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Halobacillus trueperi CT7: A Spore-Forming, Gelatinase Producing, Salt-Tolerant Bacteria Isolated from Çankırı Salt Mine

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Abstract: Halophilic microorganisms are an extreme group of organisms that can spread at high salt concentrations, and a significant part of them consists of halophilic bacteria. Salt mines are important sources where halophilic bacteria are detected. In this study, *Halobacillus trueperi* CT7, a halophilic bacterium, was isolated from Çankırı Salt Mine. It was determined that the obtained strain showed 98.1% similarity with *Halobacillus trueperi* by DNA isolation and sequence analysis as well as biochemical analysis. In addition, two-dimensional (scanning electron microscopy) and three-dimensional (atomic force microscopy) images of *Halobacillus trueperi* were performed to reveal the cell morphology. In order to determine the industrial use potential of the microorganism, the minimum and maximum salt concentrations, temperature and pH ranges in which the species can grow, as well as the enzyme activities, where the species can grow, were determined qualitatively. The interest in halophilic organisms for their use in extreme industrial processes is increasing day by day. It is thought that this study will contribute to future studies on halophilic bacteria.

Keywords: Halophilic bacteria, *Halobacillus trueperi*, Salt Mine

Öz: Halofilik mikroorganizmalar yüksek tuz konsantrasyonunda yayılım gösterebilen ekstrem bir canlı grubudur ve bunların önemli bir bölümü halofilik bakterilerden oluşmaktadır. Tuz madenleri halofilik bakterilerin tespit edildiği önemli kaynaklardandır. Bu çalışmada bir halofilik bakteri olan *Halobacillus trueperi* CT7, Çankırı Tuz Madeni'nden izole edilmiştir. Elde edilen bu strainin, biyokimyasal analizlerin yanısıra DNA izolasyonu ve sekans analiziyle *Halobacillus trueperi*'ye %98,1 benzerlik gösterdiği tespit edilmiştir. Ayrıca *Halobacillus trueperi*'nin iki boyutlu (taramalı elektron mikroskobu) ve üç boyutlu (atomik kuvvet mikroskobu) görüntülemeleri gerçekleştirilmiştir. Mikroorganizmanın endüstriyel kullanım potansiyellerinin belirlenmesi amacıyla türün gelişim gösterebildiği minimum ve maksimum tuz konsantrasyonları, sıcaklık ve pH aralıklarının yanısıra enzim aktiviteleri de kalitatif olarak tespit edilmiştir. Ekstrem endüstriyel proseslerde kullanımları açısından halofilik organizmalara olan ilgi her geçen gün artmaktadır. Bu çalışmanın da halofilik bakterilerle ilgili gelecek çalışmalara katkı sağlayacağı düşünülmektedir.

Anahtar Kelimeler: Halofilik bakteri, *Halobacillus trueperi*, Tuz Madeni

1. Introduction

Extreme microorganisms are a very interesting group among all microorganisms. They are famous for being able to adapt to harsh conditions in which the vast majority of organisms can not survive. Among the extreme microorganisms, halophilic microorganisms are a different group of organisms as they can live in environments with high salt concentrations. This group consists of organisms with various genetic characteristics, with members in both Bacteria, Archaea and Eukarya domains. Halophilic bacteria are grown in salt-containing media to simulate their natural environment. Since environments with high salt concentration are not suitable for the growth of microorganisms other than halophilic group, the risk of contamination is also very low. Considering the reasons such as the easy and fast production of microorganisms and the ease of genetic modification, the importance of these microorganisms for industrial processes emerges.

Parameters such as oxidase, catalase, Gram stain, growth in a single carbon source, indole, nitrate production are quite important parameters in terms of biochemical characteristics of microorganisms [1]. However, today's technological developments enable us to detect species similarities with the data based on the base sequences of the species. After DNA isolation of pure cultures, 16 S rRNA sequences amplified by polymerase chain reaction are sequenced with next generation sequencing technologies. Following this, the obtained base sequences obtained are checked in databases and species similarity rates are revealed.

One of the areas where halophilic bacteria have industrial potential is their wide range of pigmentation properties [2,3,4]. These pigments, which can be yellow, orange, pink or red, play an important role in many fields such as

cosmetics, food, textiles, pharmaceuticals. Pigment-rich extracts obtained from halophilic microorganisms are also investigated for their antimicrobial, anticancer, antioxidant and cytotoxic properties [5,6,7]. In this context, it is thought that the biochemical and phenotypic properties of *Halobacillus trueperi*, which is a halophilic bacterium, isolated from the salt mine are very important in creating a study area for many medical and industrial processes.

2. Material and Method

Isolation of halophilic strain

Salt sample was obtained from salt mines in Çankırı (Türkiye). For enrichment, 1 gram of salt sample was dissolved in 90 ml of sterile 15% NaCl and incubated for several hours [8]. 1 mL of brine were mixed with 50 ml of MAM JCM 168 which contained (g/L): casamino acids (5), yeast extract (5), sodium glutamate (1), trisodium citrate (3), $MgSO_4x7H_2O$ (29.5), KCl (2), NaCl (175.5), $FeCl_2x4H_2O$ (0.036), $MnCl_2x4H_2O$ (0.36 mg). The medium pH was 7.0-7.2 before autoclaving. After that the samples were incubated at 37 ºC for several weeks until the appearance of colorful colonies was occurred. In order to obtain pure cultures, the culture medium was inoculated into petri dishes with the same medium composition and added 20g/L agar. After 5 days of 37°C incubation, colonies with different pigmentation and morphology were planted in different petri dishes and pure cultures were obtained.

Identification of halophilic strain

Biochemical tests

The isolated strain which has pale yellow colony pigmentation was coded as CT7 and analyzes were carried out to reveal its biochemical characteristics. By adding 10% NaCl to the staining solutions, gram staining was carried out according to the method by Dussault [9]. The ideal growing conditions were examined using a growth medium containing varied NaCl concentrations (0–25%) and yeast concentrations (0-0.1%), as well as various incubation temperatures (10–50 ºC) and pH values (5–10). By incorporating 1% KNO_3 into the growing medium, the conversion of nitrate to nitrite and the production of gas from nitrate were examined.

The amount of gas produced by nitrate was measured using Durham tubes submerged in the broths. In the growing medium that was supplemented with a 1% (w/v) substrate, glucose, lactose, maltose, fructose, mannitol, trehalose, galactose, and sucrose were used as the sole carbon sources. By introducing Kovac's reagent to the growth medium containing 1% tryptone, the synthesis of indole from tryptone was identified. Hydrolyses of starch, gelatin, casein, and Tween 80 were evaluated for amylase, gelatinase, caseinase, and esterase activities in addition to the strains' oxidase and catalase capabilities.

DNA isolation procedure and polimerase chain reaction conditions

The strains of halophilic bacteria were identified using phylogenetic analysis. The strains' DNA was isolated using the technique described by Neumann et al. [10]. Strains grown on MAM JCM 168 agar medium were taken into 1 mL STE buffer with loop, vortexed and homogenized. The cell suspension was centrifuged at 8 000 rpm for 2 minutes and the supernatant was removed.

The cell pellet was washed twice with 400 µL of STE, and after the last supernatant was removed, the cell pellet was resuspended with 200 µL of TE buffer and vortexed by adding 100 µL of tris-saturated phenol. The suspension, which was centrifuged at 13 000 rpm for 5 min at 4 ºC, was separated into two phases and transferred to a new 160 µL tube from the upper phase. By adding 40 µL of TE buffer and 100 µL of chloroform, it was centrifuged at 13 000 rpm for 5 minutes at 4 ºC, and the extraction process with chloroform continued until the color of the lysate became clear. 160 µL of the upper phase was taken into a new tube, 40 µL of TE buffer and 5 µL of RNase enzyme (10 mg/mL) were added and incubated at 37 ºC for 10 minutes. At the end of the incubation, 100 µL of chloroform was added to the tubes and centrifuged at 13 000 rpm for 5 minutes at 4 ºC, the upper phase containing pure DNA was transferred to a clean tube and stored at -20 ºC for use in future studies. DNA purity was checked by running 1% agarose gel electrophoresis at 80 V, 100 mA for 60 minutes.

The PCR primers used to amplify the 16S rRNA gene were bacterial forward primers 5′-AGAGTTTGATCCTGGCTCAG-3′, and reverse primers Bac 1493r′ 5′-ACGGCTACCTTGTTACGACTT-3′.

Table 1. The protocol of polimerase chain reaction

Content of reaction	First concentration	Volume in Reaction	Last concentration
TaqDNA polymerase 2x master mix	2X	25 µL	1X
Forward primer	1 µM	2.5 µL	0.05 µM
Reverse primer	1 µM	2.5 µL	0.05 µM
DNA	-	2.5 µL	-
Sterile double distilled water	-	17.5 µL	-
Total reaction volume	-	50 µL	-

Table 2. The protocol of thermalcycler

Temperature	Time	Cycle
98 ºC	3 min	1
98 ºC	10 sec	30
61 ºC	30 sec	30
72 ºC	30 sec	30
72 ºC	5 min	1
4 ºC	-	∞

The PCR products of the species were sequenced using the Sanger sequencing method and the base sequences were submitted to the NCBI database.

Two and three dimensional imaging of strain

A previously described technique by Kesbiç and Gültepe [11] was used to prepare the strains for scanning electron microscope (SEM) and atomic force microscope (AFM) imaging. Fresh cultures of the halophilic bacteria strains were transferred from the agar surface to the coverslip surface after being cultured at 37 °C for three days. The coverslips were treated overnight in a 2% glutaraldehyde solution after being allowed to dry by air. After allowing the coverslips to air dry, they were treated overnight in a 2% glutaraldehyde solution. To completely remove the water, the preparations were taken from the solution and immersed in an acetone series of 30, 50, 70, and 90% for 10 minutes and 30 minutes, respectively. The preparations were coated with Au-Pd using the Cressington Sputter Coater at 40 mA for 60 seconds before being immediately placed on the FEI Quanta FEG 250 SEM (running at 10 kV). For three-dimensional imaging, the same preparation procedure was employed in Bruker's Edge3 AFM.

Determination of pigment characteristic of strain

The cultures were cultivated at 37 ºC for three days before being centrifuged at 9000 rpm for 10 minutes to yield cell pellets. The pellets were treated with 2 mL of methanol before being sonicated in an ultrasonic bath for 60 minutes and centrifuged. The extract was obtained using a two-stage extraction technique and scanned between 300 and 600 nm in a UV–Vis spectrophotometer (BioTek-Epoch 2) [12].

3. Results

The biochemical characteristics of strain CT7 were listed in Table 1. The strain has rod shape and pale yellow pigmentation. It has been determined that strain can grow in salt-free environment and also at high salt concentration up to 20% salinity. In addition, it has been determined that the temperature tolerance is on a very wide scale. According to the enzyme hydrolysis test results, it was observed that it has gelatinase enzyme but not caseinase, esterase and amylase enzymes.

Table 3. Biochemical test results of *H. trueperi* CT7

	Halobacillus trueperi CT7
Colony color	Pale yellow
Gram stain	+
NaCl tolerance (%)	0-20
pH tolerance	6-9
Temperature tolerance (˚C)	10-40
Oxidase	+
Catalase	+
Indole	-
Gas from Nitrate	-
Nitrate to Nitrite	-
Nitrate	-
Glucose	+
Lactose	+
Maltose	+
Fructose	+
Mannitol	+
Trehalose	+
Galactose	+
Sucrose	+
Gelatin Hydrolysis	+
Starch Hydrolysis	-
Casein Hydrolysis	-
Tween 80 Hydrolysis	-

The sequences of *H. trueperi* CT7 were analyzed by using NCBI Blast, aligned with other reported gene sequences and was submitted to the NCBI database (GenBank) under accession number OR352511. A phylogenetic tree was reconstructed by the neighbor-joining method with MEGA11 software (Figure 1).

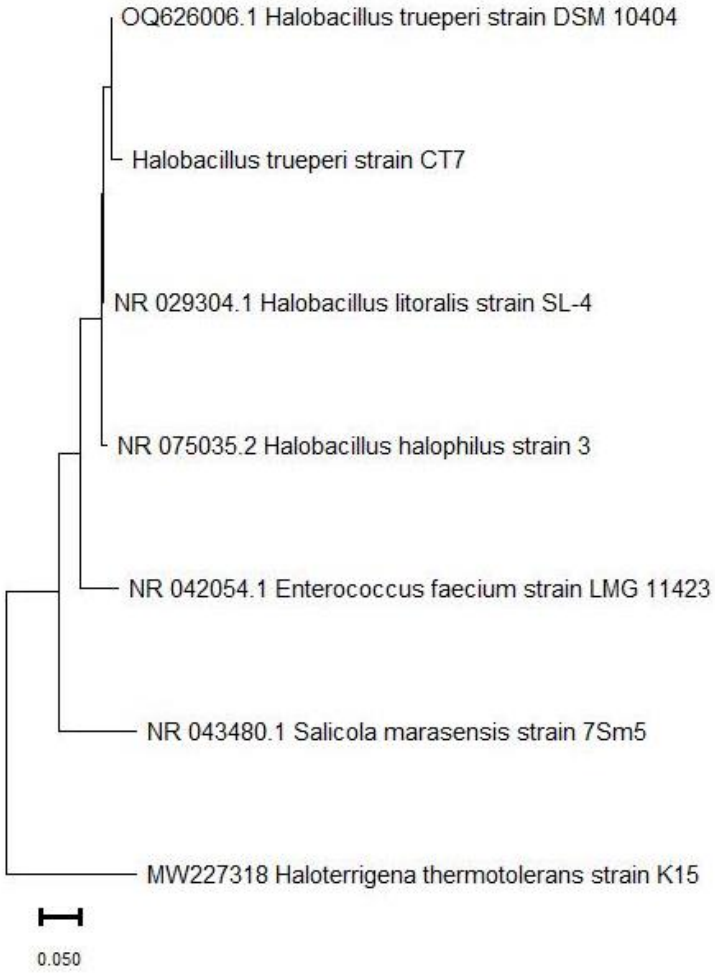

Figure 1. Phylogenetic tree of *Halobacillus trueperi* strain CT7 and its close relatives. *Haloterrigena thermotolerans* was used as an outgroup.

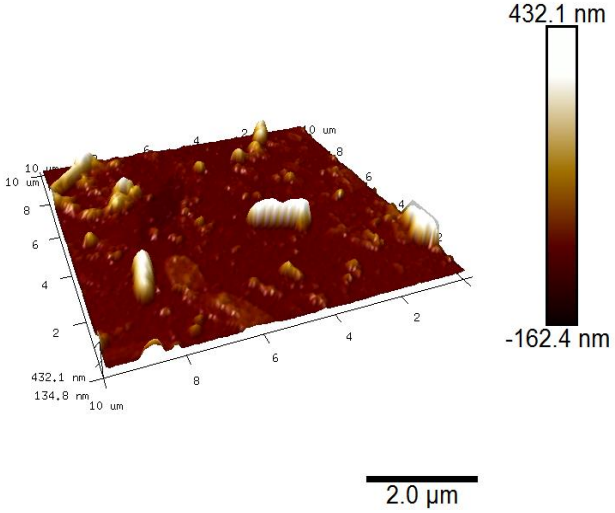

Figure 2. AFM image of *H. trueperi* CT7

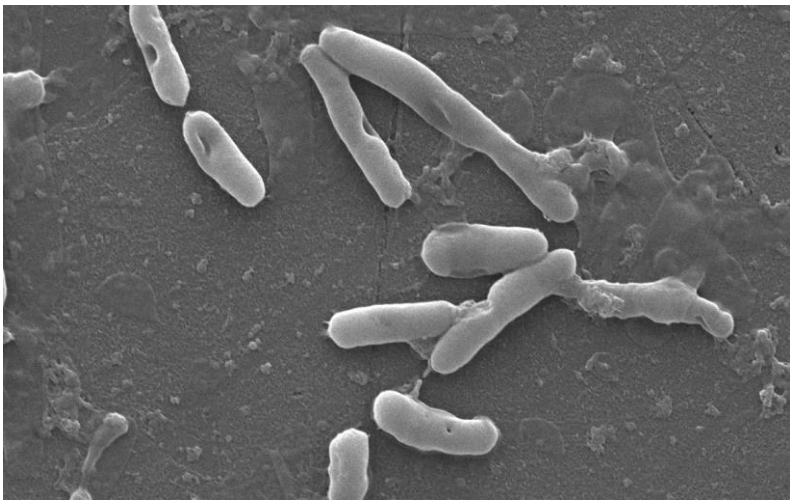

Figure 3. SEM image of spore-forming cells of *H. trueperi* CT7

The spectral scanning of *H. trueperi* extract are presented in Figure 4. According to the spectrum, maximum absorbances have been seen at 400, 425, 450, and 480 nm.

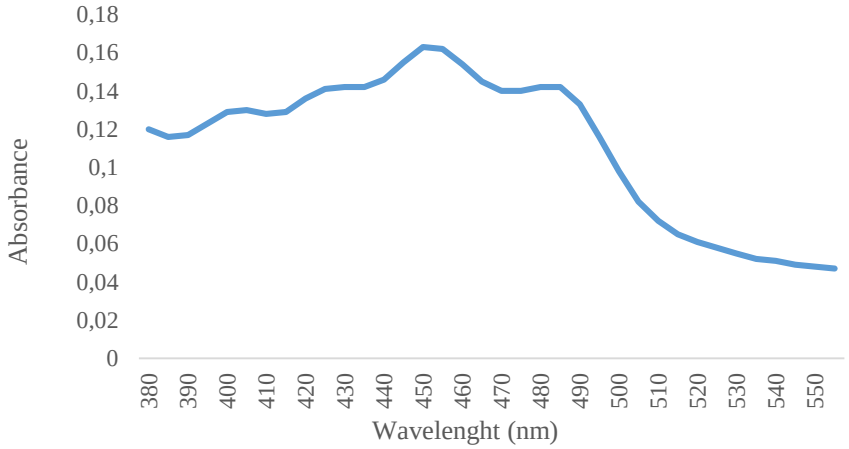

Figure 4. The spectral scanning of *H. trueperi* extract.

4. Discussion and Conclusion

It is known that *Halobacillus trueperi*, a halophilic bacterium, was first isolated from the Great Salt Lake in Utah, USA, and recorded as a new species. In the study, cells were observed with a phase contrast microscope and it was stated that the species was an endospore-forming bacterium [13]. However, in our study, *Halobacillus trueperi* species was examined for the first time with advanced technology microscopes such as SEM and AFM, and the endospore structure was detected as a hole in the SEM image in Figure 2 and the AFM image in Figure 3. Although the endospore structure is not seen in most bacteria, it is formed in some bacteria to provide resistance against adverse external conditions. Considering that the salt tolerance of *Halobacillus trueperi* is between 0-20%, it can be said that endospore structures were formed to protect itself from high salt concentrations. The nitrate production, starch hydrolysis, tween 80 hydrolysis and casein hydrolysis were determined as negative while the catalase, oxidase and gelatin hydrolysis test results were determined as positive in our research and these findings were shown similarity with the results of the research of Spring et. al. [13].

It was observed that the obtained bacterial extract obtained had a yellow-like color as well as the color of the colony. As a result of UV-VIS spectrophotometric scanning, peaks at 400, 425, 450 and 480 nm wavelengths were remarkable. The wavelengths of pigments of *Staphylococcus aureus* colonies, which were determined as yellow-orange or golden in a previous study, are similar to these wavelengths: Neo-4,4'-diaponeurosporene C, 430, 405, 428, 456 nm wavelengths, cis-4,4'-Diaponeurosporenoic acid at wavelengths of 453, 481 nm and Zeta carotene-like pigments at wavelengths of 400, 421, 452 nm [14]. It is thought that these wavelengths obtained in our study are similar to those of *Staphylococcus aureus*. It is possible to make pigment characterization with advanced chromatographic methods in future studies.

Halophilic microorganisms are a newly discovered group compared to non-extreme ones. For this reason, they continue to attract attention day by day thanks to their interesting features. There are already many patents on the use of halophilic microorganisms in medical and industrial processes [15-20]. Their enzymes, pigments and many secondary

metabolites that have not yet been identified are very promising. The wide temperature and salt tolerance range of *Halobacillus trueperi*, which was isolated and identified in our study, shows its potential to be an important brandy for industrial processes. In addition, the fact that the species has gelatinase enzyme indicates that it can be a source for gelatinase enzyme used in medicine and photography sectors [21]. With further studies, quantitative analyzes and enzyme purification of this enzyme can be performed. In addition, it is thought that it will be beneficial to shed light on the secondary metabolites of the species with advanced chromatographic methods.

Conflict of Interest

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Ethics Committee Approval

Ethics committee approval is not required.

Author Contribution

Conceptization: FIK, NG; methodology and laboratory analyzes: FIK; writing draft: FIK, NG; proof reading and editing: FIK, NG, NT. Other: All authors have read and agreed to the published version of manuscript.

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