

# Characterization of polyextremotolerant bacterial strains (*Kocuria*, *Virgibacillus*, and *Halomonas*) with industrial potential isolated from the İzmir Çamaltı Saltern

## İzmir Çamaltı Tuzlası'ndan izole edilen endüstriyel potansiyele sahip poliektremotolerant bakteri suşlarının (*Kocuria*, *Virgibacillus* ve *Halomonas*) karakterizasyonu

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**Abstract:** In this study, industrially important halotolerant bacterial strains were isolated and identified from seawater and bulk samples from the İzmir Çamaltı Saltern. Three selected isolates underwent comprehensive analyses including phenotypic, biochemical, and partial 16S rRNA gene sequencing and accession numbers were taken from GenBank. Species specific trees were constructed using MEGA 6.06 software. The isolates were classified within the phyla Firmicutes, Actinobacteria, and Gammaproteobacteria, exhibiting highest sequence similarity to the genera *Virgibacillus* (95%), *Kocuria* (99%), and *Halomonas* (99%), respectively. The strains were designated as *Virgibacillus* sp. CT-5 (KP238666), *Kocuria* sp. CT-6 (KP238667), and *Halomonas* sp. CS-5 (KP238669). Phenotypic characterization revealed that *Virgibacillus* sp. CT-5 is thermotolerant, growing at 20–55 °C; *Kocuria* sp. CT-6 is psychrotolerant, growing at 4–37 °C; and *Halomonas* sp. CS-5 exhibits both psychrotolerance and thermotolerance, growing at 4–45 °C. All strains were alkalitolerant, thriving within a pH range of 5–11, and halotolerant, with growth observed at 0–22% (w/v) NaCl for *Virgibacillus* sp. CT-5, 0–19% (w/v) for *Kocuria* sp. CT-6, and 0–25% (w/v) for *Halomonas* sp. CS-5. This study presents three polyextremotolerant bacterial strains -*Kocuria* sp. CT-6, *Virgibacillus* sp. CT-5, and *Halomonas* sp. CS-5- isolated from the İzmir Çamaltı Saltern. Their multiple tolerance traits render them highly promising candidates for various industrial applications. This is the first report documenting the polyextremotolerance of these strains from this area.

**Keywords:** Polyextremotolerant, halotolerant, alkalitolerant, thermotolerant, *Virgibacillus* sp., *Halomonas* sp., *Kocuria* sp.

**Öz:** Bu çalışmada, endüstriyel açıdan önemli halotolerant bakteri suşlarının İzmir Çamaltı Tuzlası'ndan alınan deniz suyu ve yığın örneklerinden izolasyonu, saflaştırılması ve tanılanması gerçekleştirilmiştir. Seçilen üç izolat, fenotipik, biyokimyasal ve kısmi 16S rRNA dizi analizleri ile kapsamlı şekilde incelenmiş ve türler erişim numaraları alınarak GenBank'a kayıt edilmiştir. Türe özgü filogenetik ağaçlar MEGA6.06 programı kullanılarak oluşturulmuştur. Suşların sırasıyla Firmicutes, Actinobacteria ve Gammaproteobacteria filumlarına; %95 benzerlik ile *Virgibacillus*, %99 benzerlik ile *Kocuria* ve %99 benzerlik ile *Halomonas* cinslerine ait olduğu belirlenmiştir. Suşlar *Kocuria* sp. CT-6 (KP238667), *Virgibacillus* sp. CT-5 (KP238666) ve *Halomonas* sp. CS-5 (KP238669) olarak adlandırılmıştır. Fenotipik karakterizasyona göre *Virgibacillus* sp. CT-5 termotolerant olup 20-55 °C aralığında büyüyebilmektedir; *Kocuria* sp. CT-6 psikrotolerant olup 4-37 °C aralığında yaşamaktadır; *Halomonas* sp. CS-5 ise hem psikrotolerant hem termotolerant olup 4-45 °C aralığında gelişim göstermektedir. Tüm suşlar alkalitolerant olup pH 5-11 aralığında büyüyebilmekte; halotolerant olup sırasıyla *Virgibacillus* sp. CT-5 için %0-22 (w/v), *Kocuria* sp. CT-6 için %0-19 (w/v) ve *Halomonas* sp. CS-5 için %0-25 (w/v) NaCl konsantrasyonlarında gelişim gösterebilmektedir. Bu çalışmada, İzmir Çamaltı Tuzlası'ndan izole edilen üç poliektremotolerant bakteri suşu- *Kocuria* sp. CT-6, *Virgibacillus* sp. CT-5 ve *Halomonas* sp. CS-5- bildirilmiştir. Bu suşlar, birden fazla tolerans özelliğine sahip olup, endüstriyel uygulamalar için büyük bir öneme sahiptir. Bu çalışma, bu bölgeden poliektremotolerant suşların rapor edildiği ilk çalışmadır.

**Anahtar kelimeler:** Poliektremotolerant, halotolerant, alkalitolerant, termotolerant, *Virgibacillus* sp., *Halomonas* sp., *Kocuria* sp.

## INTRODUCTION

Industrially important microbial strains have gained significant attention due to their capacity to produce valuable bioproducts such as marine-derived compatible solutes, hydrolytic enzymes, anticancer compounds, and antiviral agents (Kazak Sarılmiser et al., 2015). Salt-adapted biomolecules, particularly enzymes, offer substantial advantages in industrial applications where conventional homologs typically lose activity or stability in high salt (Moreno et al., 2013).

The halophilic species are generally classified as non-halophiles, slightly halophiles, moderately halophiles and extreme halophiles (Christian and Waltho, 1962). The first extreme halotolerant species submitted to literature was

*Halomonas elongata*. The term halotolerant was suggested as the fifth group of halophilism as organisms living in both at NaCl and non-NaCl medium (Vreeland et al., 1984). The term halophilism were reclassified with their revised optimum NaCl % (Kushner, 1992).

The halotolerant and halophilic bacteria are very common worldwide with their important properties on several biotechnologies. Studies on halophilic and halotolerant species in Türkiye are still in their infancy. One of the most important and saline environment in Türkiye is Çamaltı Saltern as the biggest solar saltern in Türkiye, and the habitats of halophilic communities (Koru, 2004). In the literature, despite its biotechnological and ecological potentials, researches on

halotolerant bacteria from the Çamaltı Saltern has been relatively limited to date.

Over the past two decades, extensive microbiological investigations have been conducted to explore the bacterial and haloarchaeal diversity of the Çamaltı Saltern (Yaşa et al., 2008). Despite the vast size of this hypersaline ecosystem, the number of studies remains limited, making it difficult to comprehensively monitor the microbial community structure. Independent studies conducted in various parts of the saltern have reported the occurrence of phylogenetically distinct microbial species. Molecular analyses have revealed the presence of diverse clusters within the families Halomonadaceae, Streptomycetaceae, and Bacillaceae. *Halomonas* sp. AAD12 (Ceylan et al., 2012), *Halomonas* sp. AAD21 (Uzyol et al., 2012), and *Halomonas smyrnensis* AAD6<sup>T</sup> (Poli et al., 2013) has been reported to date. Additionally, *Streptomyces smyrmæus* and *Streptomyces iconiensis* are the only halotolerant Actinobacteria identified from the Çamaltı Saltern to date (Tatar et al., 2014). Recent phylogenetic screening has led to the identification of new strains, including *Halomonas* sp. 110Y (KP795378.1), *Halomonas* sp. 16Y (KP795386.1), *Halomonas* sp. K15

(KP795384.1), *Virgibacillus* sp. C15 (KF863789.1), and a *Halobacillus* species (Mutlu and Güven, 2015). Some industrially important products, especially levan (Kazak Sarilmiser et al., 2015) and amylase (Uzyol et al., 2012) production have been reported from this region. Biodiversity studies in the Çamaltı Saltern are still in their early stages, and the diversity of halophilic strains remains largely unexplored. This study aims to isolate, purify, and identify industrially important bacterial strains from the Çamaltı Saltern, İzmir, Türkiye.

## MATERIALS AND METHODS

### Isolation source

Saline samples were collected from the saltwater pond (coordinates: 38°30'23" N, 26°56'15" E) and from salt bulk deposit (coordinates: 38°29'17"N, 26°54'33"E) in the İzmir Çamaltı Saltern on November 2, 2011 (Figure 1). The salinity of the pond water was measured on-site by using a floating glass hydrometer calibrated in degrees Baumé pH values were measured (Mettler Toledo) in the laboratory. Salt samples were collected in sterile 50 mL plastic tubes and transported to the laboratory within three hours, where they were stored at 4 °C (Tekin, 2015).

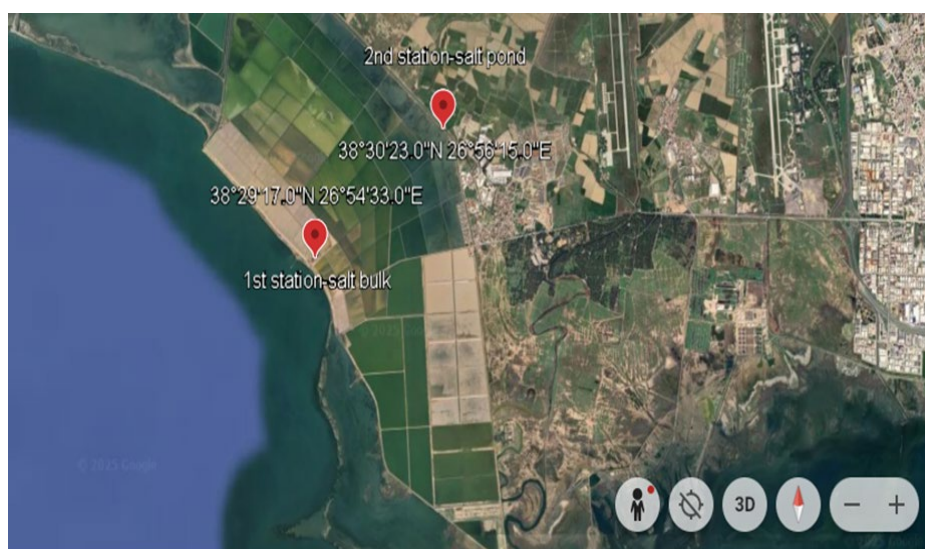


Figure 1. Satellite image of İzmir Çamaltı Saltern at the moment of 2<sup>nd</sup> November 2011 (Source: Google Earth)

### The isolation and purification of halotolerant bacteria

Halotolerant bacteria were selectively isolated in 17.8% (w/v) NaCl containing Halophilic Medium (HM) broth and HM agar media at 30 ±1 °C for 3 days (Ventosa et al., 1982). Isolation was followed by purification using serial dilution. Each purification step was confirmed by Gram staining. Three isolates named CT-5 and CT-6 (from salt bulk samples) and CS-5 (from salty water samples) were selected for further identification.

### Growth tests

Moderately Halophilic (MH) medium was prepared for growth tests. The basal formulation contained (% w/v)

proteose peptone No.3, 0.5 g; yeast extract, 1 g; glucose, 0.1 g; and a defined artificial sea salt mixture (10%) consisting of NaCl, 8.1 g; MgCl<sub>2</sub>, 0.7 g; MgSO<sub>4</sub>, 0.96 g; CaCl<sub>2</sub>, 0.036 g; KCl, 0.2 g; NaHCO<sub>3</sub>, 0.006 g; and NaBr, 0.0026 g with the pH adjusted to 7.2. Solid medium was prepared by the addition of 1.5% (w/v) agar. To determine tolerance to total sea salts, MH agar was prepared with concentrations ranging from 0.5% to 30% (w/v), with the amount of each salt adjusted proportionally to the 10% formulation above. Plates were incubated at 37 °C for 24 h. To assess halotolerance to NaCl specifically, Nutrient Agar plates containing 0-25% (w/v) NaCl (pH 7.5) were prepared and incubated at 37 °C for 5 days. pH tolerance was evaluated on Nutrient Agar with (5%, w/v) NaCl and adjusted to pH values between 5.0 and 11.0 (±0.1) using 1 M HCl or 1

M NaOH; these plates were incubated at 37 °C for 3 days (Ventosa et al., 1982).

#### Differential biochemical tests

For biochemical characterization, the MH-5 medium (Moderately Halophilic Medium containing (5%, w/v) total sea salts, as described and formulated above was used. The composition of MH-5 (5%, w/v) was as follows: proteose peptone, 0.5 g; yeast extract, 0.5 g; glucose, 0.1 g; NaCl, 4.05 g; MgSO<sub>4</sub>, 0.48 g; MgCl<sub>2</sub>, 0.35 g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.018 g; KCl, 0.1 g; NaHCO<sub>3</sub>, 0.003 g; and NaBr, 0.0013 g with the pH adjusted to 7.2. MH-5 was prepared in both broth and agar forms for the respective assays, and a carbon- and nitrogen-free salt solution was also prepared when required. Inoculated cultures were incubated at 37 °C for 24 h prior to testing (Ventosa et al., 1982).

#### Catalase test

Catalase activity was tested by spotting 15 µL actively growing cultures onto MH-5 agar followed by incubation at 37 °C for 24 h. After incubation, three percent (3%, v/v) hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was added to each spot (in triplicate). The formation of bubbles were interpreted as a positive result, while absence of bubble formation indicated a negative result (Ventosa et al., 1982).

#### Oxidase test

Fifteen microliters (15 µL) of each culture were applied to MH-5 agar plates (without glucose and nitrate), and were incubated at 37 °C for 24 h. The oxidase reagent consisted of (0.5%, w/v) N,N,N',N'-tetramethyl-p-phenylenediamine (Sigma) and (0.1%, w/v) ascorbic acid (Merck). The appearance of a purple color was interpreted as a positive result, while the absence of color change indicated a negative result. *Bacillus cereus* and *Staphylococcus aureus* was used as the positive control, and negative control, respectively (Kovács, 1956).

#### Nitrate reduction

Nitrate reduction tests were performed by replacing KCl with (0.2%, w/v) KNO<sub>3</sub> in the MH-5 broth. Two percent (2%, v/v) of actively growing cultures were inoculated into the nitrate-containing broth, which included inverted durham tubes for gas detection. To differentiate between aerobic and anaerobic conditions, one tube was left open to air, and the other was overlaid with 1 mL of sterile mineral oil and incubated at 37 °C for 5 days. After incubation, 5 drops of each of freshly prepared α-naphthylamine and sulphanilic acid reagents were added for color development. A dark red or red color indicated a positive result, while a light cream color indicated a negative result. Uninoculated medium served as negative control (Ventosa et al., 1982).

#### H<sub>2</sub>S production

Triple Sugar Iron (TSI) agar with MH-5 total sea salts was used for testing hydrogen sulfide (H<sub>2</sub>S) production. The

formation of a black precipitate in the butt of the tube indicated the reaction between H<sub>2</sub>S gas produced by the organism and ferrous ions in the medium, leading to the formation of insoluble ferrous sulfide (FeS), while its absence indicated as a negative result (Kim et al., 2007).

#### Extracellular protease enzyme activity

Extracellular protease enzyme activities were screened on plates containing (3%, w/v) skimmed milk solution (pH 7.5) and 1.5% agar. The milk solution was autoclaved separately at 120 °C for 5 minutes and stored at 65 °C, while the MH-5 total sea salts containing agar supplemented with 0.5% yeast extract was sterilized at 121 °C for 15 min. The mixture was allowed to cool, then poured into petri dishes. Active cultures (15 µL) were spotted to the plates, and were cultured at 37 °C for 48 h. The presence of a clear zone indicated positive protease, while absence of zone formation indicated a negative result (Romano et al., 2005).

#### Amylase activity

Amylase activity were screened using MH-5 total sea salts with (1%, w/v) starch, 0.2% yeast extract and 1.5% agar. Twenty microliters of cultures were cultured at 37 °C for 72 h. Following incubation, diluted Gram's iodine solution (1:10 in water) was applied to the plates to detect clear zones around the colonies, which indicated amylase activity. The absence of zone formation indicated negative result. *B. cereus* and *S. aureus* were used as positive and negative control, respectively (Cojoc et al., 2009).

#### DNase activity

DNase activity was performed using DNase agar (Merck) containing 2% tryptose, 0.5% NaCl, 0.2% DNA, 1.5% agar, and adjusted to (4.2%, w/v). The medium was modified according to Kamble and Kadu (2012) by supplementing with 0.2% yeast extract, 0.1% peptone, and MH-5 total sea salts (pH 7.4). DNase enzyme activity was assessed using media with and without 0.005% methyl green to evaluate the effect of the dye. Triplicate plates were incubated at 37 °C for 72 h and subsequently the colonies were overlaid with either methyl green, 1 N HCl, or 0.05% toluidine blue. In methyl green supplemented plates, the appearance of yellow zones around the colonies was interpreted as a positive reaction. For plates to which toluidine blue was added after incubation, the presence of clear zones indicated a positive reaction, while the absence of zones was considered as negative result. In media without methyl green, 1 N HCl was applied to detect hydrolysis. Due to the inhibitory effect of methyl green on certain strains, final interpretations were preferentially made using the HCl method (Jeffries et al., 1957).

#### Decarboxylase tests: lysine, arginine and ornithine

Decarboxylase activities, including lysine, arginine, and ornithine decarboxylation, were assessed using the respective decarboxylase broths (Merck) at pH 6.8, supplemented with MH-5 total sea salts. Active cultures (2%, v/v) were inoculated

into the broths, and 1 mL of sterile mineral oil was added to create anaerobic conditions. Plates were incubated at 37 °C, and color changes were monitored daily for up to one week, with final results evaluated at the end of the incubation period. Acidic products resulting from carbon source utilization initially turned the medium yellow. If lysine decarboxylation occurred after carbon consumption, alkaline products were generated, turning the color of the medium to purple-red, which was interpreted as a positive result. Yellow coloration or absence of color change was considered as negative result (Brooker et al., 1973; Fay and Barry, 1972; Goldschmidt and Lockhart, 1971).

#### Phenylalanine deaminase activity

Phenylalanine deaminase activity was evaluated in Nutrient Broth (Difco) supplemented with 0.2% (w/v) DL-phenylalanine and MH-5 total sea salts (pH 6.8). Following incubation at 37 °C for one week, 10% (w/v) ferric chloride and five drops of 0.1 N HCl were added. Formation of a green color after 5 min indicated a positive reaction, whereas absence of color change was considered negative (Ederer et al., 1971).

#### Acid and gas production

Acid and gas production from sole carbon and nitrogen sources was evaluated with minor modifications, including the addition of filter-sterilized thiamine (0.0005 mg/mL, w/v) (Romano et al., 2005). Carbon and nitrogen sources were sterilized by syringe filtration (0.45 µm) and added at (1%, v/v) to the respective media. The tested carbon sources included D-melezitose, L-arabinose, D-fructose, D-galactose, Bactoinositol, D-mannitol, D-ribose, D-trehalose, D-salicin, maltose, lactose, D-sorbitol, melibiose, D-raffinose, glycerol, sucrose, citrate, D-glucose, D-mannose, D-cellobiose, and arbutin. The tested nitrogen sources included L-isoleucine, L-serine, L-valine, L-methionine, L-lysine, L-threonine, L-arginine, L-cysteine, and DL-asparagine.

Marine Broth medium was prepared by dissolving 7.5 g NaCl, 0.2 g KCl, 0.02 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g KNO<sub>3</sub>, 0.03 g bromothymol blue, 0.1 g (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.05 g KH<sub>2</sub>PO<sub>4</sub>, and 50 µL of thiamine solution (10 mg/100 mL) in distilled water, supplemented with (1%, v/v) of the test carbon source, and adjusted to a final volume of 100 mL at pH 7.0. Similarly, the nitrogen source test medium contained the same basal composition (7.5 g NaCl, 0.2 g KCl, 0.02 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.03 g bromothymol blue, and 0.05 g KH<sub>2</sub>PO<sub>4</sub>) supplemented with 0.1% of the test nitrogen source, and adjusted to 100 mL with distilled water at pH 7.0 (Ventosa et al., 1982).

Active cultures were grown in 25 mL Marine Broth at 37 °C for 24 h, centrifuged, washed with basal medium, and adjusted to a (2%, v/v) inoculum. Cultures were subsequently monitored at 37 °C for 120 h to record growth (Ventosa et al., 1982).

#### Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed using the Kirby-Bauer disc diffusion method on MH-5 agar (Bauer et al.,

1966). Briefly, 100 µL of bacterial culture adjusted to a turbidity equivalent to 0.5 McFarland standard was spread uniformly onto the surface of the agar plates and commercial antibiotic discs were placed on the inoculated plates. The antibiotics tested in this study were Ampicillin 10 µg (A10), Erythromycin 15 µg (E15), Streptomycin 10 µg (S10) (BBL), Penicillin G 10 µg (P10) (BBL), Bacitracin 0.04 U (B), Tetracycline 30 µg (TE30), and Novobiocin 5 U (N5) (Ventosa et al., 1982). Plates were incubated at 37 °C for 24 h, and inhibition zone diameters were measured in millimeters. The results were interpreted as susceptible or resistant according to the Clinical and Laboratory Standards Institute (CLSI, 2015).

#### Pigment production test

Pigment production from tyrosine was evaluated on MH-5 agar with (0.5%, w/v) tyrosine. Active growing cultures were spotted to agar plates and the spotted cultures were incubated at 37 °C for 24 h. Clear zone and pigment production indicated a positive result, while the absence of zone formation and pigment production indicated a negative result. (Ventosa et al., 1982).

#### Genetic analyses

Genomic DNA Extraction Kit (Invitrogen) was used for extracting Genomic DNA's of each strains. For the amplification of the 16S rRNA genes I-star taq™ DNA polymerase was used with PCR reactions for each strain prepared by combining 20 µL of apyrogenic ultra-pure water, 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), 5 µL of 10X PCR buffer with 20 mM MgCl<sub>2</sub>, 5 µL of 10 mM dNTP mix (2.5 mM of each nucleotide), 0.5 µL of i-star taq™ polymerase (5 U/µL). Varying amounts of template DNA (1 ng to 1 µg) was adjusted to final volume for each strain. This mixture was adjusted with nuclease free-ultra-pure water to a final volume of 50 µL. The primer pairs were 27F (5'-AGAGTTTGTATCCTGGCTCAG-3') and 1542R (5'-AAGGAGGTGATCCAGCCGCA-3') (Weisburg et al., 1991). Gene amplification program was as follows: denaturation at 94 °C for 2 min (1 cycle), followed by 30 cycles of denaturation at 94 °C for 20 sec, annealing at 59 °C for 20 sec, and extension at 72 °C for 1 min. Final extension was also performed at 72 °C for 5 min (1 cycle) according to the manufacturer's instructions. Storage temperature of amplified products was at 4 °C. ABI 3130XL 16-Capillary System (Applied Biosystems) was used for sequencing the amplified products at the BIYOMER Research Center in İzmir Institute of Technology. The obtained 16S rRNA gene sequences were analyzed by using the NCBI BLAST tool to compare the strains according to the sequences available in the GenBank database. Strain identification was performed based on genus-level classification. The obtained 16S rRNA gene sequences were submitted to GenBank, and the accession numbers were provided (Altschul et al., 1990).

#### Phylogenetic analyses

Phylogenetic trees were constructed by comparing the 16S rRNA gene sequences of the strains with those of the most

similar species retrieved from the NCBI GenBank database (Stackebrandt and Goebel, 1994). Multiple sequence alignments were conducted by using the Clustal W program (Larkin et al., 2007). Maximum Likelihood method was used in order to make phylogenetic tree specific for our strains and most similar ones with bootstrap analysis conducted over 1000 replications to assess tree reliability (Tamura and Nei, 1993; Tamura et al., 2004). The initial tree was carried out using Neighbor Joining method (Saitou and Nei, 1987). Lengths of branches illustrated the substitutions per site on scale, and bootstrap values (%) were shown next to the branches in Figure 2. Less than 95% site coverages were eliminated by using MEGA 6.06, final dataset comprised 1370 positions. Phylogenetic analyses were carried out using MEGA 6.06 (Tamura et al., 2013).

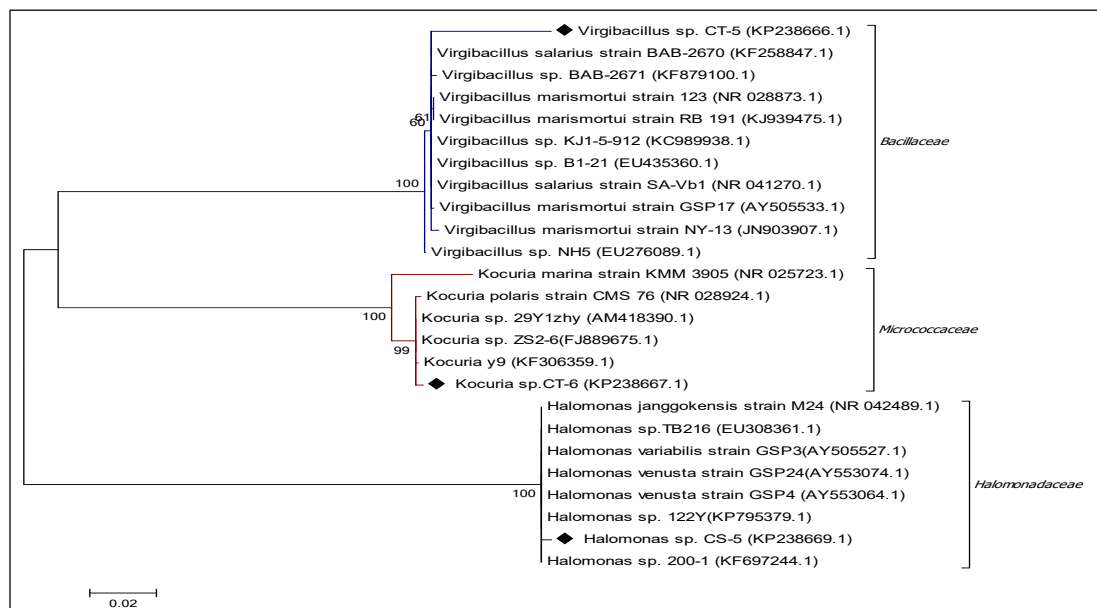
## RESULTS

### Selected and identified strains *Halomonas* sp. CS-5, *Virgibacillus* sp. CT-5 and *Kocuria* sp. CT-6

The pH of the salt samples was measured at 7.2. The salinity result was 26.5° Baumé degree. The bacterial load of the salty water from the İzmir Çamaltı Saltern was  $2.3 \times 10^7$  cfu/mL, whereas the bacterial count of the salt bulk was  $3.0 \times 10^2$  cfu/mL. The comparisons of the sequences of the 16S rRNA genes were given on Table 1. The phylogenetic positions illustrated in Figure 2. Strains were named based on the most closely related genera identified through NCBI Blast analysis of their 16S rRNA gene sequences. Accordingly, the three isolates were identified as *Virgibacillus* sp. CT-5, *Kocuria* sp. CT-6 and *Halomonas* sp. CS-5.

**Table 1.** The results of the NCBI Blast search

Strain name	16S rRNA gene	Accession Number	Similarity	Maximum Similarity
<i>Virgibacillus</i> sp. CT-5	1533 bp	KP238666	95 %	<i>Virgibacillus</i> sp. B1-21 ( <i>V. marismortui</i> ) (EU435360.1)
<i>Kocuria</i> sp. CT-6	1472 bp	KP238667	99 %	<i>Kocuria</i> sp. ZS2-6 (FJ889675.1)
<i>Halomonas</i> sp. CS-5	1469 bp	KP23866	99 %	<i>Halomonas</i> sp. TB216 ( <i>H. janggokensis</i> ) (EU308361.1)



**Figure 2.** Phylogenetic tree based on 16S rRNA gene sequences, constructed using the Maximum Likelihood method (Tamura and Nei, 1993) in MEGA 6.06 software (Tamura et al., 2013). Bootstrap values (%) (based on 1000 replications) are indicated at the branching points; only values greater than 50% are shown. The scale bar represents 2 nucleotide substitutions per 100 nucleotide positions

*Virgibacillus* sp. CT-5 is a cream-pigmented, Gram-positive bacillus growing in NaCl concentrations ranging from 0% to 22% (w/v), at temperatures between 20°C and 55°C, and pH between 5–11. *Kocuria* sp. CT-6 is a red-pigmented, Gram-positive coccus, predominantly forming tetrads, that grows in 0% to 19% (w/v) NaCl, between 4°C and 37°C, and at pH values ranging from 5 to 11. *Halomonas* sp. CS-5 is a yellow-pigmented, Gram-negative, short diplobacillus that grows in NaCl concentrations from 0% to 25% (w/v), at temperatures between 4°C and 45°C, and within a pH range of 5–11. In

summary, *Virgibacillus* sp. CT-5 exhibits thermotolerance, alkalitolerance, and halotolerance; *Kocuria* sp. CT-6 is psychrotolerant, alkalitolerant, and halotolerant; and *Halomonas* sp. CS-5 displays psychrotolerance, thermotolerance, alkalitolerance, and halotolerance.

The phenotypic, biochemical and antibiotic test results were shown in Table 2. The properties of these strains are given in the Table 2 with the comparisons of the most similar strains available on literature.

**Table 2.** Some phenotypic and biochemical test results of the strains.1. *Virgibacillus* sp. CT-5; 2. *Virgibacillus marismotui* (Arahal et al., 1999) 3. *Virgibacillus* sp. B1-21 (Essghaier et al., 2009); 4. *Kocuria* sp. CT-6; 5. *K. marina* KMM 3905<sup>T</sup> (Kim et al., 2004) 6. *Halomonas* sp. CS-5; 7. *H. janggokensis* strain M24<sup>T</sup> (*Halomonas* sp. TB216) (Tsiamis et al., 2008; Kim et al., 2007); 8. *H. smrynensis* AAD6<sup>T</sup> (Poli et al., 2013).

Characteristics	1	2	3	4	5	6	7	8
Cell shape	Rod	Rod	Rod	Coccal	Cocci	Small Rod	Rod	Rod
Colonial morphology	Irregular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Gram's reaction	+	+	+	+	+	-	-	-
Motility	+	+	+	-	-	+	+	-
Pigment production	Cream	Cream	Cream	Orange-Red	Red	Yellow	White	Dark yellow
Temperature range (°C)	20-55	15-50	30	4-37	4-43	4-45	5-45	5-40
pH tolerance	5-11	5-9	8	5-11	ND	5-11	6-10	5.5-8.5
Catalase	+	+	+	+	+	+	+	+
Oxidase	-	+	+	-	-	-	-	-
Caseinase	+-	+	-	-	+	-	-	+
Amylase	+	-	-	+	-	+	-	+
DNAse	+	+	+	+	-	+	+	-
Tyrosinase	-	-	-	-	-	-	-	+
Phenylalanine deaminase	-	-	-	-	-	-	-	-
Arginine decarboxylase	-	-	-	-	-	-	-	-
Lysine deaminase	-	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	-	-	-
Denitrification	-	-	-	-	-	-	-	-
H <sub>2</sub> S production	+	-	-	-	-	-	-	-
NaCl range (%)	0-22	5-25	5-15	0-19	0-15	0-25	1-20	3-25
<b>Acid and gas production from sole carbon source</b>								
Sucrose	-	-	-	+	-	-	+	+
Citrate	-	-	-	-	-	-	-	-
Glycerol	-	+	-	-	-	-	+	-
Arbutin	-	-	-	G	-	-	-	-
Fructose	-	+	-	-	-	-	+	-
Glucose	-	+	-	-	-	+	+	+
Mannitol	-	-	-	-	-	-	+	-
Trehalose	-	-	-	-	-	-	+	-
Salicin	-	-	-	+	-	-	-	-
Maltose	-	+	-	+	-	+	+	-
Lactose	-	-	-	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-	+	-
D-Cellobiose	-	-	-	-	-	-	-	-
D-Ribose	-	-	-	-	-	-	-	-
D-Galactose	-	-	-	-	-	-	+	-
D-Mannose	-	-	-	-	-	-	-	+
Bacto-inositol	-	-	-	G	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	+	-
Melibiose	-	-	-	-	-	-	-	-
D-Melezitose	-	-	-	-	-	-	-	-
D-Rafinose	-	-	-	-	-	-	-	-
<b>Growth on sole nitrogen sources</b>								
L-Methionine	-	-	-	-	-	-	-	-
DL-Asparagine	-	-	-	-	-	-	-	-
L-Serine	-	-	-	+	-	-	-	+
L-Isoleucine	-	-	-	-	+	-	-	-
L-Threonine	-	-	-	-	-	-	-	-
L-Cysteine	-	-	-	-	-	+	-	-
L-Arginine	-	-	-	+	-	+	-	-
L-Valine	+	-	-	-	-	+	-	-
L-Lysine	+	-	-	+	-	-	-	-
<b>Antibiotic susceptibility</b>								
Ampicillin 10 U	S	-	S	S	S	S	-	S
Bacitracin 0.04U	S	-	R	S	S	R	-	R
Tetracycline 30 U	S	S	-	S	S	R	-	-
Streptomycin 10 U	S	S	-	S	S	S	-	-
Erythromycin 15 U	S	S	S	R	-	R	-	S
Penicillin G 10 U	S	S	R	R	S	S	-	R
Novobiocin 5 U	S	R	-	S	-	-	-	R

+: Acid positive; -: Acid negative G: Gas positive (for acid and gas production test) R: Resistant for antibiotic susceptibility test; ND: Not determine

## DISCUSSION

### The comparison of *Virgibacillus* sp. CT-5 with the most similar strains

The partial 16 S rRNA gene (1533 bp) of our first strain *Virgibacillus* sp. CT-5 (KP238666) was compared with the maximum similar (95%) *Virgibacillus* sp. B1-21 (EU435360.1) (Essghaier et al., 2009). *Virgibacillus* sp. B1-21 grows at 5-15% (w/v) NaCl classified as moderately halophilic (Essghaier et al., 2009). Another similar strain is *Virgibacillus marismortui* 123<sup>T</sup>, isolated from the Dead Sea (Arahal et al., 1999). A comparison of the phenotypic, biochemical, and antibiotic resistance profiles of these three strains reveals distinct differences. *Virgibacillus* sp. CT-5 is halotolerant and susceptible to all antibiotics tested (Table 2). In contrast, both *Virgibacillus* sp. B1-21 and *Virgibacillus marismortui* 123<sup>T</sup> are moderately halophilic (Arahal et al., 1999). The antibiotic susceptibility test for *Virgibacillus* sp. B1-21 was available for ampicillin and erythromycin as sensitive, bacitracin and penicillin G as resistant (Essghaier et al., 2009). It was reported that *Virgibacillus marismortui* 123<sup>T</sup> was susceptible to tetracyclin, streptomycin, penicillin G and erythromycin but resistant to novobiocin, nalidixic acid, neomycin and rifampin (Arahal et al., 1999). These comparisons as given in Figure 2 and Table 2 apparently indicate that each strain is different from each other. Interestingly, there is only one phylogenetic report on a strain of *Virgibacillus* sp. C15 (KF863789.1), isolated from Çamaltı Saltern, which showed no phylogenetic similarities to the strains in this study (Mutlu and Güven, 2015). For species identification which was in the same genus based on 16S rRNA gene sequence similarity using NCBI, a threshold of  $\geq 97\%$  was generally accepted to indicate the same species. Similarity below the 97% threshold-such as the 95% similarity observed for *Virgibacillus* sp. CT-5 (accession no. KP238666) suggests its potential as novel species in the genus *Virgibacillus* (Tindall et al., 2010). Both phenotypic characteristics and 16S rRNA gene sequence analysis indicate that *Virgibacillus* sp. CT-5 (accession no. KP238666) represents a novel strain. This study constitutes the first report of *Virgibacillus* sp. CT-5 (KP238666) isolated from the Çamaltı Saltern.

### Comparison of *Kocuria* sp. CT-6 with related species

Our second strain was *Kocuria* sp. CT-6 (Accession No: KP238667). The partial 16 S rRNA gene (1472 bp) was compared with the maximum similar (99%) *Kocuria* sp. ZS2-6 (FJ889675.1) isolated from Antarctic sandy intertidal sediments in China. Despite this high similarity, the considerable geographic separation supports the assumption that these strains are distinct. Moreover, since the available data for *Kocuria* sp. ZS2-6 is limited to phylogenetic information and lacks phenotypic and biochemical characterization (Yu et al., 2010), no direct comparison could be made. Instead, *Kocuria marina* KMM 3905<sup>T</sup> (Kim et al., 2004) was evaluated as the reference strain for comprehensive comparison based on phenotypic, biochemical, and phylogenetic properties (Figure 2 and Table 2). As a result, this study represents the

first report of *Kocuria* sp. CT-6 isolated from the Çamaltı Saltern.

### Comparison of *Halomonas* sp. CS-5 with related species

Our third strain was *Halomonas* sp. CS-5 (Accession No. KP238669). The partial 16S rRNA gene sequence (1469 bp) was compared with the maximum similar (99%) *Halomonas* sp. TB216 (EU308361.1), which was previously identified as *Halomonas janggokensis*, moderately halophilic that grows in 4–20% (w/v) NaCl (Tsiamis et al., 2008). In contrast, *Halomonas* sp. CS-5 is halotolerant, growing across a broader NaCl concentration range of 0–25% (w/v). Moreover, the reference strain *H. janggokensis* (AM229315.1), isolated from the Janggok solar saltern, was reported to grow between 1–20% (w/v) NaCl and produces white pigmentation (Kim et al., 2007), whereas *Halomonas* sp. CS-5 produces yellow pigmentation. Detailed comparisons based on phenotypic and biochemical characteristics also differentiate *Halomonas* sp. CS-5 from *Halomonas smymensis* AAD6<sup>T</sup> (Poli et al., 2013), as shown in Table 1. Minimal standards for the detection of novel species within the family Halomonadaceae (Arahal et al., 2007), *Halomonas* sp. CS-5 represents a novel species and is being reported here for the first time from the Çamaltı Saltern.

### The overall evaluation

In summary; *Virgibacillus* sp. CT-5 is characterized as thermotolerant, alkalitolerant, and halotolerant. *Kocuria* sp. CT-6 is psychrotolerant, alkalitolerant, and halotolerant. *Halomonas* sp. CS-5 displays psychrotolerance, thermotolerance, alkalitolerance, and halotolerance. These results support the classification of these isolates as polyextremotolerant bacteria and underscore their biotechnological potential, marking the first report of these strains from the Çamaltı Saltern. Nevertheless, 16S rRNA gene sequencing remains essential for achieving accurate genus-level classification, which cannot be determined based solely on phenotypic traits. The evaluation of extracellular enzyme production, nitrate reduction, hydrogen sulfide (H<sub>2</sub>S) production, and antibiotic susceptibility plays a crucial role in differentiating closely related strains. Since conducting a full panel of biochemical tests is time-consuming, it is recommended to prioritize antibiotic susceptibility testing following isolation and 16S rRNA sequencing, as it can be highly effective in differentiating similar strains. Moreover, NaCl tolerance, pH range, temperature tolerance, Gram staining, colony morphology, nitrate reduction, and H<sub>2</sub>S production tests have proven to be highly informative for halotolerant strains in this study. Among hydrolytic enzyme assays, several demonstrated strong discriminatory power between strains. For the overall evaluation of the strains; *Virgibacillus* sp. CT-5 as thermotolerant, alkalitolerant, and halotolerant; *Kocuria* sp. CT-6 as psychrotolerant, alkalitolerant, and halotolerant, and *Halomonas* sp. CS-5 displayed psychrotolerant, thermotolerant, alkalitolerant, and halotolerant which are proposed as polyextremotolerant species for the first time from

Çamaltı Saltern can be beneficial for further biotechnological studies.

### Industrial importance of *Virgibacillus*, *Kocuria*, and *Halomonas* species

Polyextremotolerant bacteria, such as *Virgibacillus*, *Kocuria*, and *Halomonas* obtained from this study possess adaptations that enable them to live and become active under extreme conditions such as high salinity, pH fluctuations, temperature extremes, and oxidative stress. These properties make them particularly valuable for industrial usage where conventional microorganisms can not survive or function. The most important industrial application is enzyme production. These bacteria produce enzymes, including proteases, amylases, DNases, and catalases, that retain activity under robust industrial production conditions. For instance, in the detergent industry, thermotolerant and halotolerant proteases and amylases can efficiently degrade stains in high-temperature, alkaline, or saline steps, enhancing cleaning performance and reducing chemical usage, which contributes significantly to environmental protection (Lam et al., 2018).

Another significant application lies in the food industry, where extremotolerant enzymes enhance both process efficiency and product quality. Proteases, in particular, have diverse applications such as meat tenderization, protein modification in dairy products, and cheese ripening, offering substantial time and cost advantages over conventional enzymes (Tavano et al., 2018). Amylases from these bacteria convert starches into glucose or maltodextrin in order to obtain sweetener products and fermentation processes (Soto-Padilla et al., 2016). The alkali-tolerance and thermostability of the enzymes both increase product stability and allow energy savings during processes. The importance of these enzymes can also be seen in pharmaceutical applications especially the use of proteases and DNases for removing proteinaceous and nucleic acid contaminants in fermentation processes which ensure high quality products in the purification step during sterile production (Tetz et al., 2009, Lam et al., 2018). DNases are also valuable, as they are widely used in therapeutic and clinical applications, in the treatment of cystic fibrosis for reducing mucus viscosity and improving airway clearance (Hodson and Shah, 1995). It also maintains the infection control by disrupting biofilms when combined with antibiotics.

Catalase enzymes of polyextremotolerant bacteria also have great contributions to bioremediation and environmental management. They detoxify hydrogen peroxide in wastewater, and as a result allow cleaner and more controlled processes under extreme pH or salinity conditions (Yoon et al., 2007).

The cold adapted psychrotolerant species, particularly

*Kocuria* species in this study, provide advantages for applications in biotechnology and low-temperature pharmaceutical usage, both by maintaining enzyme activity and by reducing energy consumption. The production of special industrial chemicals, especially bioactive peptides, oligosaccharides, and antioxidants that need cold conditions to maintain their activity, is not suitable for conventional microorganisms but can be achieved by cold-adapted microorganisms. These polyextremotolerant and versatile microorganisms are also valuable for industrial applications, as they prevent microbial growth under harsh conditions. Their applications across food, detergent, pharmaceutical, biopolymer, and bioremediation fields increase yield and product quality, reduce costs, and enhance process flexibility (Najari et al., 2017).

### CONCLUSION

We successfully isolated, purified, and identified novel polyextremotolerant bacterial strains from the Çamaltı Saltern, İzmir, Türkiye, for the first time, which exhibit promising potential for future industrial and biotechnological applications.

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### AUTHORSHIP CONTRIBUTIONS

All authors contributed to the idea and design of the study.

### ETHICAL APPROVAL

Ethical approval was not necessary for the study.

### CONFLICT OF INTEREST

There are no conflicts of interest or competing interests between authors.

### DECLARATION OF AI USE

The authors declare the use of Open AI's Chat GPT-5 for assistance in English language editing and grammar correction during manuscript preparation. The content and scientific interpretations are solely the responsibility of the authors.

### DATA AVAILABILITY

Data is available upon request.

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