Identification, Characterization, and Hydrolase Producing Performance of Thermophilic Bacteria: Geothermal Hot Springs in Eastern and Southeastern Anatolia Region of Turkey

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Abstract

In the last twenty years, researchers have increasingly focused on the rich microorganism-based diversity of natural hot spring resources to explore the benefits of thermophiles in industrial and biotechnological fields. For this purpose, a total of 83 thermophilic bacilli were isolated from 7 different geothermal hot springs (at temperatures ranging between 40 and 85 °C) located in the east and Southeastern of Turkey. These isolates were identified by different methods such as physiological, morphological, biochemical, molecular properties. According to 16S rRNA gene sequence analysis, 5 different isolates (*Bacillus coagulans*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus thuringiensis*, and *Geobacillus kaustophilus*) were identified. It was observed that *B. licheniformis* and *B. subtilis* were the most species obtained from the researched hot spring sources. Phylogenetic relationships of isolates were evaluated with the help of a phylogenetic tree. The conditions of bacterial isolates to synthesize various hydrolytic enzymes such as protease, cellulase, lipase, and amylase were investigated. When the potential of isolates to produce hydrolytic enzymes was examined, protease 73 (88%), cellulase 34 (41%), lipase 69 (83%), and amylase 68 (82%) were detected. All isolates have at least one or more extracellular protease, cellulase, amylase, or lipase activity. Besides, 32.8% (27) of bacterial isolates were able to synthesize all of the hydrolytic enzymes.

Introduction

Geothermal hot springs generally host microorganisms that can be active and produce various enzymes under extreme conditions, depending on their environment (Mehta et al. 2016, Panosyan et al. 2020, Short 1998). The microorganisms and their enzymes, products which are the main target of alternative sources, are often used in many industrial areas. These enzymes in the biotech industry; it is widely used in many 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).

Thermophilic microorganisms can thrive in different ecological habitats, both aquatic and terrestrial. These habitats are known as hydrothermal facilities, sugar heaps, hot springs, deep seas, and volcanic regions. Hot springs are one of the most important habitats for thermophilic microorganisms (Aanniz et al. 2015). Therefore, thermophilic microorganisms attract the attention of researchers as an important source of new and high-temperature resistant enzymes such as xylanases, DNA polymerases, amylases, cellulases, phytases, chitinases, proteases, lipases, and pectinases (Falcicchio et al. 2021, Lee et al. 2010, Luo et al. 2017, Msarah et al. 2020, Tango and Islam 2002). Although the necessary equipment and approaches have changed in parallel with the development of molecular biology in the determination of microorganisms that produce these enzymes in recent years, it is still important to perform physiological, morphological, and biochemical analyzes for pure culture isolation studies (Abdollahi et al. 2021, Akmar et al. 2011, Bouacem et al. 2021, Chan et al. 2015, Ifandi and Alwi 2018, Kılınç et al. 2021, Tirawongsaroj et al. 2008). In many studies, it was demonstrated that thermophilic microorganisms have different phylogenetic varieties which include both bacteria and representatives of the archaea domain. In the bacteria domain, it is known that the most isolated thermophilic strains belonged to *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 many different hot springs around the world at different times including Turkey (Yanmis et al. 2015) India (Verma et al. 2018), Iran (Abootalebi et al. 2020), Indonesia (Safitri et al. 2020), Jordanian (Mohammad et al. 2017), Faisalabad (Saleh et al. 2020), Tunisia (Salem et al. 2020), Brazil (Bernardo et al. 2020), Egypt (Saeed et al. 2020), Malaysian (Msarah et al. 2020), Vietnam (Dang et al. 2018), Saudi Arabia (Al-Johani et al. 2016), Japan (Akita et al. 2017) and other countries.

Turkey is one of the world's most important countries because of its many thermal water reserves (Oztas Gulmus and Gormez 2020b). Hot springs in the east and southeast of Turkey are the reservoir of natural resources for biotechnological based study (Güven et al. 2018). In many studies, different bacteria and archaea have been reported analysis of microorganisms got from thermal source east and southeast of Turkey Hasanabdal (Poli et al. 2012, Savas et al. 2009, Yanmis et al. 2015), Sırnak (Derya and Ahmet 2014), Diyaradin (Baltacı et al. 2017), Dargeçit (Acer et al. 2015), Güclükonak (Poli et al. 2012), and Pasinler (Adigüzel et al. 2011, Oztas Gulmus and Gormez 2020b). It is also observed that many of the isolates belong to the thermophilic *Bacillus* and *Geobacillus* (Oztas Gulmus and Gormez 2020b, Yilmaz et al. 2016). Most of the enzymes produced by these microorganisms in extreme conditions and which are especially important in the hydrolysis of biomass have not been discovered, yet (Thakur et al. 2021). Therefore, it is necessary to isolate and investigate new bacilli in the production of biotechnologically thermostable enzymes (Margaryan et al. 2018).

In this study, it was aimed to isolate bacteria from 7 hot springs at high altitude in Eastern and Southeastern Anatolia regions, to identify them by conventional and molecular methods, to determine the optimum growth conditions of the isolates, and to analyze for their capability to produce hydrolase enzymes such as protease, amylase, lipase, and cellulase.

Materials And Methods

Collection of biological material and isolation of bacteria

The 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 Köprü; 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 (Güclükonak Lat.: 37,476818, Lng.: 41,939351) in August and September 2016. The locations of the different geothermal resources in this study are shown in the map in Fig. 1. The samples were collected from the shallow and muddy areas at the outlet of the hot springs in sterile cap bottles surrounded by an aluminum foil in a way not to receive sunlight. The temperature and pH parameters of the spring waters were determined by a portable device that can measure both pH and temperature (AZ 8685). The collected samples from each location were transferred to sterile tubes containing physiological saline water (0.85 g NaCl and 100 mL distilled water). 0.1 mL of diluent from each Eppendorf was transferred to Luria Bertani (LB) (Sigma-Aldrich) culture medium and 100 mL Erlenmeyer flasks and incubated at different temperatures (40 °C, 55 °C, 65 °C, 70 °C, 75 °C, 85 °C, and 90 °C) for 24-72 hours. Single colonies forming different colony morphologies were determined, and

these pure isolates were streaked again on solid media. The purified isolates were preserved in an LB medium containing 15% glycerol at -80 °C until use in subsequent studies (Gormez et al. 2013).

Conventional characterization of bacteria

The colony morphology and motility tests of all isolates were examined by light microscope (Best Scopes BS-2030t + BLC - 450). Morphological properties of isolated bacteria were determined by Gram staining, endospore formation, and mobility tests using standard procedures (Bernard et al. 2017). Physiological properties (temperature, pH, and NaCl concentrations) of all isolates were tested at various stages of growth under aerobic conditions (Oztas Gulmus and Gormez 2020b). For this purpose, all the isolates were incubated at different temperatures (40-90 °C), different pH levels (4.0-12.0) and variable NaCl concentrations (up to 10% w/v), and their growths were evaluated by measuring optical density (OD) at 600 nm with a spectrophotometer. Biochemical properties (oxidase, catalase, and the ability of carbon source usage) of isolates were performed according to the methods described by Harley and Prescott (2002) (Prescott 2002).

Molecular identification of bacteria

Genomic DNA isolation

Total genomic DNA was performed by modifying Sambrook's classic phenol: chloroform: isoamyl alcohol method (Sambrook 1987) and samples were stored at -80 °C (Prescott and Harley 2002).

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

PCR amplification of bacterial isolates was conducted by using a GenePro Thermal Cycler (Bioer Technology Co., Ltd) TC-E-96G Model. In order to amplify the 16S rRNA gene region of DNA samples, universal primers (forward primer 27F = 5'-GAGTTTGATCCTGGCTCA-3'; reverse primer 1385R = 5'-CGGTGTGT[A/G]CAAGGCCC-3') were used. Amplification reactions were carried out in 25 µL volumes containing: 10X PCR buffer (Applied Biosystems, Roche, California, USA), 25 µM magnesium chloride, 25 mM dNTP, 25 pmol of each primer, and 5 unit/µ Taq DNA polymerase (Applied Biosystems). We performed the PCR 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. 1% agarose gel was prepared for visualization of PCR products by gel electrophoresis. The PCR products were purified, cloned, and sequenced according to the methods described by many researchers (Jacobus and Gross 2015, Li et al. 1999). The sequence data of all isolates were analyzed by using GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) EzTaxon (<https://www.ezbiocloud.net/>) server and the results were uploaded to the NCBI GenBank sequence database.

Phylogenetic analysis

The phylogenetic tree of the bacterial isolates was formed by using the neighbor-joining method with the MEGA 7 software (Oztas Gulmus and Gormez 2020b).

Screening of enzyme activity of the bacterial isolates

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

Amylase Activity, All the isolates were inoculated on an agar plate containing 1% starch and amylase production after feeding with Lugol solution was determined as clear and large areas (Yadav et al. 2018).

Protease Activity, All the isolates were streaked on an agar medium containing 10% skimmed milk and the protease activity of the isolates that form a transparent zone on the agar plate was recorded as positive (Oztas Gulmus and Gormez 2020a).

Lipase Activity, All the isolates were streaked on an agar medium containing 1% tributyrin and the lipase activity of the isolates that form a hydrolysis zone on the agar plate was recorded as positive (Ertuğrul et al. 2007).

Cellulase Activity, All of the isolates were streaked on agar medium [carboxymethylcellulose (10 g/L), peptone (5 g/L), yeast (5 g/L), KH₂PO₄ (1 g/L), MgSO₄·7H₂O (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).

Results

The distribution of the hot springs in Turkey parallels the distribution of the fault systems and volcanic areas (Mutlu and Güleç 1998). The altitude of the studied area is between 1125 and 1925 meters 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 Hista, Köprü, and Davut hot springs (pH 8.5 and 9.1).

The results of conventional test

In the study, 83 bacterial isolates were got 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 Sirnak-Guclukonak (2 isolates) at different temperatures (40 °C, 55 °C, 65 °C, 70 °C, 75 °C, 85 °C, and 90 °C). According to the results of morphological tests, it was determined that all isolates were gram positive and rod-shaped, and formed endospores except for 9 isolates. Colonies of the isolates were cream, white and pink colors with rough, smooth, or shiny surfaces and smooth or irregular edges. The optimum temperature of isolated bacteria was determined as 55, 60, and 72 °C. Therefore, most of the isolates were considered as obligate thermophiles, which showed optimum growth at temperatures between 55–65 °C, whereas those that grew optimally at 50–55 °C were defined as thermotolerant (Panosyan et al. 2020).

Therefore, most of the isolates (97.5%) were being obligate thermophiles with optimum growth at temperatures between 55-65 °C, while some isolates (2.5%) that grow optimally at 50-55 °C were recorded as heat resistant. Most isolates were shown optimum growth in the pH 5.0 - 9.5 and 0 – 9% NaCl concentration range. The results of the physiological tests revealed that most isolates could grow in high temperature and pH ranges (Table 1).

Table 1

The characteristic features of bacterial isolates

Isolate Code	Physiological Tests				Morphological Tests				
	pH	NaCl (%)	Temperature range 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	-	+	+
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	+	+	+

The results of 16s rRNA sequencing analyses of bacterial isolates

16S rRNA gene regions were amplified with universal primers and visualized by gel electrophoresis. According to the results of the blast analysis, it was determined that all isolates contained 862-1419 base pair nucleotides (Table 2). All bacteria showing thermophilic properties were closely related to members of 2 different genera, *Bacillus* and *Geobacillus* (Fig. 2). Sequence analysis identified 68 strains of *Bacillus* (81.93%) and 15 strains of *Geobacillus* (18.07%). According to the results of blast analysis, the closest match (100% similarity) to isolate (BTX53-59, BTX72-73, and BTX79) was with *B. thuringiensis* strain KNU-07, which was isolated from Korea. Based on the 16S rRNA gene sequence similarity analysis, 33 isolates demonstrated 95 to 99% sequence similarity to species *B. subtilis*. Among the described species, the closest relatives of isolates BTX42-47, BTX49-52, BTX69-71, BTX77, and BTX78 were *Geobacilluskaustophilus* with a sequence homology rate of 99%. Among the described species, the closest relatives of isolates BTX16-19, BTX20, BTX21, BTX29, BTX36-41, BTX72, BTX73, and BTX79 were *B. licheniformis* with a sequence homology rate of 97-99%. Among the described species, the closest relatives of isolates BTX62-68, BTX74-76, and BTX83 were *B. coagulans* with a sequence homology rate of 93-98%.

The results of phylogenetic analysis

The phylogenetic tree was created with 16S gene sequencing data of identified bacteria, their type strains, and outgroup to determine the evolutionary relationships (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 = 0.34395833 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). 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 (Chakraborty 2002) and are in the units of the number of base differences per site. The analysis of 89 nucleotide sequences (83 isolates, 5 type strains, and 1 outgroup) was conducted in MEGA7 (Kumar et al. 2016). As shown in the figure, bacteria belonging to the genus *Bacillus* were divided into distinct groups from the genus *Geobacillus*, to be a single group with type strains as expected. The results showed that there was no important difference between the groups and the bacteria in terms of locations or optimum temperature of the isolates.

Table 2

Isolate Code	Identification	GenBank Number	Similarity* rate (%)	Base length** (nucleotide)	Location
				(nucleotide)	
BTX1	<i>Bacillus subtilis</i>	MH101281	99%	1397	Davut
BTX2	<i>Bacillus subtilis</i>	MH101282	99%	1387	Davut
BTX3	<i>Bacillus subtilis</i>	MH101283	99%	1385	Davut
BTX4	<i>Bacillus subtilis</i>	MH101284	99%	1389	Davut
BTX5	<i>Bacillus subtilis</i>	MH101285	99%	1386	Davut
BTX6	<i>Bacillus subtilis</i>	MH101286	99%	1384	Davut
BTX7	<i>Bacillus subtilis</i>	MH101287	99%	1382	Davut
BTX8	<i>Bacillus subtilis</i>	MH101288	99%	1381	Davut
BTX9	<i>Bacillus subtilis</i>	MH101289	99%	1386	Davut
BTX10	<i>Bacillus subtilis</i>	MH101290	99%	1386	Davut
BTX11	<i>Bacillus subtilis</i>	MH101291	99%	1380	Davut
BTX12	<i>Bacillus subtilis</i>	MH101292	99%	1386	Davut
BTX13	<i>Bacillus subtilis</i>	MH101293	99%	1385	Davut
BTX14	<i>Bacillus subtilis</i>	MH101294	99%	1383	Davut
BTX15	<i>Bacillus subtilis</i>	MH101295	99%	1387	Davut
BTX16	<i>Bacillus licheniformis</i>	MH101314	98%	1417	Davut
BTX17	<i>Bacillus licheniformis</i>	MH101315	98%	1410	Davut
BTX18	<i>Bacillus licheniformis</i>	MH101316	98%	1411	Davut
BTX19	<i>Bacillus licheniformis</i>	MH101317	98%	1414	Davut
BTX20	<i>Bacillus licheniformis</i>	MH101318	98%	1413	Davut
BTX21	<i>Bacillus licheniformis</i>	MH101319	98%	1416	Davut
BTX22	<i>Bacillus subtilis</i>	MH101296	99%	1384	Davut
BTX23	<i>Bacillus subtilis</i>	MH101297	99%	1384	Davut
BTX24	<i>Bacillus subtilis</i>	MH101298	99%	1384	Davut
BTX25	<i>Bacillus subtilis</i>	MH101299	99%	1387	Davut
BTX26	<i>Bacillus subtilis</i>	MH101300	99%	1384	Davut
BTX27	<i>Bacillus subtilis</i>	MH101301	99%	1383	Davut
BTX28	<i>Bacillus subtilis</i>	MH101302	99%	1386	Davut
BTX29	<i>Bacillus licheniformis</i>	MH101320	98%	1410	Davut
BTX30	<i>Bacillus subtilis</i>	MH101303	99%	1384	Davut
BTX31	<i>Bacillus subtilis</i>	MH101304	99%	1384	Davut
BTX32	<i>Bacillus subtilis</i>	MH101305	99%	1384	Davut
BTX33	<i>Bacillus subtilis</i>	MH101306	99%	1391	Davut
BTX34	<i>Bacillus subtilis</i>	MH101307	99%	1380	Davut
BTX35	<i>Bacillus subtilis</i>	MH101308	99%	1387	Davut
BTX36	<i>Bacillus licheniformis</i>	MH101321	98%	1410	Davut
BTX37	<i>Bacillus licheniformis</i>	MH101322	98%	1413	Pasinler
BTX38	<i>Bacillus licheniformis</i>	MH101323	98%	1419	Pasinler
BTX39	<i>Bacillus licheniformis</i>	MH101324	98%	1415	Pasinler
BTX40	<i>Bacillus licheniformis</i>	MH101325	98%	1418	Pasinler
BTX41	<i>Bacillus licheniformis</i>	MH101326	98%	1411	Pasinler

BTX42	<i>Geobacillus kaustophilus</i>	MH101338	98%	1415	Pasinler
BTX43	<i>Geobacillus kaustophilus</i>	MH101339	98%	1411	Pasinler
BTX44	<i>Geobacillus kaustophilus</i>	MH101340	98%	1410	Pasinler
BTX45	<i>Geobacillus kaustophilus</i>	MH101341	98%	1409	Pasinler
BTX46	<i>Geobacillus kaustophilus</i>	MH101342	98%	1418	Pasinler
BTX47	<i>Geobacillus kaustophilus</i>	MH101343	98%	1410	Pasinler
BTX48	<i>Bacillus subtilis</i>	MH101309	98%	1384	Pasinler
BTX49	<i>Geobacillus kaustophilus</i>	MH101344	98%	1410	Pasinler
BTX50	<i>Geobacillus kaustophilus</i>	MH101345	98%	1414	Pasinler
BTX51	<i>Geobacillus kaustophilus</i>	MH101346	98%	1412	Pasinler
BTX52	<i>Geobacillus kaustophilus</i>	MH101347	98%	1410	Hista
BTX53	<i>Bacillus thuringiensis</i>	MH101328	100%	873	Hista
BTX54	<i>Bacillus thuringiensis</i>	MH101329	100%	868	Hista
BTX55	<i>Bacillus thuringiensis</i>	MH101330	100%	866	Hista
BTX56	<i>Bacillus thuringiensis</i>	MH101331	100%	866	Guclukonak
BTX57	<i>Bacillus thuringiensis</i>	MH101332	100%	866	Guclukonak
BTX58	<i>Bacillus thuringiensis</i>	MH101333	100%	878	Dargecit
BTX59	<i>Bacillus thuringiensis</i>	MH101334	100%	864	Dargecit
BTX60	<i>Bacillus subtilis</i>	MH101310	99%	1384	Hista
BTX61	<i>Bacillus subtilis</i>	MH101311	99%	1384	Hista
BTX62	<i>Bacillus coagulans</i>	MH101353	98%	1339	Davut
BTX63	<i>Bacillus coagulans</i>	MH101354	98%	1352	Kopru
BTX64	<i>Bacillus coagulans</i>	MH101355	98%	1343	Kopru
BTX65	<i>Bacillus coagulans</i>	MH101356	98%	1346	Kopru
BTX66	<i>Bacillus coagulans</i>	MH101357	98%	1347	Hasanabdal
BTX67	<i>Bacillus coagulans</i>	MH101358	98%	1343	Kopru
BTX68	<i>Bacillus coagulans</i>	MH101359	98%	1343	Pasinler
BTX69	<i>Geobacillus kaustophilus</i>	MH101348	98%	1408	Pasinler
BTX70	<i>Geobacillus kaustophilus</i>	MH101349	98%	1410	Pasinler
BTX71	<i>Geobacillus kaustophilus</i>	MH101350	98%	1413	Pasinler
BTX72	<i>Bacillus thuringiensis</i>	MH101335	100%	867	Pasinler
BTX73	<i>Bacillus thuringiensis</i>	MH101336	100%	862	Pasinler
BTX74	<i>Bacillus coagulans</i>	MH101360	98%	1355	Pasinler
BTX75	<i>Bacillus coagulans</i>	MH101361	98%	1341	Pasinler
BTX76	<i>Bacillus coagulans</i>	MH101362	98%	1350	Pasinler
BTX77	<i>Geobacillus kaustophilus</i>	MH101351	98%	1410	Pasinler
BTX78	<i>Bacillus subtilis</i>	MH101312	99%	1394	Pasinler
BTX79	<i>Bacillus thuringiensis</i>	MH101337	100%	867	Kopru
BTX80	<i>Geobacillus kaustophilus</i>	MH101352	98%	1408	Kopru
BTX81	<i>Bacillus subtilis</i>	MH101313	99%	1387	Kopru
BTX82	<i>Bacillus licheniformis</i>	MH101327	98%	1409	Kopru
BTX83	<i>Bacillus coagulans</i>	MH101363	98%	1343	Kopru
*Percent identity with 16S rRNA sequence based on GenBank data.					
**Number of 16S rRNA nucleotides.					

Determination of enzyme production ability of bacterial isolate

In the study, the ability of all isolates was examined to produce amylase, protease, cellulose, and lipase enzymes (Table 3). According to the qualitative analysis results, it was determined that 27 (32.8%) isolates can produce amylase, protease, cellulase, and lipase (Fig. 4). In the study, 68 (82%), 73 (88%), 34 (41%), and 69 (83%) isolates were determined as producers of amylase, protease, cellulase, and lipase, respectively. Among the isolates, BTX55 had only protease activity, BTX18 and BTX27 had lipase, BTX9 isolate had amylase activity, while all the remaining isolates had over one enzyme activity (Fig 3).

Table 3

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	Prot
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</i>	+	+	-	-	+	-	-	+	+	-	+	+

	<i>subtilis</i>												
BTX29	<i>Bacillus licheniformis</i>	-	nd	+	+	nd	+	-	-	+	+	-	+
BTX30	<i>Bacillus subtilis</i>	+	+	+	+	+	+	+	+	+	+	+	+
BTX31	<i>Bacillus subtilis</i>	+	+	+	-	+	-	-	-	+	-	+	-
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 thuringiensis</i>	+	+	+	+	+	+	+	+	-	-	+	+
BTX54	<i>Bacillus thuringiensis</i>	+	+	+	+	+	+	-	+	+	+	+	+
BTX55	<i>Bacillus thuringiensis</i>	+	+	+	-	+	-	-	+	+	-	-	+
BTX56	<i>Bacillus thuringiensis</i>	+	-	+	+	-	+	+	+	+	-	-	+
BTX57	<i>Bacillus thuringiensis</i>	+	+	+	-	-	+	-	+	+	-	+	+

BTX58	<i>Bacillus thuringiensis</i>	-	+	-	nd	+	-	-	-	-	-	+	+
BTX59	<i>Bacillus thuringiensis</i>	+	+	+	+	+	+	+	+	+	+	+	+
BTX60	<i>Bacillus subtilis</i>	+	+	+	+	-	+	+	+	+	+	+	+
BTX61	<i>Bacillus subtilis</i>	-	-	+	-	+	-	-	-	+	-	+	+
BTX62	<i>Bacillus coagulans</i>	-	+	+	+	+	nd	+	+	-	-	+	+
BTX63	<i>Bacillus coagulans</i>	+	+	+	+	+	+	+	+	+	+	+	+
BTX64	<i>Bacillus coagulans</i>	+	+	-	-	-	+	-	+	+	-	+	+
BTX65	<i>Bacillus coagulans</i>	+	+	+	+	+	+	-	+	+	+	+	+
BTX66	<i>Bacillus coagulans</i>	-	+	+	+	+	+	-	+	-	-	+	+
BTX67	<i>Bacillus coagulans</i>	+	-	+	+	+	+	+	+	+	+	+	+
BTX68	<i>Bacillus coagulans</i>	+	-	+	+	+	+	+	+	+	+	+	+
BTX69	<i>Geobacillus kaustophilus</i>	-	-	+	nd	+	+	nd	+	-	-	+	+
BTX70	<i>Geobacillus kaustophilus</i>	+	+	+	+	-	+	+	+	-	+	+	+
BTX71	<i>Geobacillus kaustophilus</i>	+	+	+	-	+	-	-	+	+	-	+	+
BTX72	<i>Bacillus thuringiensis</i>	-	+	-	+	-	+	+	+	+	-	+	+
BTX73	<i>Bacillus thuringiensis</i>	+	+	+	+	+	+	-	+	-	+	+	+
BTX74	<i>Bacillus coagulans</i>	+	+	+	+	+	+	+	+	+	+	+	+
BTX75	<i>Bacillus coagulans</i>	+	+	+	-	-	+	-	+	+	-	+	+
BTX76	<i>Bacillus coagulans</i>	+	+	+	-	+	-	-	+	+	+	+	+
BTX77	<i>Geobacillus kaustophilus</i>	+	-	-	-	+	-	-	-	+	+	-	+
BTX78	<i>Bacillus subtilis</i>	-	+	nd	nd	+	-	nd	-	-	-	+	+
BTX79	<i>Bacillus thuringiensis</i>	-	+	+	+	+	+	+	+	+	-	+	+
BTX80	<i>Geobacillus kaustophilus</i>	nd	-	nd	nd	+	+	+	+	-	+	-	-
BTX81	<i>Bacillus subtilis</i>	+	+	+	+	+	+	-	-	-	+	+	-
BTX82	<i>Bacillus licheniformis</i>	+	+	+	+	-	+	-	+	+	-	+	+
BTX83	<i>Bacillus coagulans</i>	+	-	+	+	-	+	-	+	+	-	+	-
(+); observed reaction, (-); negative reaction, (nd); not detected													

Discussion

The hot springs investigated in the present study are in tectonically active regions of the Eastern and Southeastern Anatolia regions of Turkey. 83 bacterial isolates with growth potential at 40°C and 75°C were got from Davut (37 isolates), Pasinler (26 isolates), Kopru (9 isolates), and Hasanabdal (1 isolate), and coded in sequential order from BTX1 to BTX83. Sequence analysis of all isolated bacteria was performed by using 16S rRNA gene regions. Because of the BLAST analysis of the obtained isolates, it was revealed that there were 98–100% similarities with their closest matches. Although similar studies have been conducted to isolate different thermophilic bacteria species in the 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 the study, *B. subtilis* (33), *G. kaustophilus* (15), *B. licheniformis* (14 isolates), *B. coagulans* (11), and *B. thuringiensis* (10) were identified as most common bacilli in the area. Amongst the isolated bacteria, *Bacillus* was the most dominant genus isolated from hot springs in this study. Studies conducted by many researchers have also shown that the microbial diversity studied in the hot springs reports 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 Davut and Kopru hot springs are almost very close to each other, the isolated bacteria were quite different. *B. subtilis* and *B. licheniformis* were isolated from Davut, *B. coagulans* were also isolated from Kopru. The locations of other hot springs differ completely from each other. *B. coagulans* and *B. thuringiensis* were isolated from Hista for the first time. The *B. coagulans* were isolated for the first time from Diyadin and Hasanabdal. *Geobacillus* sp. and *B. licheniformis* were reported from Hasanabdal in similar studies (Poddar and Das 2018). Besides, the strains identified within the study (*B. subtilis*, *Geobacillus kaustophilus*, *B. licheniformis*, *B. coagulans* and *B. thuringiensis*) exhibit similar morphological, characteristic, physiological, and biochemical properties when compared with the literature (Baltaci et al. 2017, Oztas Gulmus and Gormez 2020a). All the isolated strains (*B. coagulans*, *B. thuringiensis*, *B. subtilis*, *Geobacillus* sp. and *B. licheniformis*) exhibited the ability to grow normally at high temperatures and pH values, which is 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 strains belonging to *B. licheniformis* isolates were able to grow in 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 many areas today. Thermophilic organisms are also microorganisms that adapt to extreme conditions, so they can create a variety of products that can be used even in unfavorable conditions. Many enzymes from thermophilic organisms such as cellulase, xylanase, catalase, lactase, lipase, sucrase, pullulanase, pectinase, amylase, and protease are purified and produced commercially. These enzymes are widely used in many fields such as textile, food, detergent, industry, beverage industry, and health (Oztas Gulmus and Gormez 2020b). *Bacillus* species, which can be isolated from many environments as well as hot water sources, are of great importance in terms of enzyme production. *Bacillus* species are one of the most effective sources of proteolytic and carbohydrates. It meets the enzyme needs especially in many areas such as starch, bread, fruit juice, paper bleaching, and brewing. For this reason, many enzymes synthesized by *Bacillus* species are used in many areas in the industry (Oztas Gulmus and Gormez 2020b). The bacteria isolated in this study can be evaluated in a helpful position in future studies because of their thermophilic properties, being highly stable enzymes that can operate at high temperatures and extreme conditions.

Conclusions

This study has documented the isolated and identified bacteria in seven hot springs from Eastern and Southeastern Anatolia regions using 16S rDNA sequencing. According to 16S rDNA sequencing results, most of the sequences were observed to belong to the genera *Bacillus* (81.93%) and *Geobacillus* (18.07%). These results suggest that all the isolates were *Firmicutes* and *Bacillus* was the most dominant genus in seven hot springs. Although the isolated bacteria from different seven hot springs were similar, they were quite diverse in their environmental attributes, encompassing a temperature range from 40 to 85°C, pH ranges from 4 to 9, and salinity 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, the presented study is designed as a preliminary study showing the diversity of bacteria that produce crucial thermophile enzymes in industrial and biotechnological terms.

Declarations

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It presented the species identification phase of the study in 2017 as an international congress summary.

Author contributions

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

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

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

Methods

Methods are incorporated into Supplementary Information

Data availability

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

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Figures

Figure 1

Referring to the Turkey map, hot springs under 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>). Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

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

Figure 3
The evolutionary relationship was inferred using the Neighbor-Joining method [58]. The optimal tree with the sum of branch length = 0.34395833 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [59]. The

analysis of 89 nucleotide sequences (83 isolates, 5 type strains, and 1 outgroup) was conducted in MEGA7 [61]. As shown in the figure, bacteria belonging to the genus *Bacillus* were divided into different groups from the genus *Geobacillus*, to be a single group with type strains as expected.

Figure 4

According to the qualitative analysis results, it was determined that 27 (32.8%) isolates can 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.

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