Research Square

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Isolation, Phenotypic Characterisation and Molecular Identification of Thermophilic and Halophilic Bacteria from the İzmir–Dikili (Bademli) Marine Hot Spring, Türkiye

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Abstract

In recent years, studies on extreme biodiversity have focused on biotechnologically important microorganisms resistant to extreme conditions such as high temperature, high salt, acid and pressure, together with their bioproducts. Hot springs are natural habitats for halophilic and thermophilic extremophiles. This study aimed to isolate and characterise thermophilic and halophilic bacteria from the İzmir/Dikili (Bademli) sea thermal spring, Turkey, and was carried out in three stages. In the first stage, samples (sea water, mud, sand) were collected from several stations of the Bademli Sea Spring and bacteria were isolated using Brown Broth and Thermus medium; microorganisms with different colony morphology were selected for phenotypic characterisation. In the second stage, strains with different morphological properties were coded and evaluated by physiological tests (motility, temperature, pH, oxygen), biochemical tests (starch and casein hydrolysis, catalase, citrate, urease, gelatine hydrolysis, oxidase), salt tolerance and antibiotic susceptibility, with results compared to reference strains. In the third stage, chromosomal DNA was isolated from selected strains and 16S rRNA analysis was performed using universal primers; sequences were compared by BLAST and aligned with homologous NCBI reference sequences using multiple sequence alignment. Phylogenetic trees were constructed in MEGA 7, with CLUSTAL W alignment and neighbor-joining distance matrices calculated under the Kimura two-parameter model. Twenty-seven strains with different colony and cell morphology were isolated, and 16S rRNA analysis showed these thermophilic and halophilic strains to be closely related to *Bacillus* and *Anoxybacillus* species.

Introduction

Extremophilic microorganisms are able to survive under extreme conditions such as temperature, pressure, acidity and salinity owing to their remarkable adaptive capacities. These extraordinary resilience properties have triggered interest in research on the diversity of life of these organisms and have intensified efforts to understand life in hot ecosystems in particular. As organisms capable of surviving under harsh environmental conditions, extremophilic microorganisms help in the development of various theories on how life began on Earth and in a better understanding of early evolutionary processes (Cavicchioli 2002; Rawat and Joshi 2019). Marine hot springs are natural habitats for microorganisms resistant to such extreme conditions. Marine hot springs, which possess significant ecological value owing to their extreme conditions, provide suitable environments for halophilic (living in saline environments) and thermophilic (favouring high temperatures) microorganisms (bacteria, archaea, fungi, etc.) and for as-yet-undiscovered prokaryotic microorganisms, as well as for many organisms that do not survive under normal conditions, and therefore play an important role in the conservation of biological diversity (Poli et al. 2017). However, the diversity and biotechnological potential of these microorganisms have still not been fully explored (Rampelotto 2013).

Unlike other conventional spa systems, marine hot springs form when seawater descends into the depths of the Earth's crust, is heated by magmatic heat, and re-emerges at the surface; because they therefore contain both seawater and various minerals, they possess unique physicochemical characteristics (Humphris and Klein 2018). These environments are particularly favourable for the isolation of thermophilic and halophilic microorganisms (Pikuta et al. 2007). Factors such as temperature, salinity ratio and high mineral content directly affect the formation of microbial diversity and the mechanisms by which organisms adapt to environmental stress conditions (Oren 2002).

Türkiye is located on the tectonically active Alpine–Himalayan Belt. This tectonic activity, which brings with it numerous young fault lines and volcanic formations, is also responsible for the country's geothermal potential. Underground spring water with a temperature above 20°C is termed thermal water. Türkiye has approximately 1000 naturally occurring thermal water sources with differing characteristics (flow rate, radioactivity, dissolved mineral ratios and accessibility) and temperatures (Çelik and Keskin 2022). This country therefore holds great opportunities in terms of newly isolated microorganisms from extreme environments, particularly thermophilic and halophilic microorganisms. In the literature, studies on thermophilic and halophilic bacteria derived from marine hot springs are limited in number. Characterisation and identification of these microorganisms will therefore fill an important knowledge gap in the field of biotechnology. The Bademli marine hot spring, one of Türkiye's important thermal sources, is located 15 km from the Dikili district of İzmir Province and is one of the rare geothermal areas where hot-water sources merge between sea and land (Güncan 2020). This region offers a natural habitat for microorganisms able to survive under extreme environmental conditions.

The main objective of this study was to obtain thermophilic and halophilic bacterial isolates from the Bademli marine hot spring, an extreme ecosystem with thermal and hypersaline characteristics located in the Dikili district of İzmir Province, and to perform phenotypic, physiological, biochemical and molecular characterisation of these isolates. In addition, the 16S rRNA gene regions of the selected isolates were amplified and sequenced, and their phylogenetic relationships were evaluated by alignment with reference sequences in the NCBI database. The data obtained aim to make a scientific contribution towards determining the taxonomic position of bacteria that have adapted to extreme conditions and towards a preliminary assessment of their potential biotechnological applications.

Materials And Methods

Culture Media

The following media were prepared to cultivate and propagate the bacterial strains used in this study. Brown Broth 1963 (BB) was prepared according to Oren and Litchfield (1999) and Birbir et al. (2004), containing (per litter): yeast extract 5 g, $C_6H_5Na_3O_7{\cdot}5H_2O$ 3 g, $MgSO_4{\cdot}7H_2O$ 20 g, KCl 2 g, and NaCl 250 g.

Thermus medium (TM) was prepared according to Ramaley and Hixon (1970), containing (per litter): tryptone 3.0 g, yeast extract 1.0 g, and Castenholz 10× basal salt solution 100 mL. The Castenholz 10× basal salt solution was prepared according to Williams and da Costa (1994), containing (per litter): nitrilotriacetic acid 1.00 g, $CaSO_4 \cdot 7H_2O$ 0.60 g, $MgSO_4 \cdot 7H_2O$ 1.00 g, NaCl 0.08 g, KNO_3 1.03 g, $NaNO_3$ 6.89 g, Na_2HPO_4 1.11 g, $FeCl_3$ 0.28 g, and Nitsch's trace element solution 10 mL. Nitsch's trace element solution (Williams and da Costa, 1994) contained: H_3BO_3 0.5 mg/L, $CoCl_2 \cdot 6H_2O$ 0.046 mg/L, $CuSO_4 \cdot 5H_2O$ 0.01 g/L, $MnSO_4 \cdot H_2O$ 0.25 g/L, $Na_2MoO_4 \cdot 2H_2O$ 0.025 mg/L, H_2SO_4 0.5 mL/L, and $ZnSO_4 \cdot 7H_2O$ 0.5 mg/L.

Nutrient Broth (NB) (Merck, Germany) was used and for all solid medium (BBA, TMA and NBA), Bacto agar (Fluka, USA) was added prior to autoclaving and dispensing into Petri dishes.

Study area and sample collection

In this study, bacteria isolated from water and muddy water samples collected at various stations from a marine hot spring in Bademli village, Dikili district, İzmir province, were used. Dikili is located in the north of İzmir, approximately 120 km from the city center. Bademli village is about 9 km from the district center. Situated at the end of Kalem Island on the seashore and accessible through olive groves, Bademli Hot Spring was formerly known as the Aiolis Hot Spring and currently takes its name from the village in which it is located. Samples were taken from three sampling stations that differed in temperature, pH, electrical conductivity and dissolved-oxygen content (Table 1; Fig. 1).

Table 1
Physicochemical characteristics of the three sampling stations at the Bademli marine hot spring

Station	Coordinates	Water temperature (°C)	pH	Conductivity (%)	Dissolved oxygen (mg/L)
Station 1 (ST1)	38°59'56.472"N, 26°48'3.672"E	69.5	7.24	39	9.31
Station 2 (ST2)	38°59'47.4714"N, 26°47'56.0394"E	46.1	7.58	38	8.62
Station 3 (ST3)	38°59'47.94"N, 26°47'58.272"E	43.9	7.94	10.86	17.55

Figure 1 Field photographs of the three sampling stations at the Bademli marine hot spring, Dikili, İzmir Province, Türkiye. a Station 1 (ST1); b Station 2 (ST2); c Station 3 (ST3). Red arrows/circles indicate the sampling points; the inset in b shows a close-up of the sampling point

Isolation and pure-culture production

Water and mud samples (50 mL each) were collected from three different stations at the Bademli marine hot spring in Dikili, İmir, and transported to the laboratory under appropriate conditions. Three different media (BB, TM, and NB) were used for the samples. One gram of each water and mud sample was weighed and transferred into Erlenmeyer flasks containing 90 mL of sterile water to obtain a 10^{-1} dilution. After homogenization by vortexing, serial dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were prepared by successive transfers. Diluted samples were inoculated onto the three different media. Additionally, diluted samples were heat-treated at 80°C for 10 min to select for spore-forming microorganisms, subsequently inoculated onto the media, and incubated at 55°C for 24 h.

Morphological, Physiological, and Biochemical Tests

From the obtained cultures, microorganisms exhibiting different colony appearances were selected and purified to obtain pure cultures. Selected colonies were characterized based on phenotypic cell morphology, including shape, size, color, surface, elevation, cell wall, opacity, and density, and different colony types were assigned codes.

Cell morphology was examined by Gram staining, performed according to the method of Dussault (1955) using crystal violet, Lugol's iodine, 96% ethanol and aqueous fuchsin, and by endospore staining using carbol fuchsin and methylene blue counterstain following cold incubation (+ 4°C, 48 h) to promote sporulation. Preparations were examined under oil-immersion light microscopy.

To assess the motility of microorganisms, 0.4% semi-solid clear agar medium was prepared and dispensed into tubes containing a glass tube (hanging drop method setup). A single colony from an overnight fresh culture was picked with an inoculating loop and stab-inoculated into the glass tube. Each isolate was incubated at its optimum temperature for 3–5 days. For this test, a drop of 1% (v/v) tetramethyl-*p*-phenylenediamine dihydrochloride solution was placed on a filter paper to moisten the center. A one-day-old bacterial colony was carefully picked with an inoculating loop and rubbed onto the moistened area. The development of a dark blue color within 10 s was recorded as positive.

Starch hydrolysis was assessed on 3% starch agar following iodine (Lugol's) staining after 1–3 days of incubation. Gelatine hydrolysis was tested in 12% gelatine broth incubated for 1–3 days followed by refrigeration (1–2 h); liquefaction was scored as positive. Catalase activity was determined by the production of gas bubbles upon exposure of colony material to 3% H2O2. Urease activity was assessed on urea agar supplemented with filter-sterilised urea, incubated for 2–5 days. Citrate utilisation was assessed on Simmons' citrate agar (Sigma-Aldrich, Germany) incubated for 2–5 days, with a colour change from green to Prussian blue scored as positive.

Antibiotic-susceptibility testing

The antimicrobial susceptibilities of the bacterial strains were determined using the disk diffusion method. The antibiotic discs used were: ampicillin (AMP, 10 µg), fusidic acid (10 µg), novobiocin (NV, 5 µg), lincomycin (15 µg), neomycin (10 µg), kanamycin (K, 5 µg), tilmicosin (TIL, 15 µg), nystatin (100 units), gentamicin (10 µg), streptomycin (S, 10 µg), penicillin (P2, 2 units), penicillin (10 units), bacitracin (10 units), and tetracycline (TE, 30 µg). One milliliter of overnight liquid culture was inoculated onto Petri dishes containing the appropriate solid medium for each bacterium, and the plates were left for 30 min to allow absorption. The antibiotic discs were then placed on the agar surface at appropriate intervals, and each isolate was incubated at its suitable temperature for 48 h. Following incubation, inhibition zone diameters were measured and evaluated.

Chromosomal DNA isolation, quantification and electrophoresis

Bacterial cultures were grown to the stationary phase in 500 mL of the appropriate liquid medium and cells were harvested by centrifugation (9,000 rpm, 15 min). Chromosomal DNA was extracted from the cell pellets using a commercial kit (Omega E.Z.N.A. Bacterial DNA Kit, USA) according to the manufacturer's protocol, including lysozyme and proteinase K lysis steps, RNase treatment, and column-based purification. DNA concentration and purity were determined spectrophotometrically (NanoDrop 2000, Thermo Scientific, USA) from A260/A280 and A260/A230 absorbance ratios. DNA integrity was verified by 1% agarose gel electrophoresis in 1× TAE buffer with ethidium bromide staining, and bands of interest were excised and purified using a gel-extraction kit (Thermo Scientific GeneJET, USA).

PCR amplification and sequencing of the 16S rRNA gene

The 16S rRNA gene was amplified using the universal primer pair 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). Reactions contained 1× PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.3 mM of each primer, 2 U Taq DNA polymerase and template DNA in a 50 µL final volume. Cycling conditions comprised an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, 57°C for 45 s and 72°C for 1 min, amplifying a fragment of approximately 1,470 bp. PCR products were purified using ExoSAP-IT™ (Thermo Fisher Scientific, USA) and visualised by agarose gel electrophoresis. Sanger sequencing was performed by BMLabosis (Ankara, Türkiye) at the Macrogen Europe (Netherlands) facility using an ABI 3730XL sequencer and BigDye Terminator v3.1 chemistry (Applied Biosystems). Forward and reverse reads were assembled into consensus sequences using the CAP contig-assembly algorithm in BioEdit.

Sequence comparison and phylogenetic analysis

Consensus 16S rRNA sequences were compared against the GenBank database using the BLAST algorithm (https://www.ncbi.nlm.nih.gov/blast/) to identify the closest relatives and corresponding sequence-similarity values. Multiple sequence alignment was performed with CLUSTAL W, and phylogenetic trees were constructed by the neighbour-joining method using CLC Sequence Viewer 8 (Qiagen, Denmark), with evolutionary distances estimated under the Kimura two-parameter model.

Results

Isolation and colony morphology

A total of 27 bacterial strains with distinguishable colony morphotypes were recovered from the three sampling stations of the Bademli marine hot spring: 12 strains from Station 1 (1AS, 1BS, 1CS, 1DS, 1SWBA–1SWBH), 2 strains from Station 2 (2AS, 2BS) and 13 strains from Station 3 (3AS, 3BS, 3CS, 3DS, 3ES, 3FS, 3GS, 3HS, 3NS, 3NS1, 3NS2, 3NS3, 3SWBNJ). Colonies varied widely in shape (circular, filamentous or irregular), colour (cream, yellowish, dark cream, light brown or pink), size (very small to large), margin (entire, undulate, lobate or filiform) and elevation/surface texture (flat, raised, convex, crateriform or umbonate), indicating considerable phenotypic diversity among isolates recovered from a single geothermal system (Tables 2–4).

Cell morphology

Gram staining indicated that isolates from Station 1 and Station 3 were predominantly Gram-positive rods, although several Gram-negative-reacting isolates were also recovered at each station (e.g., 1DS, 1SWBB, 1SWBC, 1SWBD at Station 1; 3ES and 3GS at Station 3); all isolates from Station 2 (2AS, 2BS) stained Gram-negative. Endospore staining confirmed that all 27 isolates were spore-formers, predominantly with subterminal spores, consistent with adaptation to thermal stress.

Motility, oxidase activity, and starch, gelatine, casein, urease and citrate hydrolysis were assessed for all isolates (Tables 5–7). Catalase and oxidase activities were positive in the great majority of Station 1 and Station 3 isolates, consistent with an aerobic, catalase-producing physiology typical of Bacillus-related taxa. Starch hydrolysis was strong (+++) in most Station 1 and Station 3 isolates, indicating amylolytic activity, whereas Station 2 isolates (2AS, 2BS) were largely biochemically inert, showing only weak or negative reactions across the panel. Citrate utilisation was negative for all isolates from all three stations, and gelatine hydrolysis was positive only in a minority of strains (e.g., 1SWBA, 1SWBE, 3ES).

Table 5
Morphological, physiological and biochemical properties of bacterial isolates from Station 1 (n = 12)

Strain	Gram	Spore	Catalase	Starch	Casein	Oxidase	Urease	Citrate	Motility	Gelatin
1AS	+	+	+++	+++	+	+	+	-	-	-
1BS	+	+	+++	±	+	+	+	-	+	-
1CS	+	+	+++	+++	+	+	+	-	+	-
1DS	-	+	+	+++	+	+	+	-	+	-
1SWBA	+	+	++	+++	+	+	+	-	-	+
1SWBB	-	+	+	±	+	+	+	-	+	-
1SWBC	-	+	++	+++	-	+	+	-	-	-
1SWBD	-	+	+++	+++	+	+	++	-	+	-
1SWBE	+	+	+++	+++	+	+	+	-	-	+
1SWBF	+	+	±	±	±	+	+	-	+	-
1SWBG	+	+	+	++	-	+	+	-	-	-
1SWBH	+	+	+	+++	-	+	+	-	+	±

Table 6
Morphological, physiological and biochemical properties of bacterial isolates from Station 2 (n = 2)

Strain	Gram	Spore	Catalase	Starch	Casein	Oxidase	Urease	Citrate	Motility	Gelatin
2AS	-	+	-	±	-	-	-	-	-	-
2BS	-	+	-	-	-	-	-	-	-	-

Table 7
Morphological, physiological and biochemical properties of bacterial isolates from Station 3 (n = 13)

Strain	Gram	Spore	Catalase	Starch	Casein	Oxidase	Urease	Citrate	Motility	Gelatin
3AS	+	+	++	+++	+	+	+	-	+	-
3BS	+	+	+	+++	-	+	++	-	+	-
3CS	+	+	+	±	ND	+	+	-	+	-
3DS	+	+	±	±	+	+	++	-	+	-
3ES	-	+	++	+	-	+	++	-	+	±
3FS	+	+	±	+	ND	+	+	-	-	-
3GS	-	+	±	±	ND	-	-	-	+	-
3HS	+	+	±	+++	ND	-	-	-	-	-
3NS	+	+	++	+++	-	+	+	-	+	-
3NS1	+	+	+	+++	+	+	+	-	+	-
3NS2	+	+	++	+++	-	+	++	-	-	-
3NS3	+	+	+++	+++	+	+	+	-	+	-
3SWBNJ	+	+	+	+	ND	+	+	-	-	ND

(+) positive; (-) negative; (++) strongly positive; (+++) very strongly positive; (±) weak reaction; ND not determined

Antibiotic susceptibility

Disc-diffusion testing of 11 selected isolates against 13 antibiotics revealed variable susceptibility profiles (Table 8). Several isolates (e.g., 3BS, 3GS, 3HS and 3SWBNJ, all from Station 3) showed wide inhibition zones (25–40 mm) against tetracycline, chloramphenicol, streptomycin, gentamicin and mitomycin C, indicating broad susceptibility to these agents, whereas other isolates (e.g., 1SWBD, 1SWBF, 1SWBG) were largely resistant, with small or absent inhibition zones against most antibiotics tested other than bacitracin and mitomycin C.

Table 8
Antibiotic susceptibility (inhibition-zone diameter, mm) of selected isolates by the disc-diffusion method

Strain	AMP	NV	N	FD	S	B	K	P2	TE	TIL	CN	C	MY
1CS	15	17	20	-	15	21	8	20	15	25	20	16	25
1DS	12	14	16	-	5	25	-	-	12	11	18	NA	22
1SWBD	10	NA	10	-	-	25	-	-	-	NA	NA	NA	20
1SWBF	15	-	-	-	-	23	-	-	-	NA	-	7	-
1SWBG	-	-	-	-	15	18	8	20	13	-	10	20	-
1SWBH	11	-	-	-	-	14	-	-	15	15	-	-	27
2AS	17	8	10	NA	NA	25	9	18	21	22	-	20	NA
3BS	25	-	16	-	30	-	10	-	30	NA	28	-	35
3GS	30	16	-	-	25	16	20	10	15	16	25	15	35
3HS	35	17	15	-	30	20	28	12	40	NA	25	NA	30
3SWBNJ	-	15	-	-	11	15	-	16	-	10	12	-	30

Zone diameters: 0–12 mm, resistant; 13–18 mm, intermediate; ≥18 mm, susceptible. NA not tested. AMP ampicillin, NV novobiocin, N neomycin, FD fusidic acid, S streptomycin, B bacitracin, K kanamycin, P2 penicillin (2 units), TE tetracycline, TIL tylosin, CN gentamicin, C chloramphenicol, MY lincomycin

Chromosomal DNA yield, purity and integrity

Chromosomal DNA extracted from the 13 isolates selected for molecular analysis yielded concentrations of 10.4–64.6 ng/µL with A260/A280 ratios of 1.79–2.21 and A260/A230 ratios of 1.56–3.47, indicating DNA of acceptable purity for downstream PCR (Fig. 2, Table 9). Agarose gel electrophoresis showed single, intact bands exceeding 10 kb relative to the DNA marker, with no visible RNA contamination.

Table 9
Concentration and purity of chromosomal DNA extracted from the 13 isolates selected for 16S rRNA gene sequencing

Strain	Nucleic acid conc. (ng/µL)	A260/A280	A260/A230
1CS	16.2	1.79	1.85
1DS	14.9	1.93	1.86
1SWBD	13.1	1.90	1.89
1SWBF	42.7	2.21	1.83
1SWBG	17.2	1.94	1.78
1SWBH	37.5	1.89	1.56
2AS	11.6	1.83	2.39
3BS	26.4	1.94	2.89
3ES	25.6	1.90	2.30
3FS	14.9	1.83	2.57
3GS	10.4	1.98	2.64
3HS	64.6	1.90	2.42
3SWBNJ	24.6	2.00	3.47

6S rRNA gene sequencing and phylogenetic identification

Sequencing of the 16S rRNA gene yielded fragments ranging from 772 bp (3GS) to 1,434 bp (3HS) for the 13 selected isolates. BLAST comparison against GenBank showed that all sequenced isolates were most closely related to species within the genera *Bacillus* and *Anoxybacillus*, with pairwise similarity values ranging from 96.73% to 99.88% (Table 10). Neighbour-joining phylogenetic trees constructed for each isolate confirmed clustering with these reference taxa. Several isolates (1DS, 1SWBD, 3ES, 3FS and 3HS) showed similarity values below 98% to their closest validly published relatives, a threshold commonly regarded as indicative of potential novel species or subspecies requiring further genomic confirmation.

Table 10
16S rRNA gene sequence similarity of the 13 molecularly characterised isolates to their closest GenBank relatives

Strain	Station	Closest relative(s) (GenBank accession)	Similarity (%)
1CS	ST1	*Bacillus paralicheniformis / B. piscis / B. licheniformis / B. haynesii* (KY694465 / KX753358 / AE017333)	99.35
1DS	ST1	*B. paralicheniformis* (KY694465); *B. licheniformis* (AE017333); *B. sonorensis* (AYTN01000016)	98.65–99.50
1SWBD	ST1	*B. haynesii* (MRBL01000076); *B. paralicheniformis* (KY694465); *B. sonorensis* (AYTN01000016)	97.43–97.83
1SWBF	ST1	*B. paralicheniformis / B. piscis / B. licheniformis / B. haynesii* (KY694465 / KX753358 / AE017333 / MRBL01000076)	99.19
1SWBG	ST1	*B. licheniformis* (AE017333); *B. haynesii* (MRBL01000076); *B. paralicheniformis* (KY694465)	99.19–99.88
1SWBH	ST1	*B. haynesii / B. piscis* (MRBL01000016 / KX753358); *B. glycinifermentans* (LCCW01000063)	99.55–99.64
2AS	ST2	*Anoxybacillus pushchinoensis / A. karvacharensis* (AJ831843); *A. thermarum* (KX753358); *A. gonensis* (AJ831843)	98.91–99.23
3BS	ST3	*Anoxybacillus gonensis* (CP0121552); *A. kamchatkensis* (AJ831843); *B. thermarum* (KX753358)	99.07–99.65
3ES	ST3	*B. glycinifermentans* (LCCW01000063); *B. haynesii* (MRBL01000016); *B. paralicheniformis / B. sonorensis* (KY694465 / AYTN01000016)	96.73–99.25
3FS	ST3	*B. haynesii* (MRBL01000076); *B. sonorensis* (AYTN01000016); *B. paralicheniformis* (KY694465)	97.88–98.51
3GS	ST3	*B. glycinifermentans* (LCCW01000063); *B. sonorensis* (AYTN01000016); *B. swezeyii* (MRBK0100096)	98.83–99.74
3HS	ST3	*B. thermarum* (KX753358); *Anoxybacillus ayderensis; B. salavatiensis; A. kamchatkensis; A. gonensis* (CP0121552)	98.69–99.55
3SWBNJ	ST3	*B. paralicheniformis / B. piscis / B. haynesii* (KY694465 / KX753358 / MRBL01000076); *B. glycinifermentans*	98.19–99.55

Taxonomic distribution

At the family level, the 13 sequenced isolates were distributed as 76.9% Bacillaceae and 23.1% Anoxybacillaceae. At the species level, the closest relatives were distributed as follows: *B. haynesii*, 23.1%; *B. paralicheniformis*, 15.4%; *B. glycinifermentans*, 15.4%; *B. sonorensis*, 15.4%; *A. gonensis*, 15.4%; *A. flavithermus*, 7.7%; and *B. licheniformis*, 7.7% (Fig. 3). These proportions indicate that *Bacillus*-related taxa predominated among the isolates recovered from all three stations, with *Anoxybacillus*-related taxa restricted to Stations 2 and 3.

Discussion

The isolation of 27 phenotypically diverse, spore-forming bacterial strains from three physiochemically distinct stations within a single marine hot spring underscores the microbial richness of coastal geothermal habitats, consistent with previous reports that hot-spring ecosystems support diverse thermophilic and halotolerant microbiota adapted to combined thermal and osmotic stress (Poli et al. 2017; Pikuta et al. 2007). The predominance of Gram-positive, spore-forming, catalase- and oxidase-positive isolates at Stations 1 and 3 is consistent with the physiology typically reported for members of the genus *Bacillus*, which are characteristically robust, endospore-forming, aerobic or facultatively anaerobic organisms well suited to withstand thermal and desiccation stress (Logan and De Vos 2009).

Table 11
Morphological and physiological characteristics of selected *Bacillus* species reported in the literature, compared with the isolates obtained in this study

Species	Colony shape	Colour	Opacity/matte	Surface	Appearance	Growth temperature (°C)	pH range	NaCl tolerance (%)	Source
Bacillus haynesii	ND	Creamy white	Semi-transparent	Raised	Mucoid	15–60 (optimum 37)	5.5–10 (optimum 7.0)	0–12	Dunlap et al. 2017
B. sonorensis	Irregular	Yellowish cream	Matte	Convex	Slime-like	22–55 (optimum 30)	5.7–6	0–10	Palmisano et al. 2001
B. paralicheniformis	ND	Creamy white	Semi-transparent	Raised	Mucoid	15–60	6–11 (optimum 7.0–8.0)	0–10	Dunlap et al. 2015
B. aerius	Irregular	White	ND	Raised	ND	8–37 (not 40°C)	5.5–10 (not pH 4.0 or 11)	11.6	Shivaji et al. 2006
B. glycinifermentans	ND	White	ND	Raised	ND	15–65	ND	8	Kim et al. 2015
B. piscis	ND	Pale yellow pigment	ND	ND	ND	4–30 (optimum 30)	6–10 (optimum 7.0–8.0)	0–10	Lee et al. 2016
B. spizizenii	ND	ND	ND	ND	ND	14–50	5–9	0–10	Dunlap et al. 2020
B. velezensis	Rough	Creamy white	Turbid	ND	ND	15–45	5–10	12	Ruiz-García et al. 2005
B. swezeyii	ND	Creamy white	Semi-transparent	Raised	Mucoid	15–60 (optimum 37)	5.5–10 (optimum 7.0)	0–12	Dunlap et al. 2017
B. licheniformis	Irregular	Creamy white	Matte	Rough	Mucoid	15–60 (optimum 37–50)	5.5–9.5 (optimum 7–8)	0–10	Chester 1901

ND, not determined/not reported in the original source.

Table 12
Biochemical characteristics of selected Bacillus species reported in the literature

Species	Gram	Spore	Motility	Catalase	Oxidase	Starch	Casein	Urease	Citrate	Indole	Gelatin	Source
Bacillus haynesii	+	+	+	+	-	+	+	+	+	ND	+	Dunlap et al. 2017
B. sonorensis	+	+	+	+	-	+	+	+	+	-	+/-	Palmisano et al. 2001
B. paralicheniformis	+	+	+	+	-	+	+	+	-	ND	+	Dunlap et al. 2015
B. aerius	+	+	+	+	+	+	ND	-	+	-	+	Shivaji et al. 2006
B. glycinifermentans	+	+	+	+	-	+	+	ND	+	ND	+	Kim et al. 2015
B. halotolerans	+	+	+	+	-	+	+	-	+	-	+	Kang et al. 2023
B. piscis	+	+	+	+	+	ND	ND	ND	ND	ND	ND	Lee et al. 2016
B. spizizenii	+	+	+	+	+	+	+	-	+	ND	+	Dunlap et al. 2020
B. velezensis	+	+	+	+	+	+	+	-	ND	-	+	Ruiz-García et al. 2005
B. swezeyii	+	+	+	+	-	+	+	ND	+	ND	+	Dunlap et al. 2017
B. licheniformis	+	+	+	+	-	+	+	-	+	-	+	Chester 1901

+, positive; -, negative; +/-, variable; ND, not determined/not reported in the original source.

Both the phenotypic and genotypic characteristics of all strains isolated from all station and subjected to 16S rRNA gene sequencing analysis were evaluated. The colony morphologies, Gram- and spore-staining results, and biochemical test profiles of these strains were compared with similar species reported in the literature (Tables 11 and 12).

Strain 1CS is of medium size, circular in form and cream in colour. The colony margin is undulate, the surface is flat, and the interior has a spotted, matte appearance. It is Gram-positive, spore-forming and rod-shaped. Spreading growth was observed along the agar in the motility test, indicating that the strain is motile. It gave a strongly positive reaction in the starch hydrolysis and catalase tests, the urease test was positive, and the gelatine hydrolysis and citrate tests were negative. According to 16S rRNA analysis, the strain showed its highest similarity (99.35%) to *B. paralicheniformis, B. piscis, B. licheniformis* and *B. haynesii.*

Strain 1DS is of medium size, circular in form and yellowish in colour. The colony margin is smooth, with a bubbly appearance, and the structure is sticky and translucent. This strain is Gram-negative and spore-forming, rod-shaped. It was observed to be motile in the motility test. The starch hydrolysis test gave a strongly positive result. Urease, oxidase and casein tests were positive, while gelatine hydrolysis and citrate tests were negative. According to 16S rRNA analysis, the strain showed its highest similarity to *B. sonorensis* (98.65%) and *B. licheniformis* (98.86%); a similarity of 98% indicates that the analysed isolate is highly likely to be very close to, but possibly a novel species or subspecies of, the known species, and further analyses are required.

Strain 1SWBD is of medium size, filamentous in structure and dark cream in colour. The colony margin is thread-like with a bubbly appearance; the periphery is translucent while the centre is matte. It is Gram-negative and spore-forming, rod-shaped. It was observed to be motile. Starch hydrolysis, the urease test, catalase and casein tests were positive, while the oxidase, citrate and gelatine hydrolysis tests were negative. The genetic similarity of this isolate was found to be high with *B. paralicheniformis* (97.43%), *B. haynesii* (97.83%) and *B. sonorensis* (97.63%). This degree of similarity suggests that the strain may possibly represent a novel species or subspecies, and further analyses are required.

Strain 1SWBF is of medium size, circular in form and light brown in colour. The margins are undulate with a flat appearance; the periphery is translucent and the interior is brown-spotted. Strain 1SWBF has a light brown colour and circular colony structure, and is a Gram-positive, spore-forming bacterium. The starch, casein and catalase activities of this strain were determined to be weak. In contrast, 16S rRNA analysis showed 99.19% similarity to *B. paralicheniformis, B. piscis, B. licheniformis* and *B. haynesii.*

Isolate 1SWBG displayed a cream-coloured, filamentous structure and was observed to be Gram-positive, spore-forming and non-motile. Genetic analysis of this strain showed 99.58% similarity to *B. licheniformis.*

Strain 1SWBH displayed a yellowish colour and circular colony characteristics. It is Gram-positive, spore-forming and motile, and gave positive results in the catalase and starch hydrolysis tests. According to 16S rRNA analysis, this strain showed high similarity to *B. haynesii* and *B. piscis* (99.64%).

When these findings are evaluated overall, it is understood that the bacteria obtained from Station 1 mostly show high similarity to species belonging to the genus Bacillus that carry thermophilic and mildly halophilic characteristics. The temperature- and mineral-rich structure of the Bademli marine hot spring supports the isolation of these species. In addition, the high degree of phenotypic diversity indicates that these bacteria possess different adaptation strategies against environmental stress conditions. Studies by Palmisano et al. (2001) and Dunlap et al. (2015, 2017) have reported that species such as *B. paralicheniformis* and *B. sonorensis* can display both thermophilic and halotolerant characteristics and can be successfully isolated from saline hot springs. These findings indicate that the Bademli marine hot spring offers a habitat suitable for the development of these species in terms of temperature and salinity. Similarity ratios above 98% suggest that these isolates show species-level similarity but may nonetheless carry some different genetic variations (Kim et al., 2015).

Station 2

Strain 2AS, isolated from Station 2, is very small in size, circular in form and translucent in colour, resembling a water droplet. The colony margin is smooth, the structure is raised and translucent. It is Gram-negative and spore-forming, rod-shaped. In the motility test, growth was observed along the inoculation line but no spreading occurred within the agar. It was therefore determined to be non-motile and aerobic. The starch hydrolysis test showed a partially positive reaction, while the urease test was negative. *A. pushionensis* and *A. karvacharensis* showed a high degree of similarity, at 99.23%.

These species are generally thermophilic bacterial species isolated from thermal springs, hot-water basins or volcanic environments (Belduz et al., 2003; Shivaji et al., 2006; Lee et al., 2016). *A. pushionensis* was isolated from thermal springs in India and is a bacterium that grows optimally at 55–60°C and is able to metabolise carbohydrates (Shivaji et al., 2006). *A. karvacharensis* was similarly isolated from geothermal springs in Armenia and is a thermophilic and alkalotolerant species (Lee et al., 2016). The recovery of isolate 2AS from a source with high temperature and mineral content, such as the Bademli marine hot spring, supports its environmental compatibility with these species. In addition, the species *B. thermarum* and *B. kaynarcasis* have also been found in hot-water sources and are heat-resistant bacteria with potential enzyme-producing capacity (Liu et al., 2014). *B. thermarum* is a species that grows in the range of 60–65°C and can produce biotechnologically important thermostable enzymes (particularly proteases and lipases). *B. kaynarcasis* was isolated from the Kaynarca region of Türkiye, and its genetic relatedness to *B. thermarum* has been demonstrated. Although the Gram-negative reaction of isolate 2AS appears to be a deviation from the classical *Anoxybacillus* morphology, this may be a variation explained by environmental effects and factors such as cell-wall ageing (Logan and De Vos, 2009). Its spore-forming nature and colony characteristics are, however, quite consistent with the species to which it shows genetic similarity.

Station 3

Strain 3BS is small in size, filamentous in structure and matte cream in colour. The margins are thread-like; the colony appearance is crater-shaped, with a translucent periphery and a matte cream centre. It is Gram-positive, spore-forming, rod-shaped. It was observed to be motile. The starch hydrolysis test gave a strongly positive result. Urease, oxidase and catalase tests were positive, while citrate and gelatine hydrolysis were negative. According to 16S rRNA analysis, its similarity to *A. gonensis* is 99.65%.

Strain 3ES is small in size, circular in structure and matte cream in colour. The margins are undulate, with a flat appearance and an undulate periphery. It is Gram-negative and spore-forming, rod-shaped. It has motile and aerobic characteristics. The starch hydrolysis test gave a positive result. The urease test was positive, while casein and citrate were negative. 16S rRNA analysis showed similarity to *B. paralicheniformis, B. haynesii* (98.29%), *B. sonorensis* (98.12%), *B. glycinifermentans* (97.88%) and *B. aurius* (98.04%). This degree of similarity suggests that the strain may possibly represent a novel species or subspecies, and further analyses are required.

Strain 3FS is of medium size, circular in form and light cream in colour. The colony margins are lobate, convex in structure, with a translucent, flat periphery. It is Gram-positive, spore-forming, rod-shaped. It was determined to be non-motile. The starch hydrolysis test was positive. Urease and oxidase tests were positive. It shows 98.51% similarity to *B. paralicheniformis* and B. haynesii, 98.37% to B. sonorensis, and 97.88% to *B. glycinifermentans*. These results suggest that it may represent a novel species or subspecies, and further analyses are required.

Strain 3GS is small in size, circular and yellowish in colour. The colony margins are smooth, with a raised appearance; the periphery is translucent and there is a yellowish spot in the interior. It is Gram-negative and spore-forming, rod-shaped. It has motile and aerobic characteristics. The starch hydrolysis test gave a partially positive result. The urease test was negative. Genetic analysis showed 99.74% similarity to *B. glycinifermentans*.

Strain 3HS is very small in size, circular in structure and pink in colour. The colony margins are smooth, with a raised appearance; the periphery is flat and the interior has a translucent, spotted structure. It is Gram-positive, spore-forming and rod-shaped. It was confirmed by testing to be non-motile. The starch hydrolysis test gave a strongly positive result. The urease test was negative. According to 16S rRNA analysis, it shows 98.69% similarity to *A. kamchatkensis*. These results suggest that it may represent a novel species or subspecies, and further analyses are required.

Strain 3SWBNJ is large in size, irregular in structure and off-white cream in colour. The colony margins are undulate, with a convex appearance; the surface is matte, the periphery is dark cream and the interior is translucent. It is Gram-positive, spore-forming, rod-shaped. It was observed to be non-

motile. Catalase, starch, oxidase and urease tests were positive, while the gelatine test result is unknown. In 16S rRNA analysis it shows 99.55% similarity to *B. paralicheniformis, B. piscis* and *B. haynesii.*

Overall, both the morphological and genetic diversity of the bacteria isolated from Station 3 are high, and they are understood to show similarity mostly to species belonging to the genera *Bacillus* and *Anoxybacillus.* This situation reflects the microbial habitat structure of the Bademli marine hot spring, which is characterised by high temperature, mineral content and mildly halophilic properties. It has also been reported that these species are natural sources of many industrial enzymes and, owing to their environmental adaptation capacity, have significant potential for biotechnological applications (Goh et al., 2013; Pikuta et al., 2007). Among these isolates, *A. ayderensis* and *A. gonensis* in particular are thermophilic species that have previously been isolated from other geothermal sources in Türkiye (Shivaji et al., 2006; Lee et al., 2016). This similarity indicates that the sampling point used in this study shares characteristics with other thermal habitats in the country. Members of the genus *Anoxybacillus* are generally spore-forming thermophiles that can develop successfully in environments with moderate salinity at temperatures of 50–65°C (Najar et al., 2020). The Bademli marine hot spring can therefore be said to offer a suitable ecological niche for these species. In addition, the high similarity (above 99%) to species such as *B. salavatiensis* and *B. thermarum* suggests that this isolate is either the same as, or a very close variant of, these species. However, similarity ratios that do not reach 100% also leave open the possibility of a novel species or an adaptive mutation (Chun et al., 2018).

Conclusion

In this study, a total of 27 thermophilic and halophilic bacterial strains were isolated from the İzmir/Dikili (Bademli) marine hot spring. Twenty-seven bacteria were isolated from three stations of the marine hot spring that differed in physicochemical characteristics, and 13 of these strains were subjected to molecular-level characterisation by sequencing the 16S rRNA gene region.

The presence of high temperature and salt concentration in the isolation environment has enabled the obtained strains to develop adaptations specific to extreme conditions. Thermophilic bacteria can be exploited in industry and biotechnology by producing thermostable enzymes that function at high temperatures. For example, thermostable versions of enzymes such as α-amylase, protease and lipase are used in various applications including food processing, detergents and biofuel production. Thermophiles are also known to be promising sources for processes such as biopolymer and biofuel production, wastewater treatment and bioremediation.

It was determined that the majority of the isolates resistant to hot and saline environmental conditions belong to the genera *Bacillus* and *Anoxybacillus.* Most of these bacteria showed similarity ratios ranging from 97.33% to 99.74% to species previously reported from different thermal and/or saline environments, such as *B. paralicheniformis, B. haynesii, B. glycinifermentans, B. sonorensis, A. kamchatkensis, A. gonensis* and *A. ayderensis.* In addition, similarity ratios remaining within the range of 97–99% suggest that these microorganisms contain a certain degree of genetic divergence and may potentially represent novel species or strain candidates.

In conclusion, the scientific significance of the Bademli marine hot spring lies in the fact that both thermophilic and halotolerant bacteria can naturally co-occur within this ecosystem. This means that the bacteria isolated from this site may possess versatile biotechnological properties. This study has both contributed to determining local microbial diversity and highlighted the ecological and potential biotechnological importance of extremophilic microorganisms.

Declarations

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Competing interests The authors have no relevant financial or non-financial interests to disclose.

Author contributions Both authors contributed to the study conception and design. Material preparation, sampling, laboratory analyses and data collection were performed by M.D under the supervision of F.M.B. The first draft of the manuscript was written by M.D and both authors commented on and approved the final manuscript. This study was derived from the Master's thesis of M.D at Dicle University, Turkey.

Ethics approval This study did not involve human participants, animals, or animal-derived material. The environmental sampling of water and mud was carried out with respect for local regulations and did not require institutional ethics committee approval.

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Table 2, 3, 4

Table 2,3,4 are available in the Supplementary Files section.

Figures

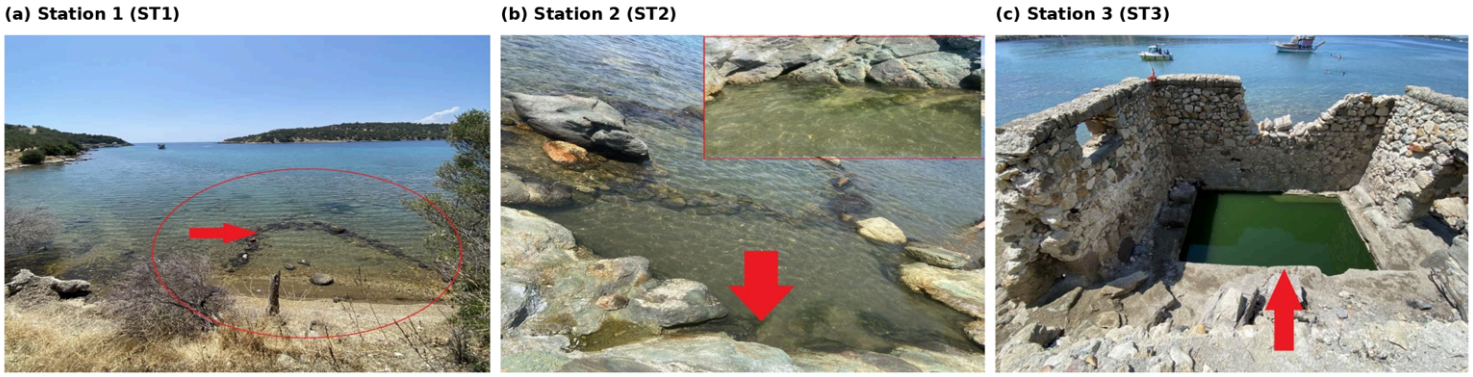


Figure 1

Field photographs of the three sampling stations at the Bademli marine hot spring, Dikili, İzmir Province, Türkiye. a Station 1 (ST1); b Station 2 (ST2); c Station 3 (ST3). Red arrows/circles indicate the sampling points; the inset in b shows a close-up of the sampling point

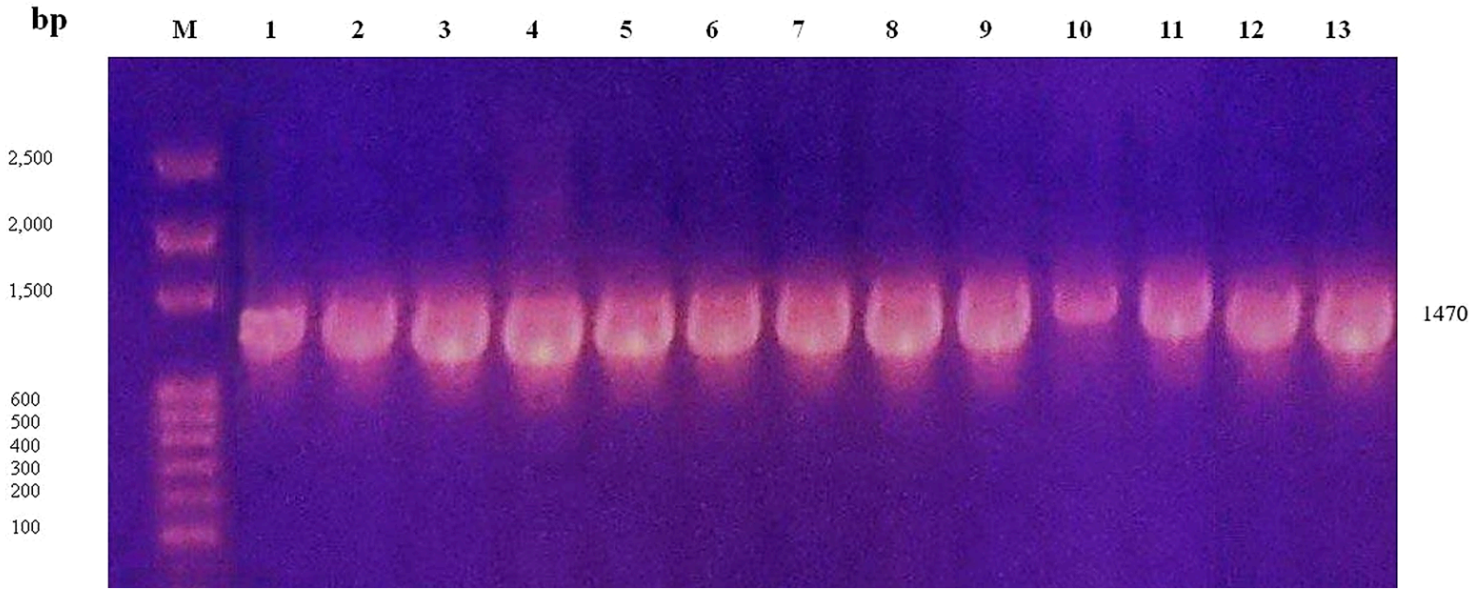


Figure 2

Agarose gel (1%) electrophoresis image of the chromosomal DNA isolated from the bacterial strains. Lane M, DNA size marker (bp); lanes 1–13, chromosomal DNA of the thirteen isolates selected for 16S rRNA gene sequencing. Relative to the DNA marker, the chromosomal DNA of all isolates was larger than 10 kb and present at concentrations exceeding 50 ng; the appearance of a single band in each lane indicates that the DNA was free of RNA contamination

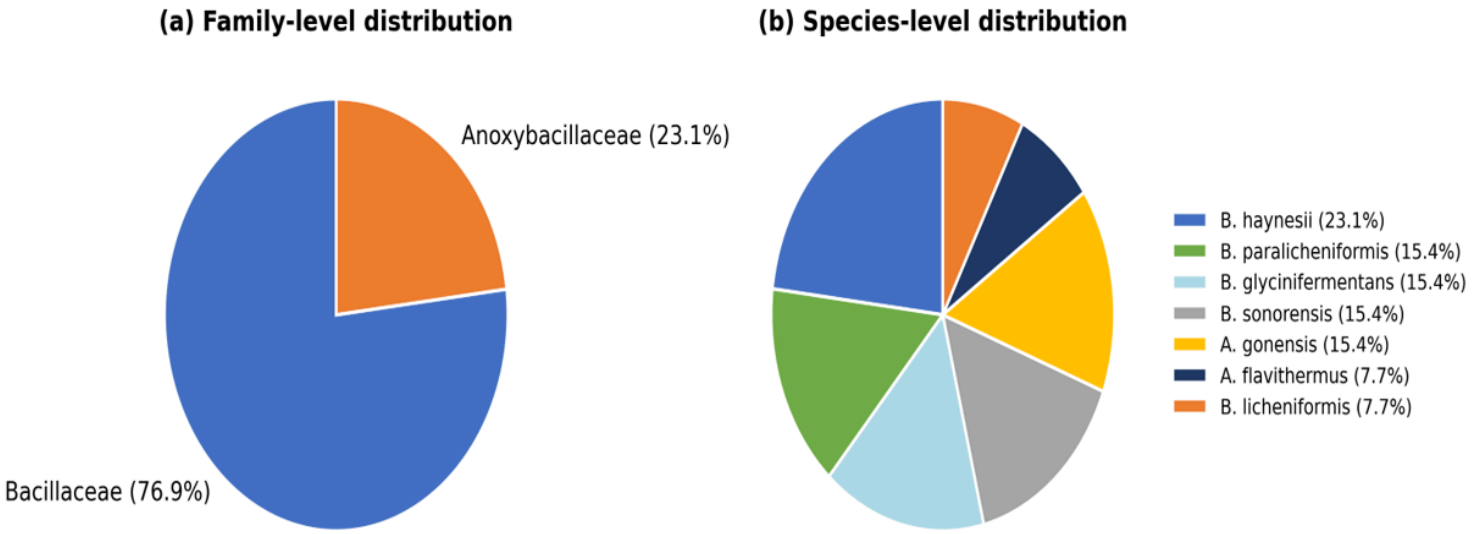


Figure 3

Taxonomic distribution of the 13 molecularly identified isolates from the Bademli marine hot spring. a Distribution at family level; b distribution at species level, based on the closest BLAST relative of each isolate

Supplementary Files

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- Table234.docx