



Identification, characterization and hydrolase producing performance of thermophilic bacteria: geothermal hot springs in the Eastern and Southeastern Anatolia Regions of Turkey

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Abstract In the last two decades, researchers have increasingly focused on the rich microorganism-based diversity of natural hot spring sources to explore the benefits of thermophiles in industrial and biotechnological fields. Within the scope of this study, a total of 83 thermophilic *Bacilli* strains were isolated from 7 different geothermal hot springs (at temperatures ranging between 40 and 85 °C) located in the Eastern and Southeastern Anatolia Regions of Turkey. The physiological, morphological, biochemical and molecular properties of the isolates were determined. As a result of the 16S rRNA gene sequence analysis, 5 different species (*Bacillus licheniformis*, *Bacillus* sp., *Bacillus subtilis*, *Geobacillus kaustophilus*, and *Weizmannia coagulans*.) were identified. *B. licheniformis*

and *B. subtilis* were the most frequently encountered species among those obtained from the researched hot spring sources. Phylogenetic analysis was conducted to evaluate the phylogenetic relationships of the isolated species. The results showed that there was no significant difference between the groups and the bacteria in terms of the locations or optimum temperatures of the isolates. The bacterial isolates were screened for amylase, cellulase, lipase and protease hydrolytic enzyme activities. The hydrolytic enzyme production potentials among the isolates were identified in 68 (82%) isolates for amylase, 34 (41%) for cellulase, 69 (83%) for lipase and 73 (88%) for protease. All isolates were found to have at least one or more extracellular enzyme activities. Additionally, it was determined that 27 of the existing isolates (32.8%) were able to produce all of the aforementioned hydrolytic enzymes.

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Introduction

Thermophilic microorganisms can thrive in aquatic and terrestrial habitats. Examples of such environments are sugar heaps, hot springs, deep seas and volcanic regions. It is known that thermophilic

microorganisms have a diversity that includes both bacteria and archaea, and the most frequently isolated thermophilic strains have also been reported to belong to the *Bacillus* genus (Abootalebi et al. 2020; Ladeira et al. 2015; Shen et al. 2021; Verma et al. 2018; Wang et al. 2006). The presence of thermophilic *Bacilli* has been investigated in hot springs and compost sources around the world (Yanmis et al. 2015), such as those in India (Verma et al. 2018), Iran (Abootalebi et al. 2020), Indonesia (Safitri et al. 2020), Jordan (Mohammad et al. 2017), Faisalabad (Saleh et al. 2020), Tunisia (Salem et al. 2020), Brazil (Bernardo et al. 2020), Egypt (Saeed et al. 2020), Malaysia (Msarah et al. 2020), Vietnam (Dang et al. 2018), Saudi Arabia (Al-Johani et al. 2016), South Africa (Tsoetsi et al. 2020) and Japan (Akita et al. 2017).

Turkey is one of the world's most significant countries because of its abundant thermal water reserves (Oztas Gulmus and Gormez 2020b). Hot springs in eastern and southeastern Turkey are reservoirs for natural resources that are attractive for biotechnological studies (Güven et al. 2018). Various thermophilic microbial species have been reported in many studies carried out in the Hasanabdal thermal springs (Poli et al. 2012; Savas et al. 2009; Yanmis et al. 2015), Sırnak (Derya and Ahmet 2014), Diyadin (Baltacı et al. 2017), Dargeçit (Acer et al. 2015), Guclukonak (Poli et al. 2012) and Pasinler (Adiguzel et al. 2011; Oztas Gulmus and Gormez 2020b). The presence of many thermophilic *Bacillus* and *Geobacillus* species has been reported in the Pasinler and Ilica thermal spring of Erzurum by different researchers (Oztas Gulmus and Gormez 2020b; Yilmaz et al. 2016).

Geothermal hot springs generally host microorganisms that can be active and produce various enzymes under extreme conditions, depending on their environment (Hogendoorn et al. 2021; Mehta et al. 2016; Panosyan et al. 2020; Short 1998). These microorganisms attract the attention of researchers as important sources of new and high-temperature resistant enzymes such as xylanases, DNA polymerases, amylases, cellulases, chitinases, lipases, pectinases, phytases and proteases (Falcicchio et al. 2021; Lee et al. 2010; Luo et al. 2017; Msarah et al. 2020; Tango and Islam 2002). These enzymes originating from microorganisms are also frequently preferred in industrial fields. In particular, enzymes are widely used in many biotechnological fields such as food processing, medicine, molecular biology, vaccine

development, genetics, renewable energy, organic synthesis and biofuel production (Eriksen 2008; Khan and Sathya 2018; Zakzeski et al. 2010).

Hot springs are some of the most important habitats for thermophilic microorganisms (Aanniz et al. 2015). At the same time, many enzymes produced by microorganisms that can survive under extreme conditions remain undiscovered (Thakur et al. 2021). Due to the need in fields such as the industry, it is necessary to isolate and investigate new genera in terms of the production of biotechnologically thermostable enzymes. In general, the production of enzymes from some bacteria such as *Bacilli* occurs mostly in a short time, with carbon and nitrogen sources that do not require much cost (Margaryan et al. 2018). Therefore, this study is important in terms of investigating new genera with the potential to produce thermozyms in different fields of research and biotechnology.

The present study, was aimed to isolate bacteria from 7 hot springs at high altitudes in the Eastern and Southeastern Anatolia Regions of Turkey, identify these isolates by conventional and molecular methods, determine the optimum growth conditions of the isolates and analyze their capability to produce hydrolase enzymes including amylase, cellulose, lipase and protease.

Materials and methods

Collection of biological material and isolation of bacteria

Soil/sediment, mud and water samples were collected from 7 hot springs in the Eastern and Southeastern provinces of Turkey including Agri (Diyadin, Davut and Koprü; Lat.: 39.493548, Lng.: 43.649362), Erzurum (Pasinler; Lat.: 39.978194 Lng.: 41.664286), Van (Hasanabdal; Lat.: 39.225049, Lng.: 43.388236), Siirt (Hista; Lat.: 37.707582, Lng.: 42.002020), Mardin (Dargeçit; Lat.: 37.546184, Lng.: 41.720381) and Sırnak (Guclukonak; Lat.: 37.476818, Lng.: 41.939351) in August and September 2016. The locations of the different geothermal sources in this study are shown on the map in Fig. 1. Samples were collected from the shallow and muddy areas at the outlet of the hot springs in sterile capped bottles. Aluminum foil was used to cover the caps to prevent exposure to sunlight. The temperature and pH values

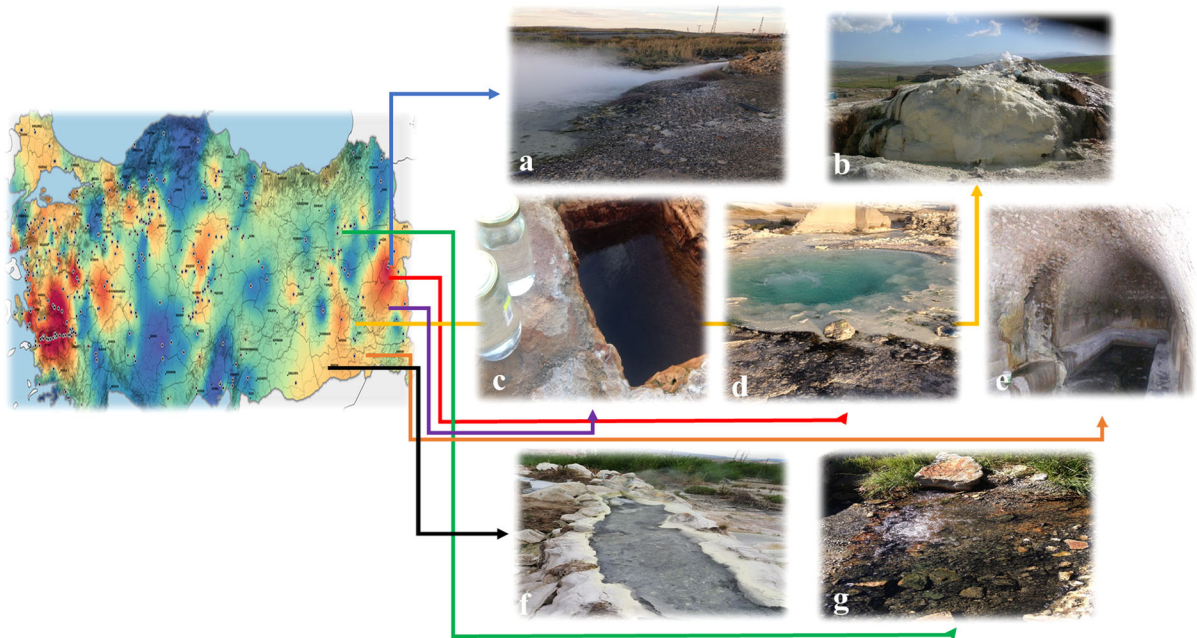


Fig. 1 Referring to the map of Turkey, hot springs under this study are indicated by arrows. Source of geothermal hot springs: **a** Kopru (Agri), see Supplemental Movie 1. **b** Hista (Siirt), **c**

Hasanabdal (Van), **d** Davut (Agri), **e** Guclukonak (Sirnak), **f** Dargecit (Mardin), **g** Pasinler (Erzurum) (<https://www.mta.gov.tr>)

of the samples were measured with a pocket device (Model No. AZ 8685, Taiwan). The digital thermometer was carefully immersed at a depth of approximately 5 cm in the area where the samples were collected, and the readings were noted. The samples were collected at 3-m intervals. The collected samples were transferred to sterile tubes as dilutions of 10^{-1} – 10^{-6} (0.1 mL sample, 0.9 mL physiological saline (0.85 g NaCl and 100 mL distilled water)). Each dilution (0.1 mL sample) was transferred to petri dishes containing Luria Bertani (LB) agar (Sigma-Aldrich). The samples were incubated for 24–72 h at different temperatures (40, 55, 65, 70, 75, 85 and 90 °C). Different colony morphologies were selected, and these pure isolates were inoculated into the LB agar. The purified isolates were preserved in an LB medium containing 15% glycerol at -80 °C for subsequent analyses (Gormez et al. 2013).

Conventional characterization of bacteria

Morphological properties

The colony morphology and motility tests of the isolates were carried out by a light microscope

(Bestscope BS-2030t + BLC - 450). The morphological properties of the isolated bacteria were determined by Gram and endospore staining, and motility tests were carried out using standard procedures (Bernard et al. 2017).

Motility

Cell motility, morphology, shape and endospore staining properties were determined using a wet mount of freshly prepared isolates via phase contrast microscopy (Bestscope BLM-280 LCD, China) (Tso-tetsi et al. 2020).

Gram staining

The bacteria were grown on the solid medium (LB agar) overnight, colonies were taken with the help of a loop, and these colonies were spread by dripping physiological saline on one side of the clean slide. The prepares were air-dried and fire-dried. They were kept in a crystal violet solution for 2 min and washed with water to allow staining. After soaking in Lugol’s solution for 1 min, the sample was washed and dried. In order to provide dechlorination in the prepare, it

was kept for 15 to 20 s in 96% alcohol. It was kept waiting and washed with water again and dried. As the final processing step, the dye was removed by waiting for 30 s with fuchsin. After the preparates were dried on blotting paper, immersion oil was dropped, and the preparates were examined with the immersion lens under the microscope. As a result of the examinations, the blue or purple appearance of the bacteria was considered Gram-positive, and if the bacteria appeared pink, they were considered Gram-negative (Oztas Gulmus and Gormez 2020b).

Endospore staining

The bacteria were grown overnight on the solid medium (LB agar). Colonies were spread on the slide with the help of a loop. The dried preparates were first stained with carbol fuchsin for 5 min. After washing, they were treated with 10% nitric acid for 10 s. The preparates that were stained with methylene blue for 2 min were washed and dried. They were then examined under a microscope with immersion oil (Oztas Gulmus and Gormez 2020b).

Physiological properties

The physiological properties (temperature, pH and NaCl concentrations) of the isolates were tested at various stages of growth under aerobic conditions (Oztas Gulmus and Gormez 2020b). The bacterial isolates were incubated at temperatures from 40 to 90 °C. The growth of the bacterial isolates was evaluated at different pH values (ranging from pH 4.0 to 12.0, with increments of pH 1.0). By adding up to 10% NaCl (w/v) to the incubation medium, the range of the NaCl concentrations for growth was determined. The growth of the bacteria was evaluated by measuring optical density (OD) at 600 nm with a spectrophotometer (GENESYS 140, Thermo Scientific). The use of various compounds at a 1% concentration (glucose, lactose, galactose, fructose, citrate, casein and starch) as a carbon source was tested.

Biochemical properties

The biochemical properties (oxidase, catalase and carbon source) of the isolates were analyzed according

to the methods described by Prescott and Harley (2002) (Prescott 2002).

Catalase test

A loop sample was taken from the culture to be examined and suspended with distilled water on a slide. Then, when a loopful of 30% H₂O₂ was added and mixed, the emergence of air bubbles showed that the test was positive.

Oxidase test

Special strips containing N, Ndimethyl-1,4-phenylenediammonium dichloride were used for the oxidase test (Bactident Oxidase). The bacterial isolates taken with the loop were inoculated to the tip of the strip, and those showing color changes were determined as positive (Prescott 2002).

Molecular identification of bacteria

Genomic DNA isolation

Total genomic DNA extraction was performed by modifying Sambrook's classic phenol: chloroform: isoamyl alcohol method (Sambrook 1987), and the samples were stored at – 80 °C (Prescott and Harley 2002). The bacterial isolates were taken into a medium containing 5 mL of LB broth and incubated at 37 °C for 24 h. The sample was transferred to Eppendorf tubes (1.5 mL) and centrifuged at 7000 rpm for 5 min, and then, dH₂O (200 µl), 0.5 M EDTA (50 µl), 20% sarkosyl (10 µl), proteinase K (10 mg/ml, 10 µl), 1 M Tris–HCl (pH: 8, 10 µl) and 5 M NaCl (5 µl) were added. The mixture was vortexed for 5 min and incubated in a 65 °C water bath for 30 min. It was vortexed every 10 min. The same volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added. The mixture was centrifuged at 13,000 rpm for 5 min, and the supernatant was taken with the help of a pipette and transferred to a new Eppendorf tube. The phenol: chloroform: isoamyl alcohol (25:24:1) treatment was performed 3 times as described above. 3 M NaOAc up to 1/10 of the supernatant volume and 2 times the volume of absolute ethanol was added to new Eppendorf tubes and incubated at -20 °C for 1 night. At the end of the incubation time, the preparate was centrifuged at 13,000 rpm for 10 min. The supernatant

was removed, and the pellet was dried. dH₂O (200 µl) was added to the pellet, and the pellet was dissolved. A 1/10 volume of 0.3 M NaOAc and ethanol (440 µl) were added to the thawed pellet and stored at -20 °C for 1 night. At the end of the storage time, the supernatant to be centrifuged at 13,000 rpm for 10 min was removed from the tube, and the pellet was left to dry. The dried pellet was dissolved with dH₂O (100 µL).

Amplification and sequencing of 16S ribosomal RNA (16S rRNA) gene of isolates

The PCR amplification of bacterial DNA was conducted by using a GenePro Thermal Cycler (Bioer Technology Co., Ltd) with the model code TC-E-96G. The 16S rRNA gene region of the DNA was amplified using universal primers (forward primer 27F = 5'-GAGTTTGATCTGGCTCA-3'; reverse primer 1385R = 5'-CGGTGTGT[A/G] CAAGGCC-3') (Oztas Gulmus and Gormez 2020b). The amplification reaction was carried out in a total volume of 25 µl containing: 10X PCR buffer (2.5 µL) (Applied Biosystems, Roche, California, USA), 25 µM magnesium chloride (2 µL), 25 mM dNTP (1.5 µL), 25 pmol primer 27F (2.5 µL), 25 pmol primer 1385R (2.5 µL), genomic DNA (1 µL), 5 unit/µ Taq DNA polymerase (0.25 µL) (Applied Biosystems), and dH₂O (12.75 µL). The PCR procedure was performed under the following conditions: 1 cycle of 94 °C for 5 min., 40 cycles of 94 °C for 1 min., 55 °C for 1 min. and 72 °C for 1 min., and 1 cycle of 72 °C for 5 min. The products that were amplified by PCR were analyzed on 1% agarose gel containing ethidium bromide (0.5 mg/ml) and a DNA (1 kb) molecular weight marker. The PCR products were purified and sequenced according to the methods described by many researchers (Jacobus and Gross 2015; Li et al. 1999). The band size formed by the product run in the gel was determined with the help of the appropriate marker. The band was carefully cut from the gel under UV light, and the purification process was carried out by applying the gel purification protocol of the manufacturer (Invitrogen PureLink Quick Gel Extraction Kit, Cat. no: K210012). The PCR products were sent for sequencing analysis with service procurement, and the data (sequencing results) were analyzed using GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequencing results were uploaded onto the NCBI

database. Accession numbers were obtained from the NCBI database.

Phylogenetic analysis

The phylogenetic analysis of the bacterial isolates was conducted using the neighbor-joining method and the MEGA 7 software (Oztas Gulmus and Gormez 2020b). A phylogenetic tree was created with the 16S gene sequencing data of the identified bacteria, their type strains and related type strains, and outgroup to determine their evolutionary relationships. The evolutionary relationships were inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with a sum of branch length of 0.43955091 was identified. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was seen next to the branches (Felsenstein 1992). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed by using the *p*-distance method (Nei and Kumar 2000) and are presented in units of the number of base differences per site.

Screening of the enzyme activity of the bacterial isolates

All isolates were screened for enzyme activity to reveal their ability to produce extracellular hydrolase enzymes including amylase, cellulase, lipase and protease.

Amylase activity

Amylolytic activity was examined by the use of the streak-plate technique, with a medium containing (w/v) soluble starch (1%), peptone (1%), KH₂PO₄ (0.5%) and agar (1.5%) and calibrated to pH 7.0. The isolates producing clear zones in this medium after flooding the plates with Lugol's solution (0.5% w/v and 1% w/v in distilled water) were considered amyolytic (Yadav et al. 2018).

Cellulase activity

Cellulase agar was used to determine cellulase activity. The isolated bacteria were streaked on cellulose agar medium. The isolates were streaked on the agar medium [carboxymethylcellulose (10 g/L), peptone (5 g/L), yeast (5 g/L), KH_2PO_4 (1 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g/L), NaCl (10 g/L) and agar (15 g/L)]. The cellulase activity of the isolates was determined by staining the petri dishes with Congo Red Dye (Baltaci et al. 2017).

Lipase activity

The LB medium contained 5.0 g peptone and 3.0 g beef (per liter). The pH of the medium was adjusted to 7 with NaOH (0.1 M). The isolation process was carried out by inoculating the samples into petri dishes in nutrient media containing tributyrin agar. The tributyrin medium contained 5.0 g peptone (per liter), 3.0 g yeast extract, 10 ml tributyrin and 15 g agar. The culture plates were incubated and examined at 12-h intervals for 2 days. Areas with clear zone formation in the petri dishes showed activity (Ertuğrul et al. 2007).

Protease activity

A medium that included 0.5% skim milk powder, 0.5% glucose and 2% agar (pH 7.0) was used to assess the proteolytic activity of the isolates. The proteolytic microorganisms developing in this agar showed a brilliant zone (spot), while the medium remained turbid if the bacterial strain was devoid of this activity (Oztas Gulmus and Gormez 2020a).

Results

Study sites

The hot springs in Turkey (in the cities of Agri, Erzurum, Mardin, Sirt and Van) are very close to volcanic fault systems (Mutlu and Güleç 1998). The altitude of the studied area is between 1125 and 1925 m above sea level. The source temperatures of the samples were measured as 38 °C (Pasinler), 60 °C

(Hista, Dargecit and Guclukonak) and 75 °C (Kopru, Davut and Hasanabdal). Most of the sources were found to be at “neutral pH”, except for the Hista, Köprü and Davut hot springs (pH 8.5 and 9.1).

Conventional analysis of the bacterial isolates

In the study, 83 bacterial isolates were isolated from Erzurum-Pasinler (26 isolates), Van-Hasanabdal (1 isolate), Siirt-Hista (6 isolates), Agri-Kopru (9 isolates), Agri-Davut (37 isolates), Mardin-Dargecit (2 isolates) and Sirt-Guclukonak (2 isolates) at different temperatures (40, 55, 65, 70, 75, 85 and 90 °C). The results of the morphological analyses showed that the bacterial isolates were Gram-positive and rod-shaped, and they formed endospores, except for 9 isolates. The colonies of the isolates had cream, white and pink colors with rough, smooth or shiny surfaces and smooth or irregular edges. The optimum temperatures of the isolated bacteria were determined at 55, 60 and 72 °C. The isolates were examined for their tolerance to varying temperatures. The majority of the isolates (97.5%) were accepted as obligate thermophiles with the optimum growth rates at temperatures in the range of 55–65 °C. The remaining part of the isolates (2.5%) was defined as thermotolerant with the optimum growth at 50–55 °C (Panosyan et al. 2020).

The isolates were shown to have the optimum growth rate at pH 5.0–9.5 and a 0–9% NaCl concentration. The results of the physiological tests revealed that most isolates could grow within high temperature and pH ranges (Supplementary Table S1).

16S rRNA sequencing

16S rRNA gene sequences were amplified with universal primers and visualized by gel electrophoresis. All isolates from the BLAST search were determined to contain 862–1419 base pair nucleotides. The accession numbers were recorded in the NCBI database (Supplementary Table S1). All bacteria showing thermophilic properties (high temperatures ranging from 40 to 85 °C) were closely related to the genera of *Bacillus*, *Weizmannia* and *Geobacillus*. In total, 33 strains of *B. subtilis* (39.76%), 15 strains of *G. kaustophilus* (18.07%), 14 strains of *B. licheniformis* (16.87%), 11 strains of *W. coagulans* (13.25%) and 10 strains of *Bacillus* sp. (12.04%) were identified

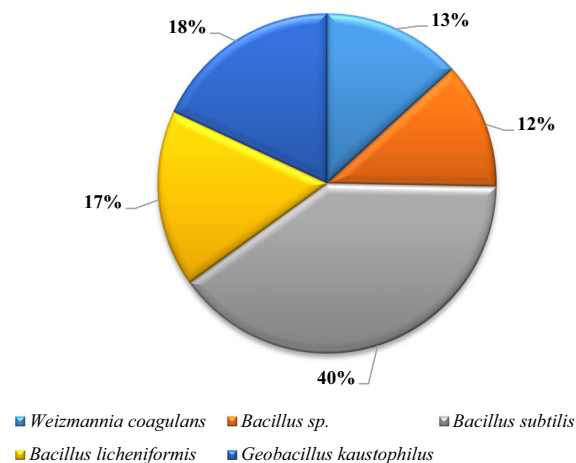


Fig. 2 Pie chart showing the distribution of bacteria resulting from sequencing in hot springs

(Fig. 2). According to the BLAST search, 10 isolates were registered as *Bacillus sp.* due to the affinity of BTX53–59, BTX72–73 and BTX79 to many different *Bacilli* with a high rate of sequence similarity. Based on the 16S rRNA gene sequence similarity analysis, 33 isolates demonstrated 95 to 99% sequence similarity to the species *B. subtilis*. Among the described species, a high sequence similarity of the isolates BTX42–47, BTX49, BTX50–52, BTX69–71, BTX77 and BTX80 to *G. kaustophilus* with a sequence homology rate of 97.59% was determined. Among the described species, the closest relative of the isolates BTX16–21, BTX29, BTX36–41 and BTX82 was *B. licheniformis* with a sequence homology rate of 98%. According to the results of the sequencing analysis, the closest relative of the isolates BTX62–68, BTX74–76 and BTX83 was *W. coagulans* with a sequence homology rate of 93.49–97.83% (Table 1).

Phylogenetic analysis

The analysis of 89 nucleotide sequences (83 isolates, 1 outgroup, 5 type strains) was conducted using the neighbor-joining method and the MEGA7 software (Kumar et al. 2016). As shown in Fig. 3, the bacteria belonging to the genera *Bacillus* and *Weizmannia* were divided into distinct groups from the genus *Geobacillus*, to become a single group with type strains as expected. The results showed that there was no significant difference between the groups and the bacteria in terms of the locations or optimum

temperatures of the isolates. The sequences of BTX1–BTX15, BTX30–BTX35, BTX48, BTX60 and BTX61 (0.038%), BTX81 (0.039%), BTX22–BTX28 (0.041%) and BTX78 (0.044%), differed from the *B. subtilis* Type Strain ATCC 6051, those of BTX62–BTX68 (0.051%), BTX74–BTX76 (0.059%) and BTX83 (0.051%) differed from the *W. coagulans* Type Strain ATCC7050, those of BTX53–BTX59 (0.010%), BTX72 (0.014%), BTX73 (0.008%) and BTX79 (0.012%) differed from the *Bacillus sp.* Type Strain ATCC 700,872, and those of BTX42–BTX47, BTX50–BTX52, BTX71 (0.010%), BTX49, BTX69, BTX77, BTX80 (0.008%) and BTX70 (0.009%) differed from the *G. kaustophilus* Type Strain NBRC 102445. As seen in the similarity index data formed with the type strains of the closely related isolates, BTX16–BTX21, BTX29, BTX36–41 and BTX82 differed in the range of 0.040–0.043% from the *B. licheniformis* Type Strain ATCC 14580. In a polyphyly study of the genus *Bacillus*, it was reported that these strains (*B. licheniformis* and *B. subtilis*) in the *subtilis* clade are very close to each other (Patel and Gupta 2020). In this context, the results of our study were similar to those reported in other studies in the literature.

Determination of the enzyme production ability of the bacterial isolates

The abilities of the isolates to produce the amylase, cellulase, lipase and protease enzymes were investigated (Table 2 and Supplementary Fig. S1, S2 and S3 “A, B, C and D”). The qualitative analysis showed that 27 (32.8%) of the isolates produced all hydrolase enzymes that were studied (amylase, cellulase, lipase and protease). In this study, 68 (82%), 34 (41%), 69 (83%) and 73 (88%) isolates were determined as producers of amylase, cellulase, lipase and protease, respectively. Except for four isolates (BTX9, BTX18, BTX27 and BTX55), all isolates were able to produce at least one of the studied enzymes (Fig. 4).

Discussion

The hot springs investigated in this study are in tectonically active parts of the Eastern and Southeastern Anatolia Regions of Turkey. Although similar studies have been conducted to isolate different

Table 1 The characteristic features of bacterial isolates

Isolate code	Physiological tests				Morphological tests				
	pH	NaCl (%)	T _{opt} (°C)/ temperature (°C)	Anaerobic growth	Movement	Morphology	Chain	Endospore	Gram test
BTX1, BTX2, BTX6, BTX7, BTX8, BTX10, BTX12, BTX13, BTX14, BTX23, BTX26, BTX27, BTX30, BTX32, BTX33, BTX34, BTX35	5–8.5	≤ 9	40–85/72	+	+	Bacil	+	+	+
BTX3, BTX4, BTX5, BTX15, BTX22, BTX28	5–8.5	≤ 9	40–85/72	+	+	Bacil	–	+	+
BTX25	5–8.5	≤ 9	40–85/72	–	+	Bacil	–	–	+
BTX9, BTX1, BTX24, BTX31	5–8.5	≤ 9	40–85/72	–	+	Bacil	–	+	+
BTX16	5–8.5	≤ 5	40–85/72	+	+	Coco Bacil	–	+	+
BTX17, BTX36	5–8.5	≤ 5	40–85/72	+	+	Bacil	–	+	+
BTX18	5–8.5	≤ 5	40–85/72	+	+	Bacil	–	–	+
BTX19	5–8.5	≤ 5	40–85/72	–	+	Bacil	+	+	+
BTX20, BTX21	5–8.5	≤ 5	40–85/72	+	+	Bacil	+	+	+
BTX29	5–7.5	≤ 5	40–85/72	+	+	Bacil	+	+	+
BTX37	5–7.5	< 5	40–75/60	–	+	Bacil	–	+	+
BTX38	5–7.5	< 5	40–75/60	+	+	Bacil	–	+	+
BTX39	5–7.5	< 5	40–75/60	+	+	Bacil	+	+	+
BTX40, BTX41	5–7.5	< 5	40–75/60	+	+	Bacil	+	+	+
BTX42, BTX50	5–7.5	< 7	40–75/60	+	+	Bacil	–	+	+
BTX43	5–7.5	< 7	40–75/60	–	+	Bacil	+	+	+
BTX44	5–7.5	< 7	40–75/60	–	+	Bacil	–	+	+
BTX45, BTX46, BTX47	5–7.5	< 7	40–75/60	+	+	Bacil	+	+	+
BTX48, BTX78	5–7.5	≤ 9	40–75/60	–	+	Bacil	–	–	+
BTX49	5–7.5	< 7	40–75/60	+	+	Bacil	–	–	+
BTX51	5–7.5	< 7	40–85/72	+	+	Bacil	–	–	+
BTX52	5–7.5	< 7	40–85/72	–	+	Bacil	–	+	+
BTX53	6–9.5	< 7	40–85/72	+	+	Bacil	+	+	+
BTX54	6–9.5	< 7	40–85/72	+	+	Bacil	+	+	+
BTX55	6–9.5	< 7	40–85/72	–	+	Bacil	–	+	+
BTX56	6–7.5	< 7	40–70/55	+	+	Bacil	+	+	+
BTX57	6–7.5	< 7	40–70/55	+	+	Bacil	–	+	+
BTX58	5–7.5	< 7	40–75/60	–	+	Bacil	–	–	+
BTX59	5–7.5	< 7	40–75/60	+	+	Bacil	+	+	+
BTX60	6–9.5	≤ 9	40–85/72	+	+	Bacil	+	+	+
BTX61	6–9.5	≤ 9	40–85/72	–	+	Bacil	–	–	+
BTX62, BTX63	5–8.5	≤ 6	40–85/72	+	+	Bacil	+	+	+
BTX64	5–8.5	≤ 6	40–85/72	+	+	Bacil	–	+	+
BTX65, BTX66, BTX67, BTX68	5–7.5	≤ 6	40–85/72	+	+	Bacil	+	+	+
BTX69, BTX71	5.5–7.5	< 7	40–75/60	+	+	Bacil	–	+	+

Table 1 continued

Isolate code	Physiological tests				Morphological tests				
	pH	NaCl (%)	T _{opt} (°C)/temperature (°C)	Anaerobic growth	Movement	Morphology	Chain	Endospore	Gram test
BTX70, BTX72, BTX73	5.5–7.5	< 7	40–75/60	+	+	Bacil	+	+	+
BTX74	5.5–7.5	< 6	40–75/60	+	+	Bacil	+	+	+
BTX75, BTX76	5.5–7.5	< 6	40–75/60	+	+	Bacil	–	+	+
BTX77	6–7.5	< 7	40–75/60	+	+	Bacil	–	–	+
BTX79	5–8	< 7	40–85/72	+	+	Bacil	+	+	+
BTX80	6.7.5	< 6	40–85/72	+	+	Bacil	+	+	+
BTX81	5–7.5	≤ 9	40–85/72	+	+	Bacil	+	+	+
BTX82	5–7.5	< 7	40–85/72	+	+	Bacil	+	+	+
BTX83	5–7.5	< 6	40–85/72	+	+	Bacil	+	+	+

T_{opt} Optimum temperature

thermophilic bacteria species in hot springs in these regions, different species have been detected in hot springs such as Hasanabdal, Kopru, Pasinler and Hista. According to the results of this study, *B. subtilis* (33), *G. kaustophilus* (15), *B. licheniformis* (14 isolates), *W. coagulans* (11) and *Bacillus* sp. (10) were identified as the most common *Bacilli* in the area. Amongst the isolated bacteria, *Bacillus* was the most dominant genus isolated from the hot springs examined in this study. In general, temperature is a very important parameter in controlling the richness and diversity of microorganisms in hot springs (Wang et al. 2013). Several studies on microbial diversity in hot springs have also reported on the abundance and diversity of *Bacillus* species (Abdollahi et al. 2021; Baltaci et al. 2017; Ifandi and Alwi 2018; Margaryan et al. 2018; Oztas Gulmus and Gormez 2020b; Panosyan et al. 2020). Although the locations of the Davut and Kopru hot springs are very close to each other, the isolated bacteria were quite different. *B. subtilis* and *B. licheniformis* were isolated from Davut, and *W. coagulans* was also isolated from Kopru. The locations of the other hot springs differ completely from each other. *W. coagulans* and *Bacillus* sp. were isolated from Hista for the first time. *W. coagulans* was isolated from Diyadin and Hasanabdal for the first time. *Geobacillus* sp. and *B. licheniformis* were reported from Hasanabdal in similar studies (Poddar and Das 2018). Besides, the strains identified within this study (*B. subtilis*, *G. kaustophilus*, *B.*

licheniformis, *W. coagulans* and *Bacillus* sp.) exhibited similar morphological, characteristic, physiological and biochemical properties in comparison to those reported in the literature (Baltaci et al. 2017; Oztas Gulmus and Gormez 2020a). All isolated strains (*W. coagulans*, *Bacillus* sp., *B. subtilis*, *Geobacillus* sp. and *B. licheniformis*) exhibited the ability to grow normally at high temperatures and pH values, which was not typical. The strains could grow at temperatures ranging from 40 to 85 °C, NaCl levels up to 9% (w/v) and pH ranges from 4 to 9. Only the strains belonging to the *B. licheniformis* isolates were able to grow on NaCl levels below 5% at 40–75 °C (optimum at 60 °C) and pH 4 to 9 (optimum at pH 7).

Microbial enzymes, especially enzymes derived from extremophile organisms, are widely used in different fields today. Thermophilic organisms are also microorganisms that adapt to extreme conditions, and so, they can create a variety of products that can be used even in unfavorable conditions. Many enzymes from thermophilic organisms such as amylase, catalase, cellulase, lactase, lipase, pectinase, pullulanase, protease, sucrase and xylanase are purified and produced commercially. These enzymes are widely used in many fields such as the textile, food, detergent, industry, beverage and health sectors (Oztas Gulmus and Gormez 2020b). The results obtained in this study are very important for further taxonomic studies and for understanding the ecological and environmental effects of microorganisms. Positive findings related to

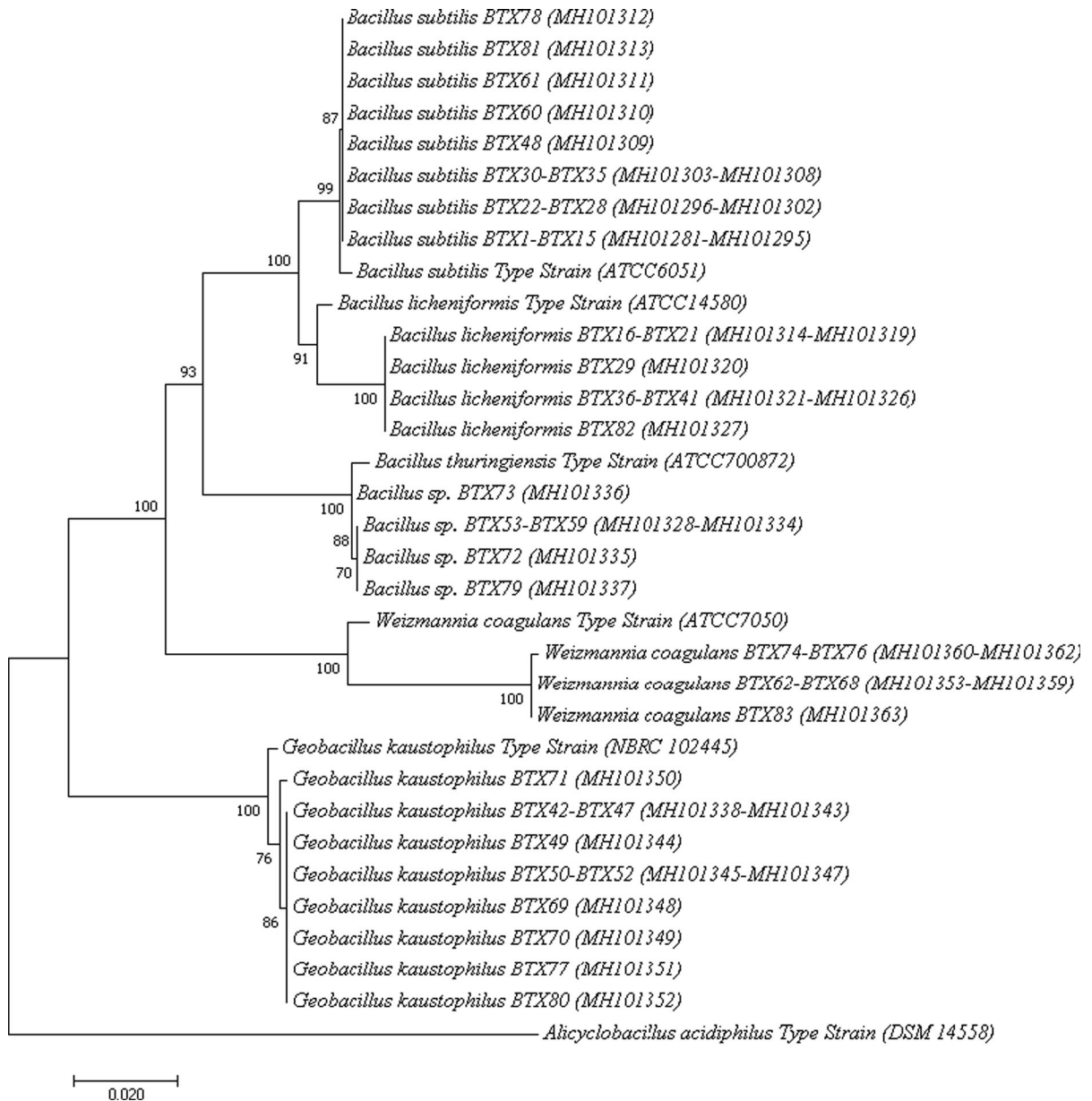


Fig. 3 The evolutionary relationship was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.34292109 is shown. The percentages of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1992). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The

evolutionary distances were computed using the p-distance method (Nei and Kumar 2000) and are in the units of the number of base differences per site. The analysis involved 33 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 754 positions in the final dataset in total. The evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016)

microorganisms and their hydrolytic activity performances are in a remarkable position in terms of biotechnological applications. In this study, the ability producing of thermostable enzymes (amylases,

cellulases, lipases and proteases) of the isolates was also determined. A number of isolates were identified throughout the study that demonstrated the activity of at least one hydrolytic enzyme. The qualitative study

Table 2 Biochemical characteristics and enzyme profiles of bacterial isolates

isolate code	Identification	Biochemical characteristics		Carbon source							Enzyme activities				
		Catalase	Oxidase	Glucose	Lactose	Galactose	Fructose	Citrate	Casein	Starch	Cellulase	Lipase	Protease	Amylase	
				+	+	+	+	+	+	+	+	+	+	+	+
BTX1	<i>Bacillus subtilis</i>	+	-	+	-	nd	+	nd	+	+	-	+	+		
BTX2	<i>Bacillus subtilis</i>	+	+	+	+	+	+	+	-	+	+	+	+		
BTX3	<i>Bacillus subtilis</i>	+	+	+	-	-	-	-	-	+	+	+	-		
BTX4	<i>Bacillus subtilis</i>	-	+	-	-	+	-	-	+	+	-	+	+		
BTX5	<i>Bacillus subtilis</i>	+	-	+	+	+	+	+	-	+	+	+	+		
BTX6	<i>Bacillus subtilis</i>	+	+	+	+	-	+	-	-	+	+	+	+		
BTX7	<i>Bacillus subtilis</i>	+	+	-	+	-	+	-	+	+	-	+	+		
BTX8	<i>Bacillus subtilis</i>	+	+	+	+	-	+	-	+	+	+	+	+		
BTX9	<i>Bacillus subtilis</i>	-	-	-	-	+	-	-	+	+	-	-	+		
BTX10	<i>Bacillus subtilis</i>	+	+	+	+	+	+	+	+	+	+	+	+		
BTX11	<i>Bacillus subtilis</i>	+	+	+	-	-	-	-	-	-	+	+	-		
BTX12	<i>Bacillus subtilis</i>	+	+	+	+	+	+	+	+	+	+	+	+		
BTX13	<i>Bacillus subtilis</i>	-	+	-	+	-	+	-	+	+	+	+	+		
BTX14	<i>Bacillus subtilis</i>	+	+	+	+	+	+	-	-	+	-	+	+		
BTX15	<i>Bacillus subtilis</i>	-	+	-	-	-	-	-	-	-	+	+	-		
BTX16	<i>Bacillus licheniformis</i>	+	+	+	-	+	-	-	-	-	+	+	+		
BTX17	<i>Bacillus licheniformis</i>	+	+	+	-	-	-	-	+	+	+	+	+		
BTX18	<i>Bacillus licheniformis</i>	-	nd	-	nd	+	-	nd	-	-	+	-	-		
BTX19	<i>Bacillus licheniformis</i>	+	+	+	+	+	+	+	+	+	-	+	+		
BTX20	<i>Bacillus licheniformis</i>	+	+	+	+	-	+	+	+	+	+	+	+		
BTX21	<i>Bacillus licheniformis</i>	-	+	-	+	+	+	-	-	-	+	+	-		
BTX22	<i>Bacillus subtilis</i>	-	+	+	+	+	+	+	+	+	+	-	+		
BTX23	<i>Bacillus subtilis</i>	+	+	+	+	-	+	+	+	+	+	+	+		
BTX24	<i>Bacillus subtilis</i>	+	+	-	-	-	+	-	+	+	+	+	+		
BTX25	<i>Bacillus subtilis</i>	-	-	-	-	+	-	-	-	-	+	+	-		
BTX26	<i>Bacillus subtilis</i>	+	+	nd	+	-	+	+	+	-	+	+	+		
BTX27	<i>Bacillus subtilis</i>	+	+	+	-	-	+	-	+	-	+	+	-		
BTX28	<i>Bacillus subtilis</i>	+	+	-	-	+	-	-	+	-	+	+	+		
BTX29	<i>Bacillus licheniformis</i>	-	nd	+	+	nd	+	-	-	+	-	+	+		
BTX30	<i>Bacillus subtilis</i>	+	+	+	+	+	+	+	+	+	+	+	+		
BTX31	<i>Bacillus subtilis</i>	+	+	+	-	+	-	+	-	-	+	-	+		

Table 2 continued

isolate code	Identification	Biochemical characteristics				Carbon source							Enzyme activities				
		Catalase		Oxidase	Glucose	Lactose	Galactose	Fructose	Citrate	Casein	Starch	Cellulase	Lipase	Protease	Amylase		
		+	+	+												+	+
BTX32	<i>Bacillus subtilis</i>	+	+	-	+	-	+	+	+	-	+	+	+	+	-	+	+
BTX33	<i>Bacillus subtilis</i>	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-
BTX34	<i>Bacillus subtilis</i>	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
BTX35	<i>Bacillus subtilis</i>	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
BTX36	<i>Bacillus licheniformis</i>	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-
BTX37	<i>Bacillus licheniformis</i>	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-
BTX38	<i>Bacillus licheniformis</i>	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-
BTX39	<i>Bacillus licheniformis</i>	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX40	<i>Bacillus licheniformis</i>	+	+	nd	+	+	+	+	-	-	-	-	-	-	-	-	-
BTX41	<i>Bacillus licheniformis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX42	<i>Geobacillus kaustophilus</i>	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+
BTX43	<i>Geobacillus kaustophilus</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX44	<i>Geobacillus kaustophilus</i>	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-
BTX45	<i>Geobacillus kaustophilus</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX46	<i>Geobacillus kaustophilus</i>	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+
BTX47	<i>Geobacillus kaustophilus</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX48	<i>Bacillus subtilis</i>	nd	+	-	-	+	-	-	nd	+	+	+	+	+	+	+	+
BTX49	<i>Geobacillus kaustophilus</i>	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BTX50	<i>Geobacillus kaustophilus</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX51	<i>Geobacillus kaustophilus</i>	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
BTX52	<i>Geobacillus kaustophilus</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX53	<i>Bacillus sp.</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX54	<i>Bacillus sp.</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX55	<i>Bacillus sp.</i>	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+
BTX56	<i>Bacillus sp.</i>	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
BTX57	<i>Bacillus sp.</i>	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+
BTX58	<i>Bacillus sp.</i>	-	+	-	nd	+	-	-	-	-	-	-	-	-	-	-	-
BTX59	<i>Bacillus sp.</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX60	<i>Bacillus subtilis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX61	<i>Bacillus subtilis</i>	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-
BTX62	<i>Weizmanni coagulans</i>	-	+	+	nd	+	+	+	+	+	+	+	+	+	+	+	+

Table 2 continued

isolate code	Identification	Biochemical characteristics				Carbon source						Enzyme activities					
		Catalase		Oxidase	Glucose	Lactose	Galactose	Fructose	Citrate	Casein	Starch	Cellulase	Lipase	Protease	Amylase		
		+	+	+													
BTX63	<i>Weizmanni coagulans</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX64	<i>Weizmanni coagulans</i>	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+
BTX65	<i>Weizmanni coagulans</i>	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
BTX66	<i>Weizmanni coagulans</i>	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
BTX67	<i>Weizmanni coagulans</i>	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX68	<i>Weizmanni coagulans</i>	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX69	<i>Geobacillus kaustophilus</i>	-	-	+	nd	+	+	+	+	+	+	+	+	+	+	+	+
BTX70	<i>Geobacillus kaustophilus</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX71	<i>Geobacillus kaustophilus</i>	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	-
BTX72	<i>Bacillus sp.</i>	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
BTX73	<i>Bacillus sp.</i>	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
BTX74	<i>Weizmanni coagulans</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX75	<i>Weizmanni coagulans</i>	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	-
BTX76	<i>Weizmanni coagulans</i>	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+
BTX77	<i>Geobacillus kaustophilus</i>	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-
BTX78	<i>Bacillus subtilis</i>	-	+	nd	nd	+	-	nd	-	-	-	-	+	+	+	+	+
BTX79	<i>Bacillus sp.</i>	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BTX80	<i>Geobacillus kaustophilus</i>	nd	-	nd	nd	+	+	+	+	+	+	+	-	-	+	+	+
BTX81	<i>Bacillus subtilis</i>	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-
BTX82	<i>Bacillus licheniformis</i>	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
BTX83	<i>Weizmanni coagulans</i>	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+

(+); observed reaction, (-); negative reaction, (nd); not detected

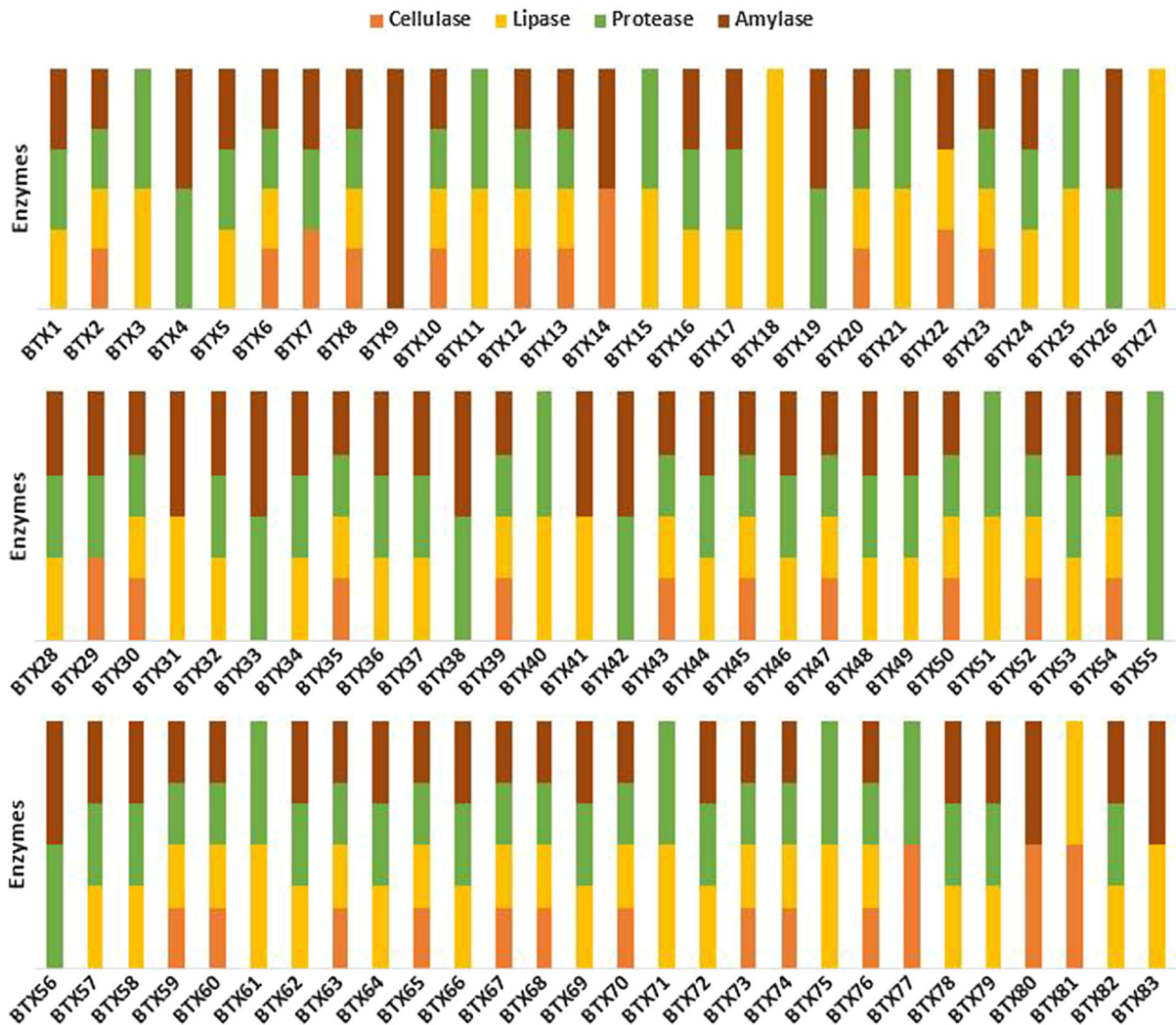


Fig. 4 According to the qualitative analysis results, it was determined that 27 (32.8%) isolates could produce amylase, protease, cellulase and lipase. In the study, 68 (82%), 73 (88%),

34 (41%) and 69 (83%) isolates were determined as producers of amylase, protease, cellulase and lipase, respectively

results revealed that 27 (32.8%) bacterial isolates had amylase, cellulase, lipase and protease production capacity. These bacterial isolates were *B. subtilis* (10 isolates), *G. kaustophilus* (6 isolates), *W. coagulans* (6 isolates), *Bacillus* sp. (3 isolates) and *B. licheniformis* (2 isolates). Similarly, there are many studies on the enzyme activities of *Bacillus*, *Geobacillus*, *Weizmannia* and *Anoxybacillus* species in the literature (Baltaci et al. 2017; Mohammad et al. 2017; Oztas Gulmus and Gormez 2020a; Yanmis et al. 2015). According to Baltaci et al., *Bacillus pumilus* and *B. licheniformis* showed amylase, cellulase, lipase and protease activities (Baltaci et al. 2017). Amylase, cellulase, lipase

and protease are in the class of hydrolytic enzymes that are very important for industrial applications. Among these, amylases are used in industrial processes such as starch hydrolysis, production of fruit juices and fruit syrups, textile, beer, ethanol fermentation, starch processing, detergents, bread production and wastewater treatment (Aiyer 2005; Lahiri et al. 2021). Lipases are frequently used in the cleaning (detergent), nutrition (food), health (pharmaceutical) and agriculture (chemicals) industries. Having a very broad place in the international market (60%), proteases have a wide range of uses in various sectors such as nutrition (food), cleaning (detergent), leather (textile),

cosmetics (perfumery) and medicine (pharmaceuticals) (Gupta et al. 2002; Laxman et al. 2005). Hydrolytic enzymes are popular and frequently used in the industry (Oztas Gulmus and Gormez 2020b). Moreover, the cellulase enzyme has a wide range of applications in biotechnological and industrial fields. Some of the important applications it has are in the food, feed, beer, wine, textile, detergent, paper and agricultural industries. Despite the commercial importance of this enzyme, its high production cost limits its use in the industry for the transformation of cellulose. Therefore, research on new and different sources of microbial cellulase for more stable and active enzymes has increased. *Bacillus* species, which can be isolated from many environments, as well as hot water sources, are of great importance in terms of enzyme production. They are also some of the most effective proteolytic and carbohydrate sources. They meet the enzyme needs in many areas such as the production of starch, bread, fruit juice, paper bleaching and brewing (Bhati and Sharma 2021; Kurt and Cekmecelioglu 2021; Singh et al. 2021). For this reason, many enzymes synthesized by *Bacillus* species are used in many areas in the industry (Oztas Gulmus and Gormez 2020b). Bacteria isolated in this study may be preferred in future studies because they can grow at high temperatures, varying salinity (NaCl) and pH ranges, and they produce stable enzymes that can work in extreme conditions.

Conclusions

In this study, bacterial isolates were identified with 16S rRNA gene sequencing from seven hot springs in the Eastern and Southeastern Anatolia Regions of Turkey. According to the 16S rRNA sequencing results, most of the bacterial isolates were observed to belong to the genera *Bacillus* (68.67%), *Weizmannia* (13.25%) and *Geobacillus* (18.07%). These results showed that *Bacillus* was the most dominant genus among all isolates in the seven hot springs from where the samples were collected.

Although the bacteria isolated from the different hot springs were similar on the genus level, they were quite diverse in terms of their environmental attributes, encompassing a temperature range from 40 to 85 °C, a pH range from 4 to 9, and a salinity range from 0 to 9%. Because of these important properties,

wide pH and temperature ranges, these enzymes can be preferred in industrial applications. In this context, this is a preliminary study showing the diversity of bacteria that produce crucial thermophilic enzymes in industrial and biotechnological terms.

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Authors' contributions OU, Conceptualization, Methodology, Validation, Investigation, Resources, Formal analysis, Writing—Original Draft, Writing—Review & Editing; AG, Methodology, Investigation, Resources, Supervision. CO, Conceptualization, Methodology, Investigation, Resources. All authors read and approved the final manuscript.

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Data availability The datasets generated and/or analyzed during the current study are available from supplementary information files and the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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