found to inhibit the enzyme activity. *Halofex APP15* protease showed highest stability in ethanol while 4 M urea, 10 mM SDS, EDTA and β-mercaptoethanol inhibited the enzyme activity by 80%, 68%, 38% and 39%, respectively. Moreover, a complete loss of the activity was recorded in the presence of 10 mM PMSF.

In our study, the characterization of protease from *Haloarcula* sp. TG1 showed that the optimum temperature, pH value and NaCl concentration for the enzyme activity were 50°C, pH 4.0, and 4 M, respectively. Although previous studies detected alkali proteases, the optimum protease activity of TG1 was determined at acidic pH value while keeping its stability at alkali pH values, as well. The enzyme was found to be highly stable at the pH range of 3.0–10.0, and the temperature range of 25–90°C. The tested inhibitors affected the enzyme activity at different levels. The use of DMSO and ethanol have caused 25% inhibition approximately, while SDS (3.5 mM) was found as a highly inefficient inhibitor of the enzyme activity. The capacity of halophilic enzymes being active and stable even in saturated amount of salt concentrations provides invaluable contributions in biotechnological applications such as food processing, leather industry, bioremediation and biosynthetic processes. Therefore, the discovery and characterization of novel enzymes exerting optimum activity and stability in a wide range of salt concentrations, pH and temperatures as well as in the presence of different inhibitors are very crucial in industrial manner.
In conclusion, to our knowledge, this is the first report showing isolation of *Haloarcula* sp. TG1 that was found to be most closely related to *H. salaria* strain HST02R on the basis of 16S rRNA sequence, from Lake Tuz, an important source of commercial salt in Turkey, and also a famous salt lake worldwide due to its valuable flora and fauna. Indeed, with its high stability and activity in different pH ranges and temperatures and salt concentrations, the characterized TG1 protease is very promising for biotechnological applications.

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**References**

1. Enache M, Neagu S, Cojoc R. Extracellular hydrolases of halophilic microorganisms isolated from hypersaline environments (salt mine and salt lakes). Scientific Bulletin. Series F Biotechnol 2014;18:20–25.

2. Ventosa A, Nieto JJ, Oren A. Biology of moderately halophilic aerobic bacteria. Microbiol Mol Biol Rev 1989;62:504–44.

3. Ventosa A. Unusual microorganisms from unusual habitats: hypersaline environments. In: Logan NA, Lppin-Scott HM, Oyston PC, editors. Prokaryotic diversity—mechanism and significance. Cambridge, UK: Cambridge University Press, 2006:223–53.

4. Parte AC. List of prokaryotic names with standing in nomenclature. Available at: http://www.bacterio.net.

5. Baliga NS, Bonneau R, Facciotti MT, Pan M, Glusman G, Deutsch EW, et al. Genome sequence of *Haloarcula marismortui*: a halophilic archaeon from the Dead Sea. Genome Res 2004;14:2221–34.

6. Liu H, Wu Z, Li M, Zhang F, Zheng H, Han J, et al. Genome Sequence of *Haloarcula hispanica*, a Model Haloarchaeon for studying genetics, metabolism, and virus-host interaction. J Bacteriol 2010;193:6086–87.

7. Oren A. Industrial and environmental applications of halophilic microorganisms. Environ Technol 2010;31:825–34.

8. Charlesworth JC, Burns BP. Untapped resources: biotechnological potential of peptides and secondary metabolites in Archaea. Archaea 2015;1–7. Available at: http://dx.doi.org/10.1155/2015/282035.

9. Dassarma P, Coker JA, Huse V, Dassarma S. Halophiles, Industrial Applications. Encyclopedia of industrial biotechnology: bioprocess, bioseparation, and cell technology. In: Flickinger MC, editors. New Jersey, USA: Wiley, 2010:1–9.

10. Moreno M de L, Pérez D, García MT, Mellado E. Halophilic bacteria as a source of novel hydrolytic enzymes. Life 2013;3:38–51.

11. Gómez J, Steiner W. The biocatalytic potential of extremophiles and extremozymes. Food Technol Biotechnol 2004;2:223–35.

12. Birbir M, Esaj C. Extremely Halophilic Bacterial Communities in Şereflikoçhisar Salt Lake in Turkey. Turkish J Biol 2003;27:7–22.

13. Birbir M, Ogan A, Calli B, Meroglu B. Enzyme characteristics of extremely halophilic *Archaal* community in Tuzkoy salt mine, Turkey. World J Microbiol Biotechnol 2004;20:613–21.

14. Elevi R, Assa P, Birbir M, Ogan A, Oren A. Characterization of extremely halophilic Archaea isolated from the Aysalik Saltarn, Turkey. World J Microbiol Biotechnol 2004;20:719–25.

15. Özcan B, Cokmus C, Coleri A, Calıskan M. Characterization of extremely halophilic archaea isolated from saline environment in different parts of Turkey. Microbiology 2006;75:379–46.

16. Özcan B, Ozcengiz G, Coleri A, Cokmus C. Diversity of halophilic archaea from six distinct parts of Turkey. J Microbiol Biotechnol 2007;17:985–92.

17. Yıldız E, Özcan B, Calıskan M. Isolation, characterization and phylogenetic analysis of halophilic archaea from a salt mine in Central Anatolia (Turkey). Pol J Microbiol 2012;61:111–7.

18. Özcan B, Ozylimaz G, Cokmus C, Calıskan M. Characterization of extracellular esterase and lipase activities from five halophilic archaeal strains. J Ind Microbiol Biotechnol 2009;36:105–10.

19. Mergen O, Karacaoglu C. Tuz lake special environment protection area, Central Anatolia, Turkey: The EUNIS Habitat classification and habitat change detection between 1987 and 2007. Ekoloji 2015;24:1–9.

20. Kılıç O, Kılıç AM. Salt crust mineralogy and geochemical evolution of the Salt Lake (Tuz Gölü), Turkey. Sci Res Essays 2010;5:1317–24.

21. Nagaoka S, Minegishi H, Echigo A, Shimane Y, Kamekura M, Usami R. *Halostagnicola alkaliphila* sp. nov., an alkalophilic haloarchaeon from commercial rock salt. Int J Syst Evol Microbiology 2011;61:1149–52.

22. Sehgal SN, Gibbons NE. Effect of some metal ions on the growth of *Halobacterium cutirubrum*. Can J Microbiol 1960;6:165–9.

23. Pathak AP, Sardar AG. Isolation and characterization of salt stable protease producing archaea from marine solar saltern of Mulund, Mumbai. Indian J of Geo-Mar Sci 2014;43:412–17.

24. Manikandan M, Pašić L, Kannan V. Purification and biological characterization of a halophilic thermostable protease from *Haloferax lentencensis* VKMM 007. World J Microbiol Biotechnol 2009;25:2247–56.

25. Delong E. *Archaee* in costal marine environments. PNAS 1992;89:5685–9.

26. Lane DJ, Pace B, Olsen GJ, Stahl D, Sogin M, Pace NR. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci USA 1985;82:695–5.

27. Thompson JD, Higgins DG, Gibson TJ. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673–80.

28. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013;30:2725–9.

29. Cojoc R, Simona M, Popescu G, Dumitru L, Kamekura M, Enache M. Extracellular hydrolytic enzymes of halophilic bacteria
isolated from a subterranean rock salt crystal. Rom Biotech Lett 2009;14:4658–64.
30. Elbanna K, Ibrahim IM, Revol-Junelles AM. Purification and characterization of halo-alkali-thermophilic protease from Halobacterium sp. strain HP2S isolated from raw salt, Lake Qarun, Fayoum, Egypt. Extremophiles 2015;19:763–74.
31. Fendrihan S, Legat A, Pfaffenhuemer M, Gruber C, Weidler G, Gerbl F, et al. Extremely halophilic archaea and the issue of long-term microbial survival. Rev Environ Sci Biotechnol 2006;5:203–18.
32. McGenity TJ, Gemmell RT, Grant WD, Stan-Lotter H. Origins of halophilic microorganisms in ancient salt deposits. Environ Microbiol 2000;2:243–50.
33. Birbir M, Calli B, Mertoglu B, Bardavid RE, Oren A, Ogmen MN, et al. Extremely halophilic Archaea from Tuz Lake, Turkey, and the adjacent Kaldırım and Kayacık salterns. World J Microbiol Biotechnol 2007;23:309–16.
34. Makhdoumi-Kakhki A, Amoozegar MA, Kazemi B, Pašić L, Ventosa A. Prokaryotic diversity in Aran-Bidgol Salt Lake, the largest hypersaline playa in Iran. Microbes Environ 2012;27:87–93.
35. Ghozlan H, Deif H, Abou Kandil R, Sabry S. Biodiversity of moderately halophilic bacteria in hypersaline habitats in Egypt. J Gen Appl Microbiol 2006;52:63–72.
36. Pinar G, Krakova L, Pangallo D, Piombino-Mascali D, Maixner F, Zink A, et al. Halophilic bacteria are colonizing the exhibition areas of the Capuchin Catacombs in Palermo, Italy. Extremophiles 2014;18:677–91.
37. Namwong S, Tanasupawat S, Kudo T, Itoh T. Haloarcula salaria sp. nov. and Haloarcula tradensis sp. nov., isolated from salt in Thai fish sauce. Int J Syst Evol Microbiology 2011;61:231–6.
38. Enache M, Neagu S, Cojoc R. Halophilic microorganisms from Romanian saline environments as a source of extracellular enzymes with potential in agricultural economy, 2013. Available at: https://mpra.ub.uni-muenchen.de/id/eprint/55006.
39. Sehar S, Hameed A. Extracellular alkaline protease by a newly isolated halophilic Bacillus sp. GjBB 2011;6:142–8.

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