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Extremely Halophilic Bacterial Communities in Şereflikoçhisar Salt Lake in Turkey

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Abstract: Şereflikoçhisar Lake is the largest salt lake in central Turkey. This lake is a major source of solar salt for food, hide and other industries locally. Due to the economic importance of salt obtained from this lake, a microbial survey has been conducted. Six salt and three brine samples were obtained from the lake for physico-chemical and microbiological analyses. Physico-chemical analysis showed that the salts and brines contain sufficient ions and hardness to support extremely halophilic bacteria. The salt collected from the lake contained 10^4-10^6 colony-forming units of extremely halophilic bacteria per gram and brine taken from the lake contained 10^3-10^5 colony-forming units of extremely halophilic bacteria per ml. Colonial pigmentation from these samples ranged from blood-red to pale-pink. A total of 82 extremely halophilic aerobic strains were isolated from the salt and brine samples, 32 of which were randomly selected strains examined in greater detail. While brick-red colonies outnumbered all other colony pigmentations, the lake does appear to support a diverse bacterial community. Most colonies were 1 to 2 mm in diameter, circular, convex, glistening and entire. Optimum growth occurred at 25% (w/v) NaCl at 40 ºC and a pH of 7.5. Seventeen strains required at least 10% (w/v) NaCl for growth. Most cells of the strains were pleomorphic and stained Gram-negative. All strains were motile. Some of these strains produced industrially important enzymes such as lipases, gelatinases, cellulases and β-galactosidases. All of the strains hydrolysed Tween 80. Twenty-three strains produced gelatinase enzymes. Cellulase enzymes were produced by 14 of these strains. Only one strain showed positive β-galactosidase activity. Ten strains produced indol from tryptophan. Twenty-three strains showed positive methyl-red reactions. All strains showed negative Voges-Proskauer reactions. Thirty-two strains were tested by the disc diffusion method for their sensitivity to 13 different antibiotics. Most of these strains were resistant to ampicillin (10 µg), chloramphenicol (30 µg), cefadroxil (30 µg), erythromycin (15 µg), penicillin G (10U) and streptomycin (25 µg). All strains were resistant to amikacin (30 µg), ciprofloxacin (5 µg), neomycin (30 µg) and spiramycin (100 µg). Most strains were sensitive to bacitracin (10 U) and novobiocin (5 µg). Half of these strains were sensitive to sulfamethoxazole and trimethoprim (25 µg). These studies showed that Şereflikoçhisar Salt Lake is an optimal environment for extremely halophilic bacteria and the lake contains a viable, diverse, potentially and industrially important bacterial community. The extremely halophilic proteolytic bacterial contents of the salt and brine samples were too high to use directly in hide preservation. The presence of proteolytic strains in the lake's salt may cause an important quality problem in the leather business.

Key Words: Şereflikoçhisar Salt Lake, extremely halophilic bacteria, antibiotic sensivity, halophilic enzymes

Türkiye'de Şereflikoçhisar Tuz Gölünde Bulunan Aşırı Halofilik Mikroorganizma Toplulukları

Özet: İç Anadolu bölgesinde bulunan Şereflikoçhisar gölü, Türkiye'nin en büyük tuz gölüdür. Bu gölden elde edilen tuz, gıda ve deri sektörü dahil olmak üzere Türkiye'deki pek çok endüstride yaygınca kullanılmaktadır. Tuz Gölünden elde edilen tuzun büyük ekonomik önemi olmasından dolayı, gölden 6 adet tuz ve 3 adet tuzlu su örneği toplanarak fiziksel, kimyasal ve mikrobiyolojik analizleri yapılmıştır. Bu örneklerde aşırı halofilik bakterilerin gelişmesini destekleyecek oranlarda iyonlara rastlanılmıştır. Bir gram tuz örneğinde aşırı halofilik bakteri sayısı 10^4-10^6 kob, bir ml tuzlu su örneğinde ise aşırı halofilik bakteri sayısı 10^3-10^5 kob olarak bulunmuştur. Bu suşlar farklı kırmızı ve pembe tonlarında koloniler oluşturmuşlardır. Gölden toplam olarak 82 adet saf, aerobik, halofilik bakteri suşun ayrımı yapılmış ancak bu suşlardan 32 adeti detaylıca incelenmiştir. Kiremit kırmızısı renkli kolonilerin tuz gölünde en fazla sayıda olduğu ve tuz gölünün farklı bakteri türleri içerdiği saptanmıştır. Suşların büyük bir çoğunluğu, 1 ile 2 mm çapında, yuvarlak, konveks, parlak ve düzgün kenarlı koloniler oluşturmuşlardır. Bu mikroorganizmalar, en iyi şekilde %25 NaCl içeren besiyerlerinde, 40ºC'de ve pH 7.5 da gelişmişlerdir. Suşların 17 adeti gelişebilmek için besiyerinde en az %10 NaCl'ye ihtiyaç duymuştur. Gölden ayırımı yapılan suşların hareketli, Gram negatif ve düzensiz çomaklar olduğu görülmüştür. Bu suşların bazıları lipazlar, jelatinazlar, selülazlar ve β-galaktosidazlar gibi endüstriyel önemi olan enzimler üretmiştir. Tüm suşlar Tween 80'i hidrolize etmiştir. Suşların 23 adeti jelatinaz enzimini, 14 adeti selülaz enzimini ve sadece 1 adeti ise β-galaktosidaz enzimini üretmiştir. Suşların 10 adeti triptofandan indol oluşturmuştur. 23 adet suş pozitif metil red reaksiyonu, suşların tümü ise negatif Voges-Proskauer reaksiyonu göstermiştir.

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Bu suşların 13 farklı antibiyotiğe duyarlılıkları disk difüzyon metodu ile tespit edilmiştir. Suşların çoğunun ampisilin (10 µg), kloramfenikol (30 µg), sefadroksil (30 µg), eritromisin (15 µg), penisilin G (10 U) ve streptomisin'e (25 µg) karşı dirençli olduğu görülmüştür. Tüm suşların amikasin (30 µg) , siprofloksasin (5 µg), neomisin (30 µg) ve spiramisin 'e (100 µg) dirençli olduğu belirlenmiştir. Suşların çoğunun ise basitrasin (10 U) ve novobiosin'e (5 µg) karşı duyarlı olduğu tespit edilmiştir. Suşların yarısının sulfametoksazol and trimetoprim'e (1.25 µg / 23.75 µg) karşı duyarlı olduğu görülmüştür. Bu araştırma, Şereflikoçhisar Tuz Gölünün aşırı halofilik bakteriler için ideal bir ortam olduğunu ve gölün canlı, çeşitli, potansiyel ve endüstriyel olarak önemli mikroorganizma topluluklarını içerdiğini göstermiştir. Tuz gölünden elde edilen tuzda ve tuzlu suda çok sayıda proteolitik bakterinin varlığı, bu tuzun deri tuzlamada direkt olarak kullanılamayacağını, kullanıldığı takdirde deri endüstrisinde önemli ölçüde kalite problemlerine neden olabileceğini ortaya koymaktadır.

Anahtar Sözcükler: Şereflikoçhisar Tuz Gölü, aşırı halofilik bakteriler, antibiyotik hassasiyetleri, halofilik enzimler

Introduction

Şereflikoçhisar Lake is a large, inland hypersaline lake occupying a depression in the dry central plateau of Turkey, 105 km north-east of Konya. For most of the year, this very shallow (1-2 m) lake has an area of about 1500 km^2 (1,2).

The lake has a total salt concentration of up to 33%. Every year more than 200,000,000 tons of salt is produced from the lake. Seventy-three percent of the salt needs of Turkey is supplied from Şereflikoçhisar Salt Lake (1).

It is known that salt lakes may contain as much as 10^7-10^8 cfu of halophilic bacteria per ml. Solar salt, without processing, often contains 10^5-10^6 cfu of halophilic bacteria per gram. Halophilic micro-organisms may cause the spoilage of heavily salted proteinaceous products, being responsible for a "pink" pigmentation in salted fish and the "red heat" of salted hides (3).

Furthermore, a wide variety of biotechnological products such as bacteriorhodopsins, halorhodopsins, compatible solutes, biopolymers, biosurfactants, exopolysaccharides, polyhydroxyalkanoates, flavouring agents, isomerases, hydrolases, nucleases, amylases, cellulases, proteases, lipases, anti-tumour drugs, and liposomes are produced by halophiles. In addition, halophilic organisms play important roles in fermenting fish sauces, modifying food textures and flavours, and in transforming and degrading waste and organic pollutants (4,5).

The salt of Şereflikoçhisar Salt Lake is commonly used directly in the brine curing of hides in the Turkish leather industry. When the halophilic bacteria containing salt is used in hide preservation, halophilic bacteria may contain proteases that can potentially digest the grain surface of the hide under extended storage conditions at elevated temperatures. This lowers the value of the leather and can represent a huge annual loss to the Turkish leather tanning industry.

There was no available information on extremely halophilicbacterial populations and the bacteria dominating Şereflikoçhisar Salt Lake. Due to the economic and industrial importance of the bacterial population of the lake, a microbial survey was conducted. The purpose of this research was to chemically analyse salt and brine samples collected from the lake, to determine the extent to which the salt and brine of the lake are contaminated with extreme halophiles, to determine populations of extremely halophilic bacteria that develop in the lake, to examine their phenotypic features and their physiological and biochemical characteristics, as well as their sensitivity to different antibiotics and to demonstrate the presence or lack of proteolytic activity of halophilic bacteria in the salt of Şereflikoçhisar Salt Lake.

It is also known that gelatinases, lipases, cellulases and β-galactosidases have diverse applications in different areas such as food technology, feed additives and chemical industries (6). For these reasons, a screening test of extremely halophilic bacteria isolated from different parts of Şereflikoçhisar Salt Lake was performed in order to select extreme halophiles which are able to produce industrially important enzymes such as gelatinases, lipases, cellulases and β-galactosidases.

Materials and Methods

Physico-Chemical Analyses

The pH, moisture content, hardness, Na^+, K^+, Ca^{2+}, Mg^{2+}, HCO_3^- and Cl^- contents of the salt and brine samples were performed according to standard methods (7).

Collection of Samples and Isolation of Halophilic Bacteria

Six salt crystal samples and three brine samples were collected from different areas of Şereflikoçhisar Salt Lake in Turkey.

Spread plate and membrane filter techniques were used to determine bacterial numbers in the lake (8,9). The membranes were transferred onto agar plates containing Brown medium with 250 g/l NaCl. After 6 weeks of incubation at 40 ºC red, blood-red, brick-red, orange-red, pink, bright-pink, pale-pink, yellowish-cream, cream, white and transparent colonies were counted. Different colonies were picked and restreaked several times to obtain pure cultures.

Morphological, Cultural and Physiological Characteristics

Thirty-two strains were chosen for detailed characterisation. Isolated strains were examined for colony and cell morphologies and cell motility. Colonial morphologies were described by using standard microbiological criteria, with special emphasis on pigmentation, diameter, colonial elevation, consistency and opacity (10). These characters were described for cultures grown at the optimum temperature, pH and salt concentration. Isolated strains were examined for motility and morphological features in wet mounts. Cell morphology was examined by light microscopy of the exponentially growing liquid cultures. Gram staining was performed by using acetic acid–fixed samples as described by Dussault (11). The salt range of growth was determined in Brown medium with 0, 30, 50, 60, 70, 80, 100, 150 and 250 g/l total salts. The pH tolerance of each isolate was tested in Brown medium with pH values of 4.5, 6.0, 7.0 and 7.5.

Biochemical Tests

Inoculants for the various biochemical tests were prepared by growing the organisms in flasks of complex growth medium at 40 ºC for 7 days. The optimal ionic content (per liter: 2 g of KCl, 20 g of $MgCl_2.6H_20$, 0.2 g of $CaCl_2.H_20$, 250 g of NaCl) was added to all the biochemical test media. Gelatinase, β-galactosidase and cellulase activities, Tween 80 hydrolysis, indol production, methyl red and Voges-Proskauer tests were performed by using standard procedures. The results were recorded after 30 days of incubation at 40 ºC (12-15).

Antibiotic Tests

The antibiotic sensitivity of 32 strains was examined by spreading bacterial suspensions on plates containing Brown medium and applying antibiotic discs (amikacin (30 µg), ampicillin (10 µg), bacitracin (10 U), cefadroxil (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), neomycin (30 µg), novobiocin (5 µg), penicillin G (10 U), spiramycin (100 µg), streptomycin (25 µg), sulphamethoxazole (23.75 µg) and trimethoprim (1.25 µg)). The results were recorded in terms of sensitivity or resistance after 14 days of incubation at 40 ºC, with sensitivity being defined as the appearance of a zone of inhibition extending at least 2 mm beyond the antibiotic disc (10,12,13,16-18).

Results

Physico-Chemical Analyses

The average temperature at the sampling sites was 32 ºC at noon. The physico-chemical characteristics of the salt and brine samples are shown in Tables 1 and 2. The pH values of the salt samples were higher than those of the brine samples. The pH of the salt samples was between 8.09 and 8.43. The pH of the brine samples was between 7.44 and 7.67. The salt samples had a slightly alkaline pH. The highest values of moisture content,

Table 1. The physico-chemical characteristics of the salt samples studied.

Sampling site	Colour of sampling site	pH	Moisture content (%)	Hardness (Fr)	Ca^{2+} g/kg	Mg^{2+} g/kg	HCO_3^- g/kg	Cl^- g/kg	Na^+ g/kg	K^+ g/kg	Total
SL1	Light pale-pink	8.20	0.68	900	1.6	1.22	34.16	528.3	342.6	0.3	908.18
SL2	Light pale-pink	8.22	0.43	1100	3.2	0.73	39.04	522.3	338.7	0.5	904.47
SL3	Dark pale-pink	8.15	7.56	1800	4.0	1.94	39.04	534.4	346.6	0.4	926.38
SL4	Dark cream	8.43	0.64	1050	2.4	1.09	31.72	543.5	352.5	0.3	931.51
SL5	Yellowish-cream	8.39	0.32	850	1.6	1.09	35.33	544.7	353.3	0.3	936.32
SL6	Dark-cream	8.09	0.38	650	2.0	0.36	38.53	533.2	385.3	0.7	960.09
Average		8.25	1.67	1058	2.47	1.07	36.30	534.4	353.16	0.42	927.83

SL1-SL6 Şereflikoçhisar Salt Lake salt samples.

Table 2. The physico-chemical characteristics of the brine samples studied.

Sampling site	Colour of sampling site	pH	Hardness (Fr)	Ca^{2+} g/l	Mg^{2+} g/l	HCO_3^- g/l	Cl^- g/l	Na^+ g/l	K^+ g/l	Total
SL7	Light pale-pink	7.44	7900	2.8	17.50	20.74	121.3	78.7	6.7	247.74
SL8	Dark-cream	7.67	5350	1.4	12.15	19.52	137.1	86.5	7.7	264.37
SL9	Cream	7.57	3000	3.6	5.10	53.68	538	348.9	1.6	950.88
Average		7.56	5417	2.6	11.58	31.31	265.47	171.37	5.33	487.66

SL7-SL9 Şereflikoçhisar Salt Lake brine samples.

hardness and Ca^{2+} content were found in the SL3 salt samples. The Mg^{2+} content of the SL7 and SL8 brine samples was considerably high. The hardness, Mg^{2+} and K^+ contents of the salt samples were low compared to the brine samples of the salt lake. The highest HCO_3^- content was found in the SL9 brine samples. Ca^{2+} content was almost the same in the salt and brine samples from Şereflikoçhisar Salt Lake. The highest hardness value was found in the SL7 brine sample. The total salt composition (g/kg) of the SL6 sampling site was higher than that of the other sampling sites. The salt samples from the lake were dominated by sodium and chloride; high levels of carbonate minerals also were present. We found that the total ionic composition of the salt lake changed according to the area. Such a salt and brine composition, with the presence of Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- and HCO_3^-, can be expected to support the growth of extremely halophilic bacteria.

Microbiological Analyses

The total number of extremely halophilic bacteria in the salt samples (10^4- 10^6 cfu/g) was higher than in the brine samples (10^3-10^5 cfu/ml) (Tables 3 and 4). The highest total extremely halophilic bacterial number (1 x 10^6 cfu/g) was seen at the SL6 sampling site. An increase in the extremely halophilic bacterial population might be due to the high salt content of the SL6 sampling site.

On Brown agar, these strains produced red, blood-red, brick-red, orange-red, pink, bright-pink, pale-pink, yellowish-cream, cream, white and transparent colonies. Colonies isolated from both salt and brine samples showed similar pigmentation. Transparent colonies were not isolated from the brine samples. Brick-red colonies were the most numerous in the lake. White colonies were the lowest in number in the lake. Due to the high number of brick-red colonies in the lake, these bacterial communities imparted a reddish-pink colour to Şereflikoçhisar Salt Lake. This explains the colour change of the lake from white to pink in recent years. During summer months, a reddish pink colour is seen on encrusted salt located around the lake.

A total of 82 extremely halophilic aerobic strains were isolated from the salt and brine samples. Fifty-two extremely halophilic aerobic strains were isolated from the salt samples of which 20 randomly selected strains were examined in greater detail. Thirty extremely halophilic aerobic strains were isolated from the brine samples of which 12 randomly selected strains were examined in greater detail.

Colonial Morphology

Most colonies on Brown agar media were 1-2 mm in diameter after 6 weeks of incubation. These colonies were circular, convex or raised, entire, transparent or translucent. The colonial pigmentation of these samples included blood-red to pale-pink, yellowish-cream and transparent. All strains produced entire colonies except for 1SL3, 2SL3, 3SL6, 6SL6, 2SL8, 3SL8 and 3SL9, which produced irregular wavy colonies. All strains produced circular colonies except for 1SL3, 2SL3, 3SL6 and 3SL8, which produced irregular, spreading colonies.

Cell Morphology

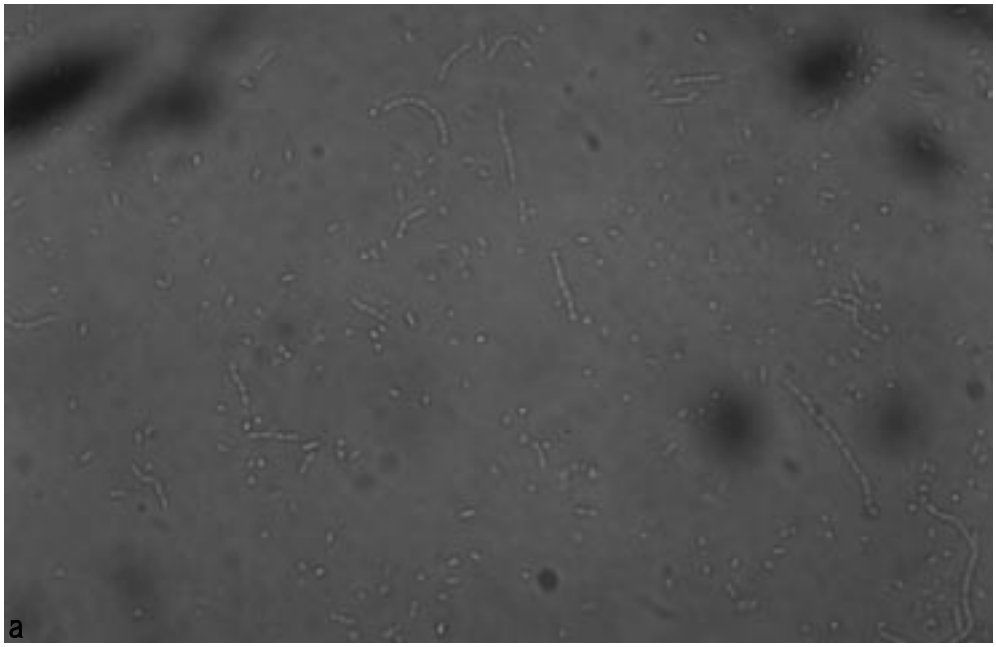
All strains isolated from the salt lake had different morphologies, physiologies and antibiotic sensitivities. The cells of all strains were extremely pleomorphic, appearing as irregular, short, long and swollen rods, bent rods, clubs, spheres and triangles and they occurred in singles, pairs or short chains (Figs. 1a, b, c). Thirty-two strains of halophiles indicated two distinctly separate clusters, one pleomorphic rod-shaped halophiles and the

Table 3. Average number of colonies (per gram salt or ml brine) obtained from different sampling sites.

Sampling site	Red	Blood red	Brick red	Orange red	Pink	Bright pink	Pale pink	Yellowish cream	Cream	White	Transparent	Total bacterial number (cfu)
SL1	7×10^4	1.3×10^3	5×10^4	-	5×10^3	-	5×10^2	4×10^4	-	-	2.3×10^5	3.96×10^5
SL2	2×10^4	-	3×10^5	1×10^4	-	-	2×10^4	1×10^4	-	-	-	3.6×10^5
SL3	2×10^4	2×10^3	-	-	8×10^3	1×10^2	1×10^2	4×10^4	-	2×10^2	-	7×10^4
SL4	1.4×10^5	1×10^2	1×10^4	1×10^2	1×10^3	2×10^2	-	1×10^2	-	-	-	1.51×10^5
SL5	4.1×10^5	1×10^2	1.3×10^5	1×10^2	2.1×10^5	2×10^4	1×10^4	1×10^4	-	-	2×10^4	8.1×10^5
SL6	1.2×10^5	2×10^4	6.1×10^5	9×10^4	1.3×10^5	1.2×10^5	-	1×10^4	-	-	1×10^4	1.1×10^6
SL7	6.7×10^3	2×10^2	-	-	6.3×10^3	1×10^2	-	-	-	-	-	1.3×10^4
SL8	3×10^2	1×10^2	1×10^2	1×10^2	5×10^3	-	-	6×10^2	-	6×10^2	-	6.8×10^3
SL9	4×10^2	-	-	2×10^4	6×10^4	7×10^4	1×10^4	1×10^4	1×10^4	5×10^2	-	1.809×10^5
Total	7.87×10^5	2.38×10^4	1.1×10^6	1.2×10^5	4.25×10^5	2.1×10^5	4.06×10^4	1.2×10^5	1×10^4	1.3×10^3	2.6×10^5	

Table 4. The total number and percentage of colonies (per gram salt or ml brine) obtained from different sampling sites.

Sampling site	Total bacterial number (cfu)	Red %	Pink %	Cream %	Non-pigmented %
SL1	3.96×10^5	31.10	1.40	10.25	57.25
SL2	3.6×10^5	91.66	5.55	2.79	-
SL3	7×10^4	31.42	11.71	56.87	-
SL4	1.51×10^5	99.14	0.79	0.07	-
SL5	8.1×10^5	68.00	29.63	0.13	2.5
SL6	1.1×10^6	76.5	23.40	0.091	0.090
SL7	1.3×10^4	52.00	48.00	-	-
SL8	6.8×10^3	8.82	73.53	17.65	-
SL9	1.809×10^5	11.27	77.39	11.34	-



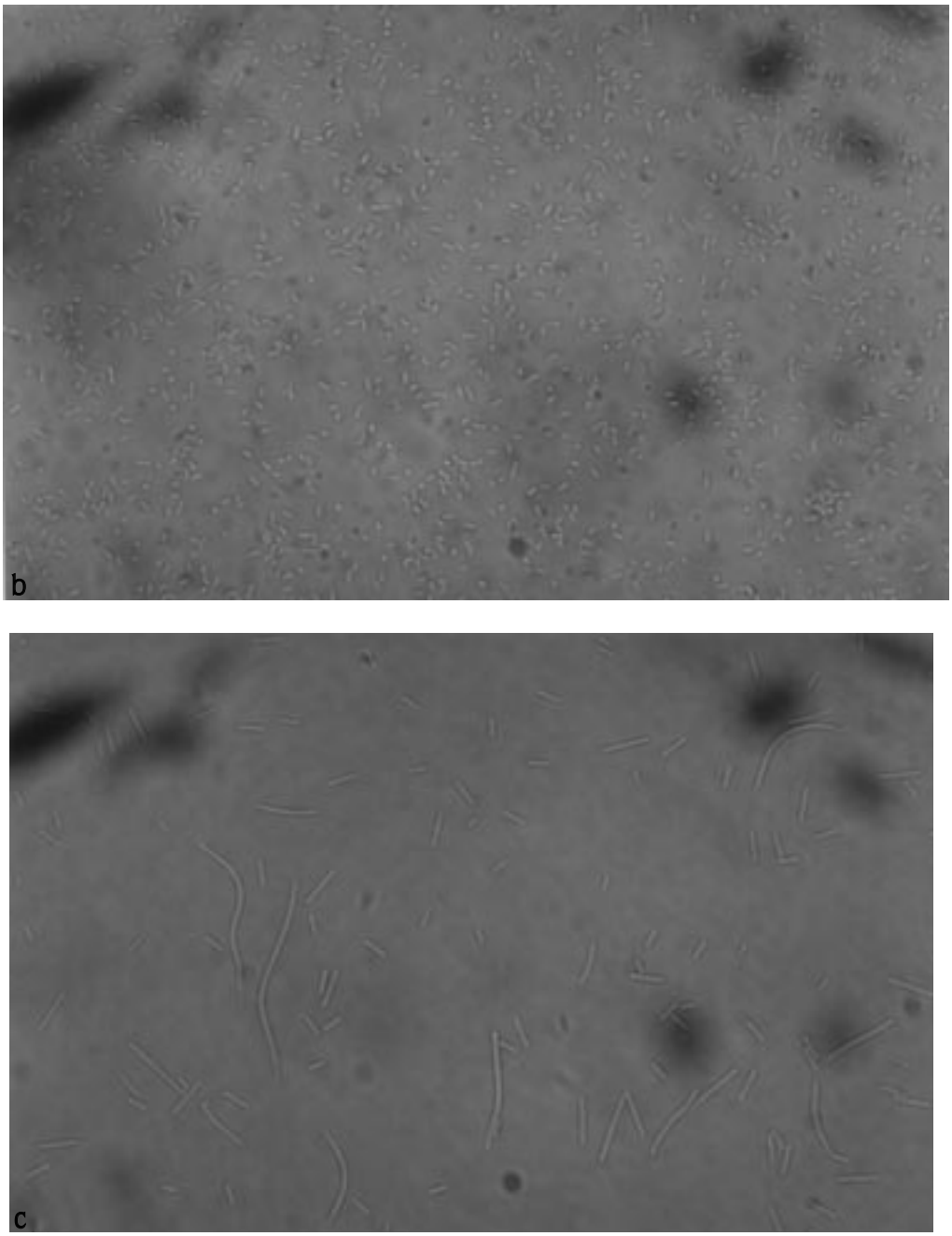


Figure 1 Examples of extremely halophilic strains as seen with a bright field light microscope. (a) 3SL2(X 696). (b) 4SL8(X 696). (c) 4SL9(X 696). Bar, 5 µm.

other of pleomorphic cell-shaped halophiles. Flexuous and flat rods were also seen. Twelve strains of extremely halophilic pleomorphic rods and 17 strains of extremely halophilic pleomorphic cells were isolated from the salt lake. Considerable diversity among these strains was seen. One strain of extremely halophilic flexuous rods (4SL9) (Fig. 1c) and two strains of extremely halophilic flat rods (1SL7 and 1SL8) were isolated from the lake. Triplicate cell structures were seen in only one sample (2SL8). Streptobacillus-like structures were seen in three samples (3SL2, 6SL6, 6SL9) (Fig. 1a). The approximate cell dimensions of the rod form were 1.0-2.5 x 2.5-7.5 µm; elongated cells up to 32 µm were observed rarely in liquid medium (4SL9) (Fig. 1c). All isolates were motile. Only one isolate was Gram positive (7SL9) .

Salt and pH Tolerance

The strains were tested for growth potential with respect to NaCl concentration in Brown medium. High salt concentrations were required to grow the strains. Seventeen out of 32 strains grew at 10, 15 and 25% (w/v) NaCl. Eight of 32 strains grew both at 15 and 25% NaCl. Seven strains grew at only 25% NaCl. No growth occurred at 0, 3, 5, 6, 7 and 8% NaCl. Optimum growth occurred at 25% NaCl, 40 ºC and a pH of 7.5. All 32 strains grew between pH 6 and 7.5, but none exhibited growth at pH 4.5.

Biochemical Tests

Twenty-three strains produced gelatinase enzymes. Only one strain showed positive β-galactosidase activity. Fourteen strains produced cellulase enzymes. Thirty-two strains hydrolysed Tween 80. Ten strains produced indol from tryptophan. Twenty-three strains showed positive methyl-red reactions. All strains showed negative Voges-Proskauer reactions. The detailed results of all cultural, morphological, physiological and biochemical tests are summarised in Table 5. The feature frequency of the morphological, physiological and biochemical characteristics of 32 extremely halophilic strains studied is shown in Table 6.

Antibiotic Sensivities

Thirty-two strains were tested by the disc diffusion method for their sensitivity to 13 different antibiotics. Eleven strains were found to be sensitive to ampicillin (10 µg) and chloramphenicol (30 µg). Three strains were sensitive to cefadroxil (30 µg). Nine strains were found to be sensitive to erythromycin (15 µg). Twenty strains were sensitive to bacitracin (10 U). Ten strains were sensitive to penicillin G (10 U). Twenty-three strains were found to be sensitive to novobiocin (5 µg). Only two strains were found to be sensitive to streptomycin (25 µg). Sixteen strains were resistant to sulfamethoxazole and trimethoprim (25 µg). All strains were resistant to amikacin (30 µg), ciprofloxacin (5 µg), neomycin (30 µg) and spiramycin (100 µg) (Tables 7 and 8).

Discussion and Conclusion

In this study, the limited number of strains (32) produced 11 different colonial pigments. A wide variation in their individual antibiotic sensitivities was seen. Seven morphological varieties of bacteria were observed, most of them being Gram-negative rods. These strains were found in larger numbers in salt crystal samples than in brine samples. These strains might be passively concentrated by brine evaporation. We concluded that the high content of total salts and organic substances in salt crystals might support the growth of extremely halophilic bacteria.

This research showed the presence of a variety of extremely halophilic red, pink, cream and colourless bacteria in the lake. The most abundant halophilic micro-organisms in the salt crystals were brick-red strains and the dominant type consisted of extremely pleomorphic cells. Flat and flexuous rods were found in very low numbers in the lake. Their distribution in the salt lake might be limited by competition from other bacteria. The brine samples contained low numbers of extremely halophilic red bacteria compared to salt crystals but extremely halophilic pink bacteria were dominant in the brine samples. Our research results showed that the predominant species in the lake were brick-red bacteria. The high salinity of the lake supports dense communities of extremely halophilic bacteria.

Aksöz and Kolonkaya (19) explained the isolation of a moderate halophilic bacterium from Şereflikoçhisar Salt Lake. The bacterium showed optimal growth in 8% NaCl containing Dundas' complex medium. The bacterium was Gram negative and pleomorphic cell shaped.

One of the principal findings was the complete absence of halotolerant micro-organisms. More than 53% of Şereflikoçhisar Salt Lake bacteria require at least 10% salt to grow and the entire bacterial community could not grow without salt. Since almost all strains had salt requirements above 10% and grew optimally at 25%, they can be considered to be extremely halophilic based upon Larsen's definitions (20).

Four different enzymes, protease, lipase, amylase and cellulase, are used in many modern washing detergents. These enzymes allow a lowering of the washing temperature to 40 ºC, without the loss of cleaning efficiency. These enzymes can be used as substitutes for less desirable chemicals in detergents resulting in smaller packaging, less waste and a reduced environmental impact. Furthermore, industrial enzymes obtained from halophiles might be used for improving garments during textile processing. Proteolytic enzymes from *Halobacterium* also play an important role in the brine

Table 5. Phenotypic features of the 32 strains studied.

	Strain							
Characteristics	1SL1	2SL1	1SL2	2SL2	3SL2	1SL3	2SL3	3SL3
Colonial morphology	circular	circular	circular	circular	circular	irregular and spreading	irregular and spreading	circular
Colony size	1 mm	3 mm	3 mm	1 mm	2 mm	5 mm	5 mm	1-2 mm
Colony elevation	convex	slightly umbonate	slightly umbonate	slightly raised	slightly raised	flat	flat	slightly raised
Colony density	transparent glistening	translucent matt	translucent matt	translucent glistening	translucent matt	translucent matt	translucent matt	transparent matt
Colony edge	entire	entire	entire	entire	entire	irregular and wavy	irregular and wavy	entire
Pigmentation	blood-red	yellowish-cream	yellowish-cream	brick-red	yellowish-cream	yellowish-cream	yellowish-cream	white
Cell shape	extremely pleomorphic cells	pleomorphic rods	pleomorphic cells	pleomorphic cells	pleomorphic rods	pleomorphic rods	pleomorphic rods	pleomorphic rods
Pointed ends	+	+	-	-	-	-	+	-
Squared ends	+	+	+	+	-	-	+	-
Rounded ends	+	+	+	+	+	+	+	+
Cell arrangement	single and paired cells	single and paired cells	single, paired and irregularly clustered cells	single and paired cells	single, paired cells and long chains	single, paired and irregularly clustered cells	single and paired cells	single, paired and irregularly clustered cells
Chains	-	+	+	-	+	+	+	+
Gram negative	+	+	+	+	+	+	+	+
Motile	+	+	+	+	+	+	+	+
Cell size; width and length (µm)	2.5 x 5.0-12.5	2.5 x 2.5-7.5	2.5 x 2.5-5.0	2.5 x 5.0-10	2.5 x 2.5-5.0	2.5 x 5.0	2.5 x 5.0-7.5	1.0-2.5x2.5-5.0
Growth at 40 ºC, pH 7.5								
0% NaCl	-	-	-	-	-	-	-	-
3% NaCl	-	-	-	-	-	-	-	-
5% NaCl	-	-	-	-	-	-	-	-
6% NaCl	-	-	-	-	-	-	-	-
7% NaCl	-	-	-	-	-	-	-	-
8% NaCl	-	-	-	-	-	-	-	-
10% NaCl	-	+	-	-	+	-	-	+
15% NaCl	-	+	+	+	+	+	+	+
25% NaCl	+	+	+	+	+	+	+	+
Growth at								
pH 4.5	-	-	-	-	-	-	-	-
pH 6.0	+	+	+	+	+	+	+	+
pH 7.0	+	+	+	+	+	+	+	+
pH 7.5	+	+	+	+	+	+	+	+
Gelatinase activity	-	+	+	-	+	+	+	+
β-galactosidase activity	-	-	-	-	-	-	-	-
Cellulase activity	-	-	-	-	-	-	-	+
Tween 80 hydrolysis	+	+	+	+	+	+	+	+
Indol production	-	-	-	-	-	-	-	-
Metil-red	+	+	-	+	+	+	+	+
Voges-Proskauer	-	-	-	-	-	-	-	-

Table 5. Continued.

	Strain							
Characteristics	4SL3	1SL4	2SL4	3SL4	1SL5	1SL6	2SL6	3SL6
Colonial morphology	circular	circular	circular	circular	circular	circular	circular	irregular and spreading
Colony size	1 mm	2 mm	1 mm	2 mm	1 mm	0.5-1 mm	1 mm	5 mm
Colony elevation	slightly raised	convex	convex	slightly raised	slightly convex	convex	convex	flat
Colony density	transparent glistening	transparent glistening	transparent matt	translucent glistening	translucent glistening	transparent glistening	transparent glistening	translucent matt
Colony edge	entire	entire	entire	entire	entire	entire	entire	irregular and wavy
Pigmentation	reddish-pale pink	blood-red	reddish-orange	light-pink	brick-red	blood-red	light-pink	yellowish-cream
Cell shape	pleomorphic rods	extremely pleomorphic cells	pleomorphic cells	pleomorphic rods	extremely pleomorphic cells	extremely pleomorphic cells	extremely pleomorphic cells	pleomorphic rods
Pointed ends	-	+	-	-	+	+	+	-
Squared ends	-	+	-	+	-	+	+	-
Rounded ends	+	+	+	+	+	+	+	+
Cell arrangement	single and paired cells	single and paired cells	single and paired cells	single and paired cells	single and paired cells	single and paired cells	single and paired cells	single and paired cells
Chains	-	-	+	-	-	+	+	+
Gram negative	+	+	+	+	+	+	+	+
Motile	+	+	+	+	+	+	+	+
Cell size; width and length (µm)	1.0-2.5 x 2.5-3.0	2.5 x 5.0-12.5	2.5 x 3.0-5.0	2.5 x 5.0	1.0-2.5 x 2.5-5.0	2.5 x 5.0-12.5	2.5 x 2.5-5.0	2.5 x 5.0
Growth at 40 °C, pH 7.5								
0% NaCl	-	-	-	-	-	-	-	-
3% NaCl	-	-	-	-	-	-	-	-
5% NaCl	-	-	-	-	-	-	-	-
6% NaCl	-	-	-	-	-	-	-	-
7% NaCl	-	-	-	-	-	-	-	-
8% NaCl	-	-	-	-	-	-	-	-
10% NaCl	-	-	+	-	+	-	+	+
15% NaCl	+	-	+	-	+	+	+	+
25% NaCl	+	+	+	+	+	+	+	+
Growth at								
pH 4.5	-	-	-	-	-	-	-	-
pH 6.0	+	+	+	+	+	+	+	+
pH 7.0	+	+	+	+	+	+	+	+
pH 7.5	+	+	+	+	+	+	+	+
Gelatinase activity	+	+	+	+	-	-	+	+
β-galactosidase activity	-	-	-	-	-	-	-	-
Cellulase activity	-	-	+	+	+	+	+	+
Tween 80 hydrolysis	+	+	+	+	+	+	+	+
Indol production	+	-	-	-	+	-	+	-
Metil-red	-	+	+	+	+	-	-	+
Voges-Proskauer	-	-	-	-	-	-	-	-

Table 5. Continued.

	Strain							
Characteristics	4SL6	5SL6	6SL6	7SL6	1SL7	1SL8	2SL8	3SL8
Colonial morphology	circular	circular	circular	circular	circular	circular	circular	irregular and spreading
Colony size	1 mm	1-2 mm	2 mm	1-1.5 mm	1.5-2 mm	1 mm	2 mm	3-6 mm
Colony elevation	slightly umbonate	slightly convex	slightly convex	convex	convex	convex	raised	slightly umbonate
Colony density	transparent glistening	transparent glistening	transparent matt	translucent glistening	opaque glistening	opaque glistening	opaque glistening	transparent glistening
Colony edge	entire	entire	irregular and wavy	entire	entire	entire	irregular and wavy	irregular and wavy
Pigmentation	light-pink	reddish-orange	yellowish-cream	brick-red	blood-red	blood-red	reddish-orange	brownish-yellow
Cell shape	extremely pleomorphic cells	pleomorphic rods	pleomorphic rods	pleomorphic rods	flat rods	flat rods	pleomorphic cells	pleomorphic cells
Pointed ends	+	-	-	-	-	-	+	-
Squared ends	+	-	-	+	-	-	+	+
Rounded ends	+	+	+	+	+	+	+	+
Cell arrangement	single and paired cells	single and paired cells	single paired cells and long chains	single and paired cells	single and paired cells	single and paired cells	single paired and triplicate cells	single paired and irregularly clustered cells
Chains	-	-	+	-	-	-	+	-
Gram negative	+	+	+	+	+	+	+	+
Motile	+	+	+	+	+	+	+	+
Cell size; width and length (µm)	2.5 x 2.5	2.5 x 5.0-7.5	2.5 x 5.0	2.5 x 5.0-7.5	2.5 x 5.0-7.5	2.5-5.0 x 5.0-7.5	2.5-5.0 x 5.0-12.5	1.0 x 5.0
Growth at 40 °C, pH 7.5								
0% NaCl	-	-	-	-	-	-	-	-
3% NaCl	-	-	-	-	-	-	-	-
5% NaCl	-	-	-	-	-	-	-	-
6% NaCl	-	-	-	-	-	-	-	-
7% NaCl	-	-	-	-	-	-	-	-
8% NaCl	-	-	-	-	-	-	-	-
10% NaCl	-	+	+	-	+	-	-	+
15% NaCl	-	+	+	-	+	-	+	+
25% NaCl	-	+	+	+	+	-	+	+
Growth at								
pH 4.5	-	-	-	-	-	-	-	-
pH 6.0	+	+	+	+	+	+	+	+
pH 7.0	+	+	+	+	+	+	+	+
pH 7.5	+	+	+	+	+	+	+	+
Gelatinase activity	+	+	+	-	+	-	-	+
β-galactosidase activity	-	-	-	-	-	-	-	-
Cellulase activity	+	-	+	-	-	+	-	+
Tween 80 hydrolysis	+	+	+	+	+	+	+	+
Indol production	+	+	-	-	+	-	+	-
Metil-red	-	+	+	+	-	+	+	-
Voges-Proskauer	-	-	-	-	-	-	-	-

Table 5. Continued.

	Strain							
Characteristics	4SL8	1SL9	2SL9	3SL9	4SL9	5SL9	6SL9	7SL9
Colonial morphology	circular	circular	circular	circular	circular	circular	circular	circular
Colony size	2 mm	1.5-2 mm	0.5 mm	3 mm	2 mm	2 mm	2 mm	1-2 mm
Colony elevation	slightly raised	slightly convex	slightly raised	slightly convex	slightly raised	convex	slightly convex	convex
Colony density	transparent glistening	translucent glistening	opaque matt	opaque glistening	transparent glistening	transparent glistening	transparent glistening	transparent glistening
Colony edge	entire	entire	entire	irregular wavy	entire	entire	entire	entire
Pigmentation	brownish-yellow	dark-reddish orange	light-pink	dark-cream	dark-light pink	light-red	white	yellowish-cream
Cell shape	pleomorphic rods	pleomorphic cells	extremely pleomorphic cells	extremely pleomorphic cells	long flexuous rods	extremely pleomorphic cells	extremely pleomorphic cells	pleomorphic cells
Pointed ends	-	+	-	+	-	+	+	-
Squared ends	-	-	+	+	-	+	+	-
Rounded ends	+	+	+	+	+	+	+	+
Cell arrangement	single, paired and irregularly clustered cells	single and paired cells	single and paired cells	single and paired cells	single and paired cells	single and paired cells	single, paired cells and long chains	single, paired and irregularly clustered cells
Chains	+	+	-	-	-	-	+	-
Gram negative	+	+	+	+	+	+	+	-
Motile	+	+	+	+	+	+	+	+
Cell size; width and length (µm)	1.0-2.5 x 2.5- 5.0	2.5 x 2.5-7.5	1.0-2.5 x 5.0-6.0	2.5 x 2.5-5.0	2.5 x 17.5- 32	2.5 x 5.0-7.5	2.5 x 2.5	2.5 x 2.5-7.5
Growth at 40 °C, pH 7.5								
0% NaCl	-	-	-	-	-	-	-	-
3% NaCl	-	-	-	-	-	-	-	-
5% NaCl	-	-	-	-	-	-	-	-
6% NaCl	-	-	-	-	-	-	-	-
7% NaCl	-	-	-	-	-	-	-	-
8% NaCl	-	-	-	-	-	-	-	-
10% NaCl	+	+	-	+	+	-	+	+
15% NaCl	+	+	+	+	+	-	+	+
25% NaCl	+	+	+	+	+	+	+	+
Growth at pH 4.5	-	-	-	-	-	-	-	-
pH 6.0	+	+	+	+	+	+	+	+
pH 7.0	+	+	+	+	+	+	+	+
pH 7.5	+	+	+	+	+	+	+	+
Gelatinase activity	-	+	+	+	+	+	-	+
β-galactosidase activity	-	-	-	-	-	+	-	-
Cellulase activity	-	-	-	-	+	+	-	+
Tween 80 hydrolysis	+	+	+	+	+	+	+	+
Indol production	-	-	+	-	+	-	-	+
Metil-red	-	+	+	-	-	+	-	+
Voges-Proskauer	-	-	-	-	-	-	-	-

Table 6. Feature frequency of the morphological and biochemical characteristics of 32 extremely halophilic strains.

Characteristic	%
Morphological	
Pleomorphic rod shaped	37.50
Pleomorphic cell shaped	21.87
Extremely pleomorphic cell shaped	31.25
Flexuous rods formed in late log-stationary phase	3.12
Flat rods	6.25
Single and paired cells	68.75
Single, paired and irregularly clustered cells	18.75
Single, paired and triplicate cells	3.12
Chains (fewer than five cells)	37.50
Chains (fewer than 12 cells)	9.37
1.0-2.5 x 2.5-7.5 µm	71.87
Growth on agar	
Colony 1-2 mm (6 weeks)	71.87
Colony 3-6 mm (6 weeks)	21.87
Circular colonies	87.50
Irregular and spreading colonies	12.50
Blood-red colonies	15.62
Light-red colonies	3.12
Brick-red colonies	9.37
Reddish-orange colonies	12.50
Reddish-pale pink colonies	3.12
Light-pink colonies	15.62
Brownish-yellow colonies	6.25
Yellowish-cream colonies	25.00
Dark-cream colonies	3.12
White colonies	6.25
Glistening colonies	68.75
Matt colonies	31.25
Transparent colonies	50.00
Translucent colonies	34.37
Opaque colonies	15.62
Entire edges	78.12
Irregular and wavy edges	21.87
Convex colonies	50.00
Raised colonies	28.12
Flat colonies	9.37
Umbonate colonies	12.5
Growth at pH 6.0 to 7.5 in 0, 3, 5, 6, 7, and 8% NaCl	0
Growth at pH 6.0 to 7.5 in 25% NaCl	100
Motile	100
Physiological	
Gram negative	97
Biochemical	
Gelatinase activity	72
β-galactosidase activity	7.0
Cellulase activity	14.00
Tween 80 hydrolysis	100
Indol production	31.2
Methyl-red	65.60
Voges-Proskauer	0

Table 7. Antibiotic sensitivity of 32 extremely halophilic strains.

	Strain															
	1SL1	2SL1	1SL2	2SL2	3SL2	1SL3	2SL3	3SL3	4SL3	1SL4	2SL4	3SL4	1SL5	1SL6	2SL6	3SL6
Antibiotic																
Amikacin (30 µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ampicillin (10 µg)	R	S	S	R	S	S	S	R	S	R	S	R	R	R	R	R
Bacitracin(10 U)	S	R	S	S	R	R	R	R	R	S	S	S	S	S	S	R
Cefadroxil (30 µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Chloramphenicol(30 µg)	R	S	S	R	S	S	S	R	R	R	R	R	R	R	R	S
Ciprofloxacin (5 µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Erythromycin(15 µg)	R	S	S	R	S	S	S	R	R	R	R	R	R	R	R	R
Neomycin (30 µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Novobiocin (5 µg)	S	S	R	S	S	R	R	R	S	S	S	S	S	S	S	R
Penicillin G (10 U)	R	R	S	R	S	S	S	R	S	R	R	R	R	R	R	S
Spiramycin (100 µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Streptomycin (25 µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Sulpfamethaxazole (23,75 µg) and Trimethoprim (1.25 µg)	S	S	R	S	R	R	R	R	R	S	R	S	R	S	R	R

	Strain															
	4SL6	5SL6	6SL6	7SL6	1SL7	1SL8	2SL8	3SL8	4SL8	1SL9	2SL9	3SL9	4SL9	5SL9	6SL9	7SL9
Antibiotic																
Amikacin (30 µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ampicillin (10 µg)	R	S	R	R	R	R	R	S	R	R	R	R	R	R	S	S
Bacitracin (10 U)	S	S	R	S	S	S	S	R	S	S	S	R	S	S	R	R
Cefadroxil (30 µg)	R	S	R	R	R	R	R	R	S	S	R	R	R	R	R	R
Chloramphenicol(30 µg)	R	R	S	R	R	R	R	S	S	R	R	S	R	R	R	S
Ciprofloxacin (5 µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Erythromycin(15 µg)	R	S	R	R	R	R	R	S	R	R	R	S	R	R	R	S
Neomycin (30 µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Novobiocin (5 µg)	S	S	S	S	S	S	S	S	S	S	S	R	S	R	R	R
Penicillin G (10 U)	R	R	R	R	R	R	R	S	R	R	R	S	R	R	S	S
Spiramycin (100 µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Streptomycin (25 µg)	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	S
Sulpfamethaxazole (23.75 µg) and Trimethoprim (1.25 µg)	R	R	R	S	R	S	S	S	R	S	S	S	S	R	S	S

Resistant R, Sensitive S

Table 8. Susceptibility rates of 32 extremely halophilic strains to 13 different antibiotics.

	Antibiotics												
	AK (30 µg)	AMP (10 µg)	B B (10 U)	CFR (30 µg)	C (30 µg)	CIP (5 µg)	E (15 µg)	N (30 µg)	NV (5 µg)	P (10 U)	SP (100 µg)	S (25 µg)	SXT (25 µg)
Resistance	100%	66%	37%	91%	66%	100%	75%	100%	28%	69%	100%	94%	50%
Sensitivity	-	34%	63%	9%	34%	-	35%	-	72%	31%	-	6%	50%

fermentation of one type of traditional fish sauce (21,22).

In 1981, Aksöz (23) emphasised that the presence of halophilic bacteria degrading organic polymers in the Şereflikoçhisar Salt Lake could assist in cleaning the lake.

Due to the industrial and economic importance of halophilic enzymes, the gelatinase, β-galactosidase, cellulase and lipase activities of 32 extremely halophilic bacteria were screened. Interestingly, all of the examined strains produced industrially important enzymes such as gelatinase, β-galactosidase, cellulase or lipase. Twenty-six strains produced two or more enzymes. Strain 5SL9 was selected as the best producer of the different enzymes. This strain produced gelatinase, β-galactosidase, cellulase and lipase enzymes.

In our research, 14 extremely halophilic bacteria showed positive cellulase activity, and this enzyme can be used in different chemical industries.

The most interesting and significant conclusion of these experiments is that all of the extremely halophilic strains showed positive lipase activity. The lipase enzyme of these strains can especially be used in laundry and automatic dish washing detergents to improve oil cleaning capabilities.

Another important aspect of extremely halophilic bacterial contamination in salt is whether all the bacteria have the same destructive action on the collagen of hide or not. Previous research results demonstrated that halophilic organisms isolated from brine-cured hides contain proteases that could potentially digest the grain surface of a hide under extended storage conditions at elevated temperatures (17,24-27).

Our results showed that the source of halophilic bacterial contamination in Turkish brine-cured hides was the salt obtained from Şereflikoçhisar Salt Lake. Proteolytic strains were present in almost all salt and brine samples of the lake. A limited survey of 32 different extremely halophilic strains from the lake showed that 23 of these strains were able to digest gelatin. This means that more than half of these strains can break down hide proteins. If the unprocessed salt is used directly in the brine curing of hides, these strains will digest the collagen of hide lowering the value of the leather. Therefore, before using the salt in hide preservation, the halophilic bacterial content and their gelatinolytic activity should be restricted to prevent hide damage. It is possible to reduce halophilic bacterial numbers by heating and treating salt with effective bactericides containing naphthalene and 1-2 dichlorobenzene, trichloro-s-triazinetrione and sodium sulphate or methylene bis (thiocyanate) and 2-thiocyanomethylthio benzothiazole (28).

If hide is treated with salt as free as possible from extremely halophilic bacteria and is stored and transported at low temperatures, the halophilic bacteria left in the salt will not matter. However, if the temperature of the hide in storage rises too much or if the atmosphere becomes too moist, the growth of extremely halophilic bacteria will be encouraged.

The *Halobacteriaceae* are generally resistant to ampicillin, chloramphenicol, cycloserine, kanamycin, polymyxin, erythromycin, neomycin, penicillin G, tetracycline and streptomycin and are sensitive to anisomycin, bacitracin and novobiocin (3,4,16).

All of our strains were resistant to amikacin, ciprofloxacin, neomycin and spiramycin. In general cream and yellowish-cream colonies were sensitive to ampicillin, erythromycin, penicillin and chloramphenicol but resistant to novobiocin and bacitracin. According to these antibiotic results, it is thought that these cream and yellowish-cream colonies are members of the Eubacteria. In addition, blood-red, red, brick-red, light-pink, and reddish-orange colonies were found to be resistant to ampicillin, chloramphenicol, erythromycin, penicillin and streptomycin but sensitive to bacitracin and novobiocin. According to these antibiotic results, it is thought that blood-red, red, brick-red, light-pink and reddish-orange colonies are members of the Archaebacteria. The separation into Archaebacterial and Eubacterial strains was supported by their growth in the presence of antibiotics. Archaebacterial strains grew in the presence of penicillin G, whereas Eubacterial strains could not grow in the presence of penicillin.

A high salt requirement and resistance to ampicillin, chloramphenicol, erythromycin, penicillin and streptomycin but sensitivity to bacitracin and novobiocin place these strains in the *Halobacteriales*. The placement of these strains in the *Halobacteriaceae* was supported by several shared morphological, cultural and physiological characteristics, biochemical tests and antibacterial sensitivities.

This research on Şereflikoçhisar Salt Lake proved that the lake contains a fairly wide diversity of proteolytic, lipolytic and cellulolytic species and that the chemical

composition of the lake supported the growth of extremely halophilic bacteria. The morphological, physiological, biochemical characteristics of these strains and antibiotic sensitivities to different antibiotics and chemical analyses of the salt lake may provide useful information about the nature of the Archael and Eubacterial communities in Şereflikoçhisar Salt Lake. The characteristic sensitivity pattern of these strains to antibiotics may serve as a guideline in the search for further biochemical and molecular differences between Archaebacteria and Eubacteria and it may also be helpful in identifying new species in the lake. Enzymatic test results of these strains present promising features for future biotechnological applications.

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