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Uncovering High-Yield Ectoine Producers from Extreme Environments: Insights from Tuz Lake

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ABSTRACT

Ectoine, a compatible solute produced by halophile microorganisms, is widely used in various industries due to its protective properties against environmental stress. This study aimed to isolate and characterize bacterial strains capable of producing high levels of ectoine without medium optimization from Tuz Lake, an extremely saline environment in Turkey. Bacterial isolates were obtained by selective culturing on meat peptone agar containing NaCl concentrations ranging from 3% to 21%, using water and soil samples collected from randomly selected sites in the Şereflikoçhisar and Cihanbeyli regions. A total of 22 isolates were screened for salt tolerance and their potential for ectoine production. Among them, 10 isolates with the highest salt tolerance and distinctive colony morphology were selected for further analysis. Molecular characterization via 16S rRNA sequencing identified these isolates as belonging to the genera *Halomonas*, *Chromohalobacter*, and *Salinivibrio*. High-performance liquid chromatography (HPLC) analysis revealed that *Salinivibrio* sp. (Isolate 12) and *Halomonas* sp. (Isolate 21) exhibited the highest ectoine production, yielding 296.88 µg mL^{-1} and 202.49 µg mL^{-1}, respectively. To optimize ectoine yield, a Plackett-Burman experimental design was applied, evaluating the effects of different nitrogen sources. Peptone was identified as a statistically significant factor ($p < 0.05$) for enhancing ectoine production in *Salinivibrio* sp. This study highlights the biotechnological potential of halophilic bacteria from Tuz Lake for industrial ectoine production and emphasizes the importance of medium optimization in improving ectoine yields. Further optimization may enable the development of scalable processes for commercial ectoine production.

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Aşırı Çevrelerden Yüksek Verimli Ektoin Üreten Mikroorganizmaların Keşfi: Tuz Gölü Örneği

ÖZET

Ektoin, halofil mikroorganizmalar tarafından üretilen ve çevresel streslere karşı koruyucu özellikleri nedeniyle çeşitli endüstrilerde yaygın olarak kullanılan uyumlu bir çözünendir. Bu çalışmada, Türkiye'deki aşırı tuzlu bir ortam olan Tuz Gölü'nden, besiyeri optimizasyonu yapılmaksızın yüksek düzeyde ektoin üretebilen bakterilerin izolasyonu ve karakterizasyonu amaçlanmıştır. Şereflikoçhisar ve Cihanbeyli bölgelerinde rastgele seçilen alanlardan toplanan su ve toprak örneklerinden, %3 ila %21 arasında değişen NaCl konsantrasyonlarına sahip et-pepton agar kullanılarak yapılan seçici kültürleme ile bakteri izolatları elde edilmiştir. Toplamda 22 izolat tuz toleransı ve ektoin üretim potansiyeli açısından taranmış, en yüksek tuz toleransına ve belirgin koloni morfolojisine sahip 10 izolat detaylı analize alınmıştır. 16S rRNA dizilemesiyle yapılan moleküler karakterizasyon, bu izolatların *Halomonas*, *Chromohalobacter* ve *Salinivibrio* cinslerine ait olduğunu göstermiştir. Yüksek performanslı sıvı kromatografisi (HPLC) analizleri, *Salinivibrio* sp. (İzolat 12) ve *Halomonas* sp. (İzolat 21) türlerinin sırasıyla 296.88 µg mL^{-1} ve 202.49 µg mL^{-1} ektoin üretimiyle en yüksek verimi gösterdiğini ortaya koymuştur. Ektoin verimini artırmak için farklı azot kaynaklarının etkisini değerlendiren Plackett-Burman deney tasarımı uygulanmış ve *Salinivibrio* sp. için peptonun

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ektoin üretimini anlamlı şekilde artıran bir faktör olduğu ($p < 0.05$) belirlenmiştir. Bu çalışma, Tuz Gölü'nden izole edilen halofilik bakterilerin endüstriyel ektoin üretiminde biyoteknolojik potansiyelini vurgulamakta ve ektoin verimini artırmada besiyeri optimizasyonunun önemini göstermektedir. İleri optimizasyon çalışmaları, ticari ektoin üretimi için ölçeklenebilir süreçlerin geliştirilmesine olanak sağlayabilir.

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INTRODUCTION

Compatible solutes are small organic molecules that can accumulate in high concentrations within cells, safeguarding against environmental stresses without impairing cellular functions (Brown, 1976). As natural osmoprotectants, these solutes, including sugars, amino acids, and specialized molecules like ectoine, enable cells to counteract the adverse effects of high osmolarity by stabilizing hydration and volume (Yancey et al., 1982; Le Rudulier et al., 1984; Kempf & Bremer, 1998; Sahin Dogan & Kocabaş, 2023). Additionally, compatible solutes contribute to cellular defense mechanisms against extreme temperature shifts, hydrostatic pressure, desiccation, and even freezing, enhancing resilience in fluctuating environments (Roessler & Muller, 2001; Burg & Ferraris, 2008; Gunde-Cimerman et al., 2018). Through their unique properties, compatible solutes are vital for maintaining cellular integrity, with roles extending beyond osmotic regulation to preserve protein structure and function, providing significant promise in various biotechnological applications (Bourot et al., 2000; Czech et al., 2018).

Among these solutes, ectoine stands out for its versatility and effectiveness. Ectoine was first isolated from the extreme halophile *Halorhodospira halochloris*, a bacterium that thrives in high-salinity environments, where ectoine functions to protect cellular components from osmotic stress (Galinski, 1995). The significance of ectoine lies not only in its natural role as a cell stabilizer but also in its protective attributes, which make it a valuable commercial product. Ectoine's utility spans industries, including cosmetics, where it enhances skin hydration and acts as a barrier against surfactants in skin care formulations (Graf et al., 2008), as well as cryopreservation, where it preserves cell integrity during freeze-thaw cycles (Lippert & Galinski, 1992). Additionally, ectoine is under study for its neuroprotective properties, with promising results showing potential applications in treating neurodegenerative diseases, such as Alzheimer's, by inhibiting amyloid-beta aggregation (Kanapathipillai et al., 2005).

The commercial demand for ectoine has driven an increasing focus on the isolation and optimization of ectoine-producing microorganisms, particularly halophiles (Çiçek et al., 2025). Despite the production capabilities of microbial isolates, ectoine's market price still remains high, and in response to this demand, recent studies have sought to optimize ectoine yield through nutrient and process modifications. For instance, initial isolates typically produce ectoine at levels around 100–120 mg/L^{-1}, but medium optimization can boost production to approximately 700 mg/L^{-1} (Elbialy et al., 2019). Through genetic modifications, production can reach impressive concentrations of 8–14 g L-1 (W. C. Chen et al., 2018; Ma et al., 2020). These advances highlight the importance of both selecting high-producing strains and refining growth conditions to maximize yield.

Efforts to increase ectoine production have centered on optimizing medium components critical to the biosynthetic pathway (Canlı Taşar & Taşar, 2023). Studies suggest that nitrogen and carbon sources, as well as micronutrients like glutamate, aspartate, and iron, are influential in enhancing ectoine synthesis (W. C. Chen et al., 2018; Ayadi et al., 2020; Ma et al., 2020). The nitrogen source, particularly the ratio of yeast extract to ammonium sulfate, has proven essential for optimizing production (W. C. Chen et al., 2018), as has the addition of urea, which contributes to higher ectoine yields (Ma et al., 2020; Y. Chen et al., 2024). These optimizations not only offer strategies for improving ectoine yield but also illustrate the complex biochemical interactions that govern compatible solute production.

In Turkey, Tuz Lake offers a unique opportunity to explore natural reservoirs of halophilic microorganisms capable of producing ectoine. Previous studies and metagenomic analyses indicate that this hypersaline environment is rich in microbial diversity, providing a fertile ground for isolating novel ectoine-producing strains (Doğan & Kocabaş, 2021; Doğan & Kocabaş, 2024). Building on this foundation, the present study focuses on isolating and identifying high-ectoine-producing bacteria from Tuz Lake and determining the main components affecting ectoine production in culture medium by the Plackett-Burman (Plackett & Burman, 1946) statistical design method. This design will allow for the systematic evaluation of culture medium components, identifying those most influential

on ectoine production and paving the way for scalable production.

MATERIAL and METHOD

Sample Collection

Water and soil samples were collected from various locations around the Tuz Lake, specifically in the Cihanbeyli and Şereflikoçhisar regions. Using sterile containers, water samples were obtained by direct immersion, while soil samples were collected from a depth of 5 cm after clearing surface debris. A total of 10 samples, consisting of 5 water and 5 soil samples, were collected in sterile plastic containers and transported to the laboratory at 4°C, where subsequent analysis was initiated immediately upon arrival.

Screening and Isolation of Ectoine-Producing Bacterial Isolates

Soil and water samples were suspended in a 25% (w v^{-1}) sterile sodium chloride (NaCl) solution and thoroughly homogenized. The homogenized samples were then serially diluted and inoculated onto meat peptone agar (MPA) plates [composition (g/L^{-1}): peptone, 5; malt extract, 5; agar, 1.5%] containing NaCl concentrations ranging from 3% to 21%, in 3% increments. Inoculated plates were incubated at 28°C for 5 days (Van Thuoc et al., 2019).

At the end of the 5-day incubation period, the relative growth of the bacterial isolates was scored on a scale from 0 to 5, where 0 indicated no growth at the corresponding salt concentration and 5 represented maximum growth. The resulting growth profiles were visualized as a heatmap using the R software. Bacterial isolates demonstrating robust growth across a wide range of NaCl concentrations were selected for further study. Isolates were evaluated based on their salt tolerance range, colony morphology (i.e., shape, margin, texture, and elevation), and pigmentation. Selected isolates were purified through repeated subculturing on the same medium. Ultimately, 10 distinct isolates were selected and used for further analysis (Mahansaria et al., 2015; Van Thuoc et al., 2019).

The selected isolates were cultivated in meat peptone broth (MPB) [composition (g/L^{-1}): peptone, 5; malt extract, 5] supplemented with 9% NaCl. Cultures were incubated at 28°C with agitation at 180 rpm on a rotary shaker. After a 48-hour incubation, bacterial samples were collected for molecular characterization, cell dry weight (CDW) measurement, and ectoine production potential analysis.

Molecular Characterization of Isolates

For the molecular characterization of the selected 10 isolates, 16S rRNA gene sequence analysis was performed. To amplify the target gene region, the Colony PCR approach was employed. Briefly, bacterial samples were collected by touching the colonies grown on agar plates with a sterile micropipette tip and suspended in 20 µL of sterile dH_2O. The suspension was then boiled for 10 minutes, followed by centrifugation at 16,000 × g for 5 minutes. The resulting supernatant was used as a template for PCR. The amplification was carried out using the universal primer pair 515F (5'-GTGYCAGCMGCCGCGGTAA-3') (Parada et al., 2016) and 806R (5'-GGACTACNVGGGTWTCTAAT-3') (Apprill et al., 2015). The PCR conditions followed were as follows: an initial denaturation at 94 °C for 3 minutes, 30 cycles of 94 °C for 30 seconds, 52 °C for 30 seconds, and 72 °C for 1.5 minutes, followed by a final extension at 72 °C for 7 minutes (Van Thuoc et al., 2019).

Amplified DNA fragments were subjected to one-way sequencing using the forward primer. Sequence data were analyzed using the BLAST tool on the NCBI against the nucleotide database (nr/nt) for molecular characterization (Altschul et al., 1990; Sayers et al., 2022). Multiple sequence alignment of the retrieved sequences was performed using MAFFT v7 with default parameters (Katoh, 2002; Katoh & Standley, 2013). A maximum likelihood phylogenetic tree was then constructed using RAxML v8 under the GTR+GAMMA model with 1,000 bootstrap replicates to assess branch support (Stamatakis, 2014). The resulting tree was visualized and annotated as a dendrogram using custom Python scripts employing the Biopython and Matplotlib libraries. The dendrogram was constructed using hierarchical clustering (Ward's method) from a pairwise distance matrix derived from aligned 16S rRNA gene sequences. Isolate codes were mapped to genus-level taxonomic assignments based on BLAST results.

Determination of Cell Dry Weight (CDW)

To measure cell dry weight, 3 mL of culture was centrifuged at 5000 ×g for 15 minutes in pre-weighed centrifuge tubes. The supernatant was discarded, and the resulting pellet was washed with 3 mL of sterile water, recentrifuged, and dried at 80°C until a constant weight was achieved (Van Thuoc et al., 2019).

Ectoine Quantification

Pellets (5 mg) were extracted with 570 µL of methanol:chloroform (10:5:4 v v-1), followed by shaking for 5 minutes.

Equal volumes of a chloroform mixture (1:1 v v-1) were added, followed by 10 minutes of shaking. Samples were centrifuged at 5000 ×g for 10 minutes, and the hydrophilic upper layer containing ectoine was collected and dried (Van Thuoc et al., 2019).

Dried residues were dissolved in 100 µL of water and 400 µL of acetonitrile. Ectoine concentrations were determined using an Agilent 1200 Series high-performance liquid chromatography (HPLC) system with a C18 column. The mobile phase consisted of acetonitrile: water (8:2 v v-1) at a flow rate of 0.5 mL min-1. Detection was performed at 210 nm, and ectoine quantification was based on a calibration curve from ectoine standards (Sadeghi et al., 2014). Based on these analyses, two isolates with the highest ectoine production potential were selected for further optimization.

Optimization of Media Components

A Plackett-Burman experimental design (Plackett & Burman, 1946) with two levels and 12 experimental sets was employed to evaluate the effects of various media components (Table 1). Concentrations marked as '+1' corresponded to levels used in ectoine production media, while '-1' represented minimal concentrations expected to have negligible effects. Bacterial cultures were grown in a rotary shaker at 28°C and 180 rpm in a medium containing 15% NaCl, with the pH adjusted to 7.2. After 48 hours of incubation, samples were collected for CDW measurement and ectoine analysis.

Table 1. Medium components and their levels for the Plackett-Burman experimental design.
Çizelge 1. Plackett-Burman deneysel tasarımı için besiyeri bileşenleri ve seviyeleri.

Chemical Code	Name	Codes	- 1*	+ 1*
A	Peptone	Pe	0	5
B	Yeast Extract	YE	0	5
C	Malt Extract	ME	0	5
D	Ammonium Sulfate	AS	0	5
E	Urea	Ur	0	3
F	Ammonium Nitrate	AN	0	3

*The concentrations of medium components are expressed in g L^{-1}.
**Besiyeri bileşenlerinin konsantrasyonları g L^{-1} cinsinden ifade edilmiştir.*

RESULTS

Exploration of Potential Ectoine-Producing Bacteria

A total of 22 isolates, obtained as pure cultures from water and soil samples collected from 10 different sites in the Şereflikoçhisar and Cihanbeyli regions of Tuz Lake, Türkiye, were selected based on their colony morphology and pigmentation characteristics. These selected isolates were evaluated for their ability to tolerate different salt concentrations. The relative growth responses of the bacteria, grown in media containing NaCl concentrations ranging from 3% to 21% with 3% increments, are presented in Figure 1.

Based on the results, isolates numbered 1, 3, 6, 9, 10, 11, 12, 17, 21, and 22 were selected due to their ability to grow across a wide range of salt concentrations and/or their distinct growth patterns observed at varying salt concentrations. For the molecular characterization of the selected isolates, their 16S rRNA gene sequences were analyzed. Figure 2 presents the agarose gel electrophoresis image of the 16S rRNA gene region amplified using the Colony PCR approach for sequencing analysis

One-way sequence analysis was performed using the forward primer, and the resulting sequences were analyzed with the BLAST tool on the NCBI platform. The results identified the isolates at the genus level as *Halomonas*, *Chromohalobacter*, and *Salinivibrio* (Table 2). The BLAST analysis of the sequencing data is presented in Supplementary Table 1. High sequence similarity within these genera precluded species-level identification due to limitations of the 16S rRNA region (García-López et al., 2014; Kalia et al., 2016). To further illustrate the phylogenetic relationships among the isolates, a dendrogram based on sequence similarity was constructed and is shown in Figure 3. The figure illustrates the phylogenetic relationships among isolates belonging to the genera *Halomonas*, *Chromohalobacter*, and *Salinivibrio*.

Assessing the Ectoine Biosynthesis Potential of Selected Isolates

To evaluate the ectoine production potential of the selected isolates, the bacteria were incubated at 28 °C in MPA medium containing 9% NaCl. At the end of the incubation period, the cell dry weights (CDW) of the harvested bacteria were determined (Table 2). The results showed that under the applied incubation conditions, the highest

CDW was observed in isolate 12 (3.22 g), while the lowest values were found in isolates 9 and 10 (0.24 g).

Ectoine extraction was performed using 5 mg of CDW from the isolates, following the protocol described by Van Thuoc et al. (2019). The ectoine content was detected using high-performance liquid chromatography (HPLC) with a C18 column at 210 nm, and quantification was achieved by referencing a calibration curve constructed from standard ectoine solutions of known concentrations (Sadeghi et al., 2014). Figure 3 presents the standard curve generated from HPLC data for ectoine standards at concentrations of 100, 200, 300, and 400 µg mL-1, which was used for the quantification calculations.

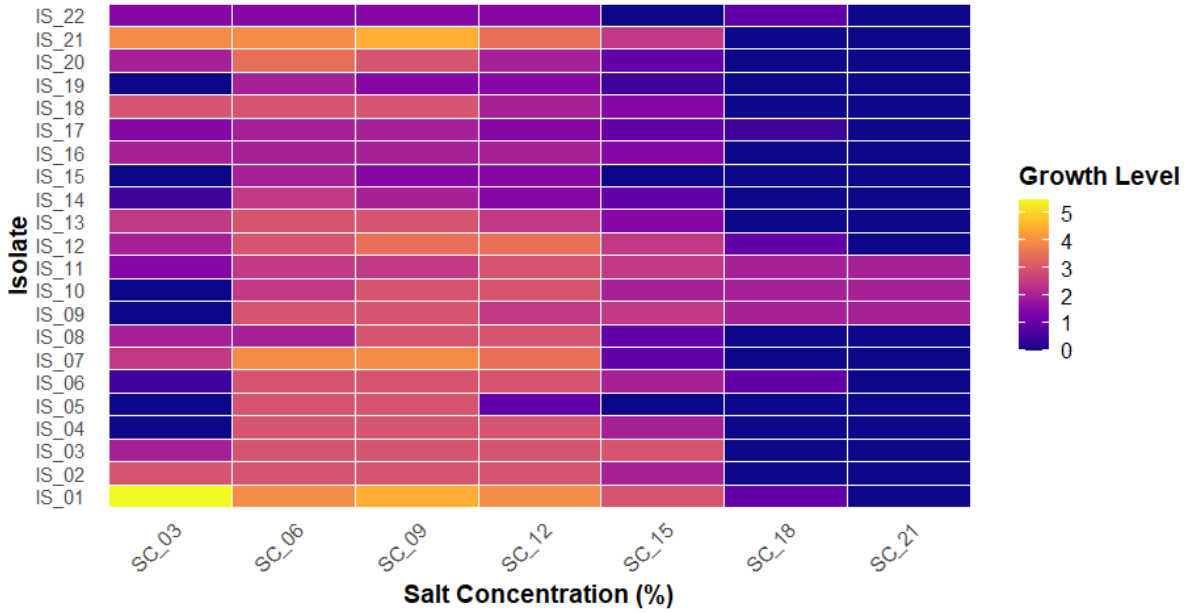

Figure 1. Relative growth responses of isolates at different NaCl concentrations. In the Growth Level chart, a score of 5 indicates optimal growth, whereas a score of 0 indicates that the isolate did not grow under the tested conditions

Şekil 1. Farklı NaCl konsantrasyonlarında izolatların nispi çoğalma tepkileri. Çoğalma Düzeyi çizelgesinde 5 en iyi çoğalma durumunu ifade ederken 0 puanı izolatın test edilen koşullarda hiç çoğalma göstermediğini ifade etmektedir.

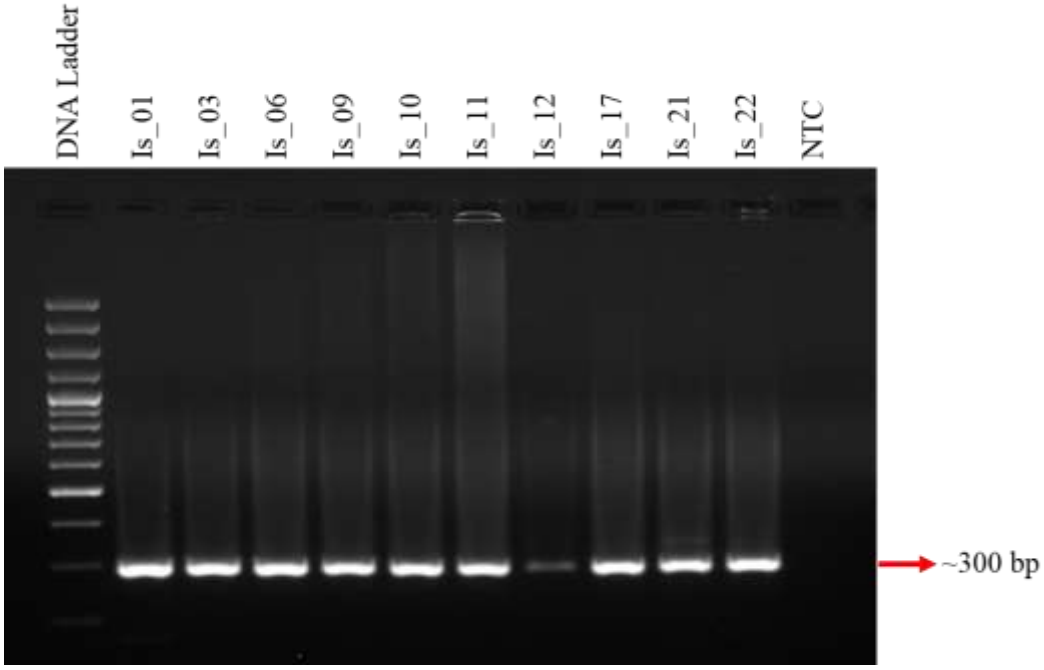

Figure 2. Agarose gel electrophoresis of PCR products amplified with the 16S rRNA primers.
Şekil 2. 16S rRNA primerleri ile çoğaltılan PCR ürünlerinin agaroz jel elektroforezi.

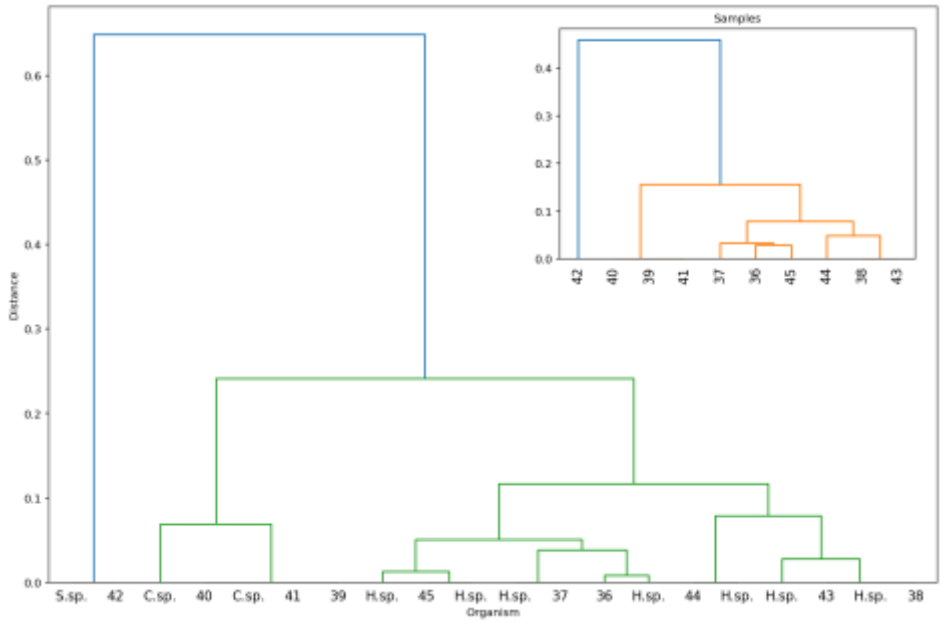

Figure 3. Phylogenetic dendrogram of bacterial isolates based on 16S rRNA gene sequence similarity
Şekil 3. 16S rRNA gen dizisi benzerliğine dayalı bakteri izolatlarının filogenetik dendrogramı

Table 2. Bacterial species identified through 16S rRNA sequence analysis.
Çizelge 2. 16S rRNA dizi analizi ile tanımlanan bakteri türleri.

Sample	Organism	Cell Dry Weight (g)
Isolate 1	*Halomonas* sp.	0.27
Isolate 3	*Halomonas* sp.	0.74
Isolate 6	*Halomonas* sp.	0.36
Isolate 9	*Chromohalobacter* sp.	0.24
Isolate 10	*Chromohalobacter* sp.	0.24
Isolate 11	*Chromohalobacter* sp.	0.26
Isolate 12	*Salinivibrio* sp.	3.22
Isolate 17	*Halomonas* sp.	0.37
Isolate 21	*Halomonas* sp.	2.10
Isolate 22	*Halomonas* sp.	1.44

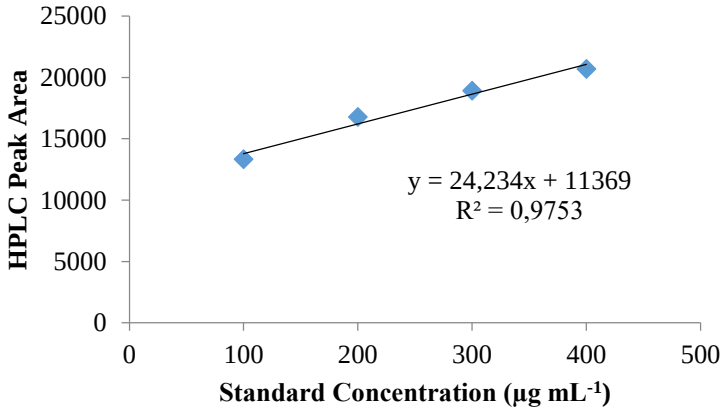

Figure 3. Calibration curve of ectoine standard*
*Şekil 3. Ektoin standardı kalibrasyon eğrisi**
*LOD = 67.7997 µg/mL LOQ = 205.4538 µg/mL

The ectoine concentrations in the isolates were calculated using the peak areas obtained from the HPLC measurements and the linear equation derived from the standard curve shown in Figure 3. The results demonstrated that isolates 12 and 21 exhibited the highest ectoine production, with concentrations of 296.88 µg

mL-1 and 202.49 µg/mL^{-1}, respectively, under the tested conditions. The evaluation of ectoine production levels in conjunction with the CDW results presented in Table 2 suggests that isolates 12 and 21 exhibit a higher level of salt adaptation compared to the other isolates. Consequently, these two isolates were selected for the subsequent Plackett-Burman experimental design phase, aimed at evaluating the effects of medium components on ectoine production potential.

Assessment of Medium Components on Ectoine Production

The Plackett-Burman experimental design was employed to assess the effects of different nitrogen sources – peptone, yeast extract, malt extract, ammonium sulfate, urea, and ammonium nitrate – on the ectoine production potential of isolates 12 and 21. The ectoine production levels, calculated through HPLC measurements, are presented in Table 3.

Based on the analysis of the Plackett-Burman experimental design, the results indicated that the inclusion of peptone as a nitrogen source had a statistically significant effect ($p < 0.05$) on ectoine production of isolate 12, as detailed in Table 4.

Table 3. Plackett-Burman experimental design and ectoine quantification by HPLC
Çizelge 3. Plackett-Burman deney tasarımı ve HPLC ile ektoin kantifikasyonu

	Nitrogen Sources						Ectoine Production (µg mL^{-1})	
	Pe	**YE**	**ME**	**AS**	**Ur**	**AN**	**Isolate 12**	**Isolate 21**
1	-1	1	1	-1	1	1	291.26	0
2	1	-1	1	1	1	-1	486.20	412.59
3	1	1	1	-1	-1	-1	401.00	246.93
4	-1	1	1	1	-1	-1	0	348.80
5	1	1	-1	1	1	1	374.80	0
6	-1	-1	1	-1	1	1	45.73	402.89
7	-1	-1	-1	1	-1	1	147.31	267.92
8	1	-1	1	1	-1	1	166.74	422.17
9	1	-1	-1	-1	1	-1	496.54	482.85
10	-1	-1	-1	-1	-1	-1	0	0
11	-1	1	-1	1	1	-1	0	220.24
12	1	1	-1	-1	-1	1	285.85	0

Table 4. Plackett-Burman design analysis results for Isolates 12 and 21.
Çizelge 4. İzolat 12 ve 21 için Plackett-Burman Tasarım Analizi Sonuçları.

	Isolate 12		**Isolate 21**	
	F value	p	F value	p
Pe	12.767	0.01599116*	0.302	0.606
YE	0.000	0.984	3.935	0.104
ME	0.032	0.865	2.129	0.204
AS	0.511	0.507	0.832	0.404
Ur	2.060	0.211	0.155	0.710
AN	0.022	0.887	1.095	0.343

DISCUSSION and CONCLUSION

In this study, water and soil samples were collected from Tuz Lake, and pure bacterial cultures were obtained from these samples using meat-peptone agar (Van Thuoc et al., 2019). The growth potential of the isolated strains across a range of salt concentrations was assessed, and 10 isolates were selected for further study. To identify these isolates at the genus level, 16S rRNA sequence analysis was conducted. Ectoine production potential was then evaluated using High-Performance Liquid Chromatography (HPLC) after growth on meat-peptone liquid medium containing 9% NaCl. Among the 10 isolates tested, the bacteria with the highest ectoine production potential were identified as Salinivibrio sp. (Isolate 12, 202 µg mL-1) and *Halomonas sp.* (Isolate 21, 297 µg mL-1), and these were further analyzed using a Plackett-Burman experimental design with various nitrogen sources.

Ectoine production from microorganisms isolated from high-salinity environments is typically low in the initial stages. For example, *Aestuariispira ectoiniformans*, isolated by Kang et al. (2022), produced 35 mg/L^{-1} of ectoine,

while *Halomonas elongata* produced 7 mg/L^{-1}. By cloning genes from *A. ectoiniformans*, ectoine production was increased to 1.67 g L^{-1} in *E. coli*. Furthermore, ectoine yields can reach levels comparable to commercial production without the need for genetic modifications, simply by optimizing the nutrient medium. For instance, Orhan *et al.* (2023) achieved an increase in ectoine production from 30 mg/L^{-1} to 710 mg/L^{-1} after nitrogen source optimization using classical methods and further increased it to 1090 mg/L^{-1} through Response Surface Methodology (RSM). Their studies focused on ammonium sources, and they found that ammonium phosphate provided the best results.

In the current study, two bacterial isolates from the genera *Salinivibrio* and *Halomonas* were found to produce approximately 200 and 300 mg L^{-1} of ectoine, respectively, in the initial phase. These values are notably high when compared to the typical initial production capacities of novel isolates, which generally range from 100 to 120 mg L^{-1} (Ayadi et al., 2020; Kang et al., 2022; Orhan et al., 2023), suggesting their promising potential for commercial ectoine production.

Similarly, Zhang et al. (2022) isolated *Halomonas qaidamensis* and reported ectoine production of 270 mg/g^{-1} CDW in a medium containing 2 mol/L^{-1} (approximately 9%) NaCl, proposing this strain as a new candidate for commercial ectoine production. High initial ectoine production levels indicate that optimization could significantly increase yields. In a study by Wei et al. (2011) involving the *Marinococcus genus*, the initial ectoine production of 600 mg L^{-1} was increased to 2500 mg L^{-1} after culture optimization. An additional example is provided by Chen et al. (2018), who successfully increased ectoine production from 3.65 g L^{-1} to 13.96 g L^{-1}.

In this study, the ectoine production of *Salinivibrio sp.* (Isolate 12) and *Halomonas sp.* (Isolate 21) was determined to be approximately 500 mg L^{-1} for both isolates following nitrogen source optimization using the Plackett-Burman experimental design.

The Plackett-Burman (PB) experimental design method is used to assess whether evaluated variables influence production outcomes. In this study, various nitrogen sources, including peptone, yeast extract, malt extract, ammonium sulfate, urea, and ammonium nitrate, were tested for their impact on ectoine production by *Salinivibrio sp.* and *Halomonas sp.* Among the nitrogen sources tested, only peptone was found to significantly affect ectoine production in *Salinivibrio sp.*. Two key conclusions can be drawn from these findings. First, media optimization is critical for the efficient production of metabolites by microorganisms. Second, while the effect of peptone was statistically significant, it suggests that ectoine production may not be solely influenced by the nitrogen source, provided an adequate nitrogen supply is available for these two microorganisms. In other words, ectoine production can occur independently of the specific nitrogen source if sufficient nitrogen is present in the medium. This observation contrasts with the findings of Wei et al. (2011) and Chen et al. (2018), where optimal ectoine production was achieved by varying organic and inorganic nitrogen sources. For instance, Wei et al. (2011) reported the highest ectoine production when 40 g/L^{-1} of yeast extract was used, and Chen et al. (2018) found that 84 g/L^{-1} of yeast extract and 28 g/L^{-1} of ammonium sulfate led to the highest yield, while inorganic nitrogen sources generally provided better ectoine production. Similarly, Orhan et al. (2023) noted that ammonium phosphate was more effective than ammonium sulfate for ectoine production.

Bacteria exhibit extensive diversity in their nitrogen assimilation abilities and regulatory mechanisms. They can utilize a broad range of nitrogen sources—from inert molecules like atmospheric dinitrogen (N_2) to complex synthetic compounds (e.g., atrazine)—often within the same organism, under tight regulatory control (Balderrama-Subieta & Quillaguamán, 2013; Deantas-Jahn et al., 2024; Xie et al., 2023). This organism-dependent variation may arise from differences in nitrogen uptake mechanisms, metabolic pathways, or regulatory networks influencing ectoine biosynthesis. Therefore, a one-size-fits-all approach to medium formulation is unlikely to yield optimal results across diverse microbial strains, underscoring the necessity of organism-specific optimization strategies in biotechnological applications. In other words, these discrepancies highlight the importance of tailoring nutrient optimization to the specific needs of each organism.

Given the diverse applications of ectoine in various industries and the untapped potential of ectoine production, the discovery of new microorganisms capable of synthesizing ectoine remains highly relevant. In this context, the halophilic microorganisms from Tuz Lake offer significant promise. This preliminary study identified three distinct genera among the 10 isolates collected from Tuz Lake, contributing to the growing body of knowledge regarding ectoine-producing organisms.

Furthermore, during the Plackett-Burman analysis phase, which serves as the foundation for subsequent optimization studies, it was observed that ectoine production increased nearly twofold under laboratory conditions. This suggests that further optimization through statistical methods could enhance ectoine production in the isolates obtained in this study, potentially reaching levels competitive with commercially utilized microbial strains. The widespread application of ectoine in various industrial sectors and the untapped potential of the market underscore the importance of discovering novel ectoine-producing microorganisms. In this regard, the extreme environment of Tuz Lake, along with the halophilic microorganisms inhabiting it, offers significant prospects. As

part of this preliminary investigation, microorganisms from three distinct genera were identified from ten isolates obtained from Tuz Lake, advancing our understanding of ectoine-producing organisms.

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Contribution Rate Statement Summary of Researchers

The authors declare that they have equally contributed to the article.

Conflict of Interest

The authors declare that there is no conflict of interest among them.

Ethics Statement

This study does not require approval from an ethics committee.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Apprill, A., Mcnally, S., Parsons, R., & Weber, L. (2015). Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquatic Microbial Ecology*, *75*(2), 129–137. https://doi.org/10.3354/ame01753

Ayadi, H., Frikha-Dammak, D., Fakhfakh, J., Chamkha, M., Hassairi, I., Allouche, N., Sayadi, S., & Maalej, S. (2020). The saltern-derived Paludifilum halophilum DSM 102817T is a new high-yield ectoines producer in minimal medium and under salt stress conditions. *3 Biotech*, *10*(12), 533. https://doi.org/10.1007/s13205-020-02512-x

Balderrama-Subieta, A., & Quillaguamán, J. (2013). Genomic studies on nitrogen metabolism in Halomonas boliviensis: Metabolic pathway, biochemistry and evolution. *Computational Biology and Chemistry, 47*, 96–104. https://doi.org/10.1016/j.compbiolchem.2013.08.002

Bourot, S., Sire, O., Trautwetter, A., Touzé, T., Wu, L. F., Blanco, C., & Bernard, T. (2000). Glycine betaine-assisted protein folding in a lysA mutant of Escherichia coli. *Journal of Biological Chemistry*, *275*(2), 1050–1056. https://doi.org/10.1074/jbc.275.2.1050

Brown, A. D. (1976). Microbial water stress. *Bacteriological Reviews*, *40*(4), 803. https://doi.org/10.1128/br.40.4.803-846.1976

Burg, M. B., & Ferraris, J. D. (2008). Intracellular organic osmolytes: Function and regulation. In *Journal of Biological Chemistry*, *283*(12), 7309–7313. https://doi.org/10.1074/jbc.R700042200

Canli Taşar, Ö., Erhan Taşar, G., Teknoloji Uygulama ve Araştırma Merkezi YÜTAM, Y., Teknik Üniversitesi, E., Meslek Yüksek Okulu, K., & Üniversitesi, A. (2023). Optimization of Keratinase Enzyme synthesized by Micrococcus luteus using Taguchi DOE Method. *Kahramanmaraş Sütçü İmam Üniversitesi Tarım ve Doğa Dergisi*, *26*(5), 1027–1033. https://doi.org/10.18016/ksutarimdoga.vi.1128064

Chen, W. C., Hsu, C. C., Lan, J. C. W., Chang, Y. K., Wang, L. F., & Wei, Y. H. (2018). Production and characterization of ectoine using a moderately halophilic strain Halomonas salina BCRC17875. *Journal of Bioscience and Bioengineering*, *125*(5), 578–584. https://doi.org/10.1016/j.jbiosc.2017.12.011

Chen, Y., Liu, Y., Meng, Y., Jiang, Y., Xiong, W., Wang, S., Yang, C., & Liu, R. (2024). Elucidating the salt-tolerant mechanism of Halomonas cupida J9 and unsterile ectoine production from lignocellulosic biomass. *Microbial Cell Factories*, *23*(1), 1–12. https://doi.org/10.1186/s12934-024-02515-w

Czech, L., Hermann, L., Stöveken, N., Richter, A. A., Höppner, A., Smits, S. H. J., Heider, J., & Bremer, E. (2018). Role of the Extremolytes Ectoine and Hydroxyectoine as Stress Protectants and Nutrients: Genetics, Phylogenomics, Biochemistry, and Structural Analysis. *Genes*, *9*(4). https://doi.org/10.3390/genes9040177

Çiçek, M., Çiçek, E., & Kocabaş, A. (2025). Ektoin: Mikrobiyal Hayatta Kalma Sırrından Biyoteknolojik Uygulamalara. *Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi*, *15*(1), 92–109. https://doi.org/10.21597/jist.1522694

Deantas-Jahn, C., Mendoza, S. N., Licona-Cassani, C., Orellana, C., & Saa, P. A. (2024). Metabolic modeling of Halomonas campaniensis improves polyhydroxybutyrate production under nitrogen limitation. *Applied Microbiology and Biotechnology*, *108*(1), 310. https://doi.org/10.1007/s00253-024-13111-8

Doğan, S. Ş., & Kocabaş, A. (2021). Metagenomic Assessment of Prokaryotic Diversity within Hypersaline Tuz Lake, Turkey. *Microbiology (Russian Federation)*, *90*(5), 647–655. https://doi.org/10.1134/s0026261721050118

Elbialy, H. A., Omara, A., Sharaf, A. M., el-hela, A., Shahin, A., & El-Fouly, M. (2019). Isolation and characterization of new ectoine-producers from various hypersaline ecosystems in Egypt. *Journal of Nuclear Technology in Applied Science*, *7*(1), 221–236. https://doi.org/10.21608/jntas.2019.16407.1005

Galinski, E. A. (1995). Osmoadaptation in Bacteria. *Advances in Microbial Physiology*, *37*(C), 273–328. https://doi.org/10.1016/s0065-2911(08)60148-4

García-López, M. L., Santos, J. A., Otero, A., & Rodríguez-Calleja, J. M. (2014). Psychrobacter. *Encyclopedia of Food Microbiology: Second Edition*, 261–268. https://doi.org/10.1016/b978-0-12-384730-0.00285-8

Graf, R., Anzali, S., Buenger, J., Pfluecker, F., & Driller, H. (2008). The multifunctional role of ectoine as a natural cell protectant. *Clinics in Dermatology*, *26*(4), 326–333. https://doi.org/10.1016/j.clindermatol.2008.01.002

Gunde-Cimerman, N., Plemenitaš, A., & Oren, A. (2018). Strategies of adaptation of microorganisms of the three domains of life to high salt concentrations. In *FEMS Microbiology Reviews*, *42*(3), 353–375. https://doi.org/10.1093/femsre/fuy009

Kalia, V. C., Kumar, R., Kumar, P., & Koul, S. (2016). A Genome-Wide Profiling Strategy as an Aid for Searching Unique Identification Biomarkers for Streptococcus. *Indian Journal of Microbiology*, *56*(1), 46–58. https://doi.org/10.1007/s12088-015-0561-5

Kanapathipillai, M., Lentzen, G., Sierks, M., & Park, C. B. (2005). Ectoine and hydroxyectoine inhibit aggregation and neurotoxicity of Alzheimer's β-amyloid. *FEBS Letters*, *579*(21), 4775–4780. https://doi.org/10.1016/j.febslet.2005.07.057

Kang, J. Y., Lee, B., Kim, J. A., Kim, M. S., & Kim, C. H. (2022). Identification and characterization of an ectoine biosynthesis gene cluster from Aestuariispira ectoiniformans sp. nov., isolated from seawater. *Microbiological Research*, *254*, 126898. https://doi.org/10.1016/j.micres.2021.126898

Katoh, K. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, *30*(14), 3059–3066. https://doi.org/10.1093/nar/gkf436

Katoh, K., & Standley, D. M. (2013). MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. *Molecular Biology and Evolution*, *30*(4), 772–780. https://doi.org/10.1093/molbev/mst010

Kempf, B., & Bremer, E. (1998). Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Archives of Microbiology*, *170*(5), 319–330. https://doi.org/10.1007/s002030050649

Le Rudulier, D., Strom, A. R., Dandekar, A. M., Smith, L. T., & Valentine, R. C. (1984). Molecular biology of osmoregulation. *Science*, *224*(4653), 1064–1068. https://doi.org/10.1126/science.224.4653.1064

Lippert, K., & Galinski, E. A. (1992). Enzyme stabilization be ectoine-type compatible solutes: protection against heating, freezing and drying. *Applied Microbiology and Biotechnology*, *37*(1), 61–65. https://doi.org/10.1007/bf00174204

Ma, H., Zhao, Y., Huang, W., Zhang, L., Wu, F., Ye, J., & Chen, G.-Q. (2020). Rational flux-tuning of Halomonas bluephagenesis for co-production of bioplastic PHB and ectoine. *Nature Communications*, *11*(1), 3313. https://doi.org/10.1038/s41467-020-17223-3

Mahansaria, R., Choudhury, J. D., & Mukherjee, J. (2015). Polymerase chain reaction-based screening method applicable universally to environmental haloarchaea and halobacteria for identifying polyhydroxyalkanoate producers among them. *Extremophiles*, *19*(5), 1041–1054. https://doi.org/10.1007/s00792-015-0775-9

Orhan, F., Ceyran, E., & Akincioğlu, A. (2023). Optimization of ectoine production from Nesterenkonia xinjiangensis and one-step ectoine purification. *Bioresource Technology*, *371*, 128646. https://doi.org/10.1016/j.biortech.2023.128646

Parada, A. E., Needham, D. M., & Fuhrman, J. A. (2016). Every base matters: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environmental Microbiology*, *18*(5), 1403–1414. https://doi.org/10.1111/1462-2920.13023

Plackett, R. L., & Burman, J. P. (1946). The Design of Optimum Multifactorial Experiments. *Biometrika*, *33*(4), 305–325. https://doi.org/10.1093/biomet/33.4.305

Roessler, M., & Muller, V. (2001). Osmoadaptation in bacteria and archaea: common principles and differences. *Environmental Microbiology*, *3*(12), 743–754. https://doi.org/10.1046/j.1462-2920.2001.00252.x

Sadeghi, A., Soltani, B. M., Nekouei, M. K., Jouzani, G. S., Mirzaei, H. H., & Sadeghizadeh, M. (2014). Diversity of the ectoines biosynthesis genes in the salt tolerant Streptomyces and evidence for inductive effect of ectoines on their accumulation. *Microbiological Research*, *169*(9–10), 699–708. https://doi.org/10.1016/j.micres.2014.02.005

Sahin Dogan, S., & Kocabaş, A. (2023). Profiling the genes associated with osmoadaptation and their variation by seasonally in Tuz Lake. *Communications Faculty of Science University of Ankara Series C Biology Geological Engineering and Geophysical Engineering*, *32*(2), 174–191. https://doi.org/10.53447/communc.1206230

Şahïn Doğan, S., & Kocabaş, A. (2024). Seasonal Gene Profiling in Tuz Lake with Regard to Biogeochemical Cycling. *Kahramanmaraş Sütçü İmam Üniversitesi Tarım ve Doğa Dergisi*, *27*(2), 273–284. https://doi.org/10.18016/ksutarimdoga.vi.1212062

Sayers, E. W., Bolton, E. E., Brister, J. R., Canese, K., Chan, J., Comeau, D. C., Connor, R., Funk, K., Kelly, C., Kim, S., Madej, T., Marchler-Bauer, A., Lanczycki, C., Lathrop, S., Lu, Z., Thibaud-Nissen, F., Murphy, T., Phan, L., Skripchenko, Y., … Sherry, S. T. (2022). Database resources of the national center for biotechnology information. *Nucleic Acids Research*, *50*(D1), D20–D26. https://doi.org/10.1093/nar/gkab1112

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, *30*(9), 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Van Thuoc, D., Hien, T. T., & Sudesh, K. (2019). Identification and characterization of ectoine-producing bacteria isolated from Can Gio mangrove soil in Vietnam. *Annals of Microbiology*, *69*(8), 819–828. https://doi.org/10.1007/s13213-019-01474-7

Wei, Y. H., Yuan, F. W., Chen, W. C., & Chen, S. Y. (2011). Production and characterization of ectoine by Marinococcus sp. ECT1 isolated from a high-salinity environment. *Journal of Bioscience and Bioengineering*, *111*(3), 336–342. https://doi.org/10.1016/j.jbiosc.2010.11.009

Xie, Y., Tian, X., He, Y., Dong, S., & Zhao, K. (2023). Nitrogen removal capability and mechanism of a novel heterotrophic nitrification–aerobic denitrification bacterium Halomonas sp. DN3. *Bioresource Technology*, 387, 129569. https://doi.org/10.1016/j.biortech.2023.129569

Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D., & Somero, G. N. (1982). Living with water stress: Evolution of osmolyte systems. *Science*, *217*(4566), 1214–1222. https://doi.org/10.1126/science.7112124

Zhang, T., Cui, T., Cao, Y., Li, Y., Li, F., Zhu, D., & Xing, J. (2022). Whole genome sequencing of the halophilic Halomonas qaidamensis XH36, a novel species strain with high ectoine production. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *115*(4), 545–559. https://doi.org/10.1007/s10482-022-01709-9.

Supplementary Table 1. The BLAST analysis of the sequencing data

Scientific Name	Max Score	Total Score	Query Cover	E value	Percent identity	Accession #	Sample #
Halomonas sp. BPA-5	313	313	99%	6E-81	100	KT324973.1	1
uncultured bacterium	313	313	99%	6E-81	100	GU212540.1	1
Halomonas sp. 2029	302	302	100%	1E-77	100	GU253999.1	3
Halomonas sp.	302	302	100%	1E-77	100	MK610751.1	3
Halovibrio variabilis	392	392	100%	1E-104	99.54	AM945680.1	6
Halomonas sp.	392	392	100%	1E-104	99.54	MT588425.1	6
uncultured bacterium	305	305	100%	1E-78	100	GU437281.1	9
Chromohalobacter sp. 06	305	305	100%	1E-78	100	EU442370.1	9
uncultured bacterium	303	303	100%	4E-78	100	GU437607.1	10
Chromohalobacter israelensis DSM 3043	303	303	100%	4E-78	100	NR_074225.1	10
uncultured bacterium	305	305	100%	1E-78	100	GU437281.1	11
Chromohalobacter sp. 06	305	305	100%	1E-78	100	EU442370.1	11
Salinivibrio budaii	298	298	100%	2E-76	100	JQ336963.1	12
Salinivibrio costicola	298	298	100%	2E-76	100	KU064692.1	12
Salinivibrio sharmensis	298	298	100%	2E-76	100	JQ336961.1	12
Halovibrio variabilis	300	300	100%	4E-77	100	AM945680.1	17
Halomonas sp.	300	300	100%	4E-77	100	MT588425.1	17
Halomonas sp. S83-1	300	300	100%	4E-77	100	EU868850.1	17
Halomonas sp. LN15KBR	300	300	100%	4E-77	100	KX185692.1	21
Halomonas sp.	300	300	100%	4E-77	100	MZ292935.1	21
Halomonas taeheungii	300	300	100%	4E-77	100	HF678759.1	22
Halomonas sp.	300	300	100%	4E-77	100	MW757273.1	22