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Isolation and Characterization of Thermophilic Bacteria from Geothermal Areas in Turkey and Preliminary Research on Biotechnologically Important Enzyme Production

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ABSTRACT
In this study, the isolation, identification and characterization of the thermophilic bacteria from different hot springs in Turkey were carried out by conventional (morphological, physiological and biochemical tests) and molecular methods (fatty acid methyl esters, GTG5-PCR and 16S rRNA sequencing). These thermophilic bacteria were then tested for their capability to produce enzymes such as lipase, protease, amylase and cellulase. O20 strain is a novel species according to identification studies. All of its isolates were capable of producing industrially valuable enzymes based on screening. In fact, most of them could produce at least two of these enzymes.

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Introduction

Temperature is a vital parameter for microbial growth, and microorganisms may prefer different ranges of temperature to survive. Therefore microorganisms are categorized as psychrophiles, mesophiles, thermophiles and hyperthermophiles (Kristjonsson and Stetter 1991). Among them, thermophilic organisms have received the attention of microbiologists and biochemists since the first thermophilic bacterial isolation by Miquel (1888). The main reason for this is their stable protein structure and resistance to various chemical reagents (Madigan and Martinko 2006). One of the thermophilic regions is hot water resource or geothermal region. Hot water resources, which consist mostly of oceans and marine ecosystems, are located in different parts of the world (Kristjonsson and Stetter 1991; Madigan and Martinko 2006). The attractive feature of hot water resources is the ecosystem wholeness, i.e. the diversity of the organisms (Adiguzel et al. 2011) and the molecular stability of the biochemical components (Genc et al. 2015). Some of the most important and comprehensive researches have been carried out in the Yellowstone National Park in the USA (Miller et al. 2006; Boomer et al. 2009) which has more than one thousand marine surface formations such as waterfalls and hot springs. Greece (Sievert et al. 2000), Italy (Maugeri et al. 2001), Iceland (Takacs et al. 2001), Bulgaria (Atanassova et al. 2008; Derekova et al. 2008), China (Lau et al. 2009) and India (Sharma et al. 2009) have attracted the attention of researchers. Turkey is one of the world's richest countries in terms of geothermal regions as there are 133 hot water resources (Akkaya and Kivanc 2009) whose temperatures are higher than 40°C. There are also a remarkable number of researches about the geothermal microbiota in Turkey (Belduz et al. 2003; Dulger et al. 2004; Cihan et al. 2012; Cihan 2013; Yanmis and Adiguzel 2014; Yanmis et al. 2015).

Enzymes are proteins catalyzing biochemical reactions that regulate vital metabolic functions in cells. They are involved in various processes of daily life. Although animals, plants and microorganisms are enzyme producers, microorganisms are more attractive due to higher catalytic activity of their enzymes. Furthermore, microbial enzymes are more stable and cheaper as well as obtainable in excessive amounts (Niehaus et al. 1999). Thermostable enzymes that are produced by thermophilic microorganisms are very important for industrial applications due to their resistance under different operation conditions. These enzymes have been reported to be more stable against many solvents, detergents, acidic and alkaline pH, and high temperatures. Because of these properties, the isolation and identification of thermophilic bacteria, and the determination and characterization of the enzymes from these bacteria are very essential (Bhalla et al. 2013; Dettmer et al. 2013).

The aim of the study was the isolation and identification of the thermophilic bacteria from some geothermal parts of Turkey like Bademli Bahce Hot Spring (Bursa), Sulusaray Hot Spring (Tokat), Sicak Cermik Hot Spring (Sivas), Bostanci Hot Spring (Balıkesir) and Diyadin Hot Spring (Agri), and determination of the thermophilic bacteria which could be grown within a wide range of pH and temperatures. For this purpose, water and sludge samples from different geothermal resources were collected. Firstly the bacterial isolations and characterizations were carried out by conventional methods (growth

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temperature, pH and salt concentration, colony morphology, Gram staining, endospore formation, and biochemical tests), and some molecular methods (fatty acid methyl esters [FAMEs], GTG5-PCR and 16S rRNA sequencing). Then, the assays for lipase, protease, amylase and cellulase were carried out in Petri dishes, and all of the isolates were evaluated according to their enzyme production ability to find out the biotechnologically important isolates.

Materials and methods

The water and sludge samples were aseptically collected from Bademli Bahce Hot Spring (58°C – pH 6.6), Sulusaray Hot Spring (50°C – pH 7.2), Sicak Cermik Hot Spring (53°C – pH 6.6), Bostanci Hot Spring (51°C – pH 7.2) and Diyadin Hot Spring (70°C – pH 6.8). The samples were used to inoculate the growth medium in order to propagate the microbes in Nutrient Agar (NA) medium and were incubated (Thermo Scientific Heratherm, the USA) at 55°C for 24–48 h, and the growing colonies were re-spread on the NA to obtain pure cultures. The colonies were stored in the nutrient broth (NB) with 15% glycerol content at −86°C for further studies (Harley and Prescott 2002), and these colonies were subsequently referred to as "test isolates."

Conventional analysis of the test isolates

The test isolates were subjected to conventional tests. The pH (Mettler Toledo) and the temperature range for bacterial growth were tested between pH 4.0 and 12.0 and 35 and 85°C, respectively. The NaCl requirement for growth was also tested in the NB medium containing 2.0–10.0% (w/v) NaCl. Cell and colony morphology, Gram stainings, and the presence of catalase and oxidase were also investigated according to the methods described by Harley and Prescott (2002).

Analysis of the fatty acid methyl ester (FAME) profiles

The fatty acid extraction of the bacterial cells was carried out according to a method by Adiguzel et al. (2012a). For this purpose, the cells were streaked in a quadrant pattern and grown overnight on Trypticase Soy Broth Agar (TSBA) at 56°C, and 40 mg of the bacterial cells was extracted at the end of the incubation. After the extraction, gas chromatography was performed with a Hewlett Packard Gas Chromatograph with fused-silica capillary column (25 m × 0.2 mm) and cross-linked with 5% phenyl methyl silicone (HP6890, Hewlett Packard, Palo Alto, CA, USA) by using the aerobe method TSBA40 and the library TSBA Version 4.0, followed by the analysis of the data by the MIS software package, from MIDI Inc. (Newark, Del., USA). The identity of the bacterial strains was displayed by the comparison of the FAME profiles of the unknown test strains with those in the library using a computer.

DNA extraction from the test isolates

To extract the total genomic DNA, the cells were firstly washed with 1 mL of Sodium Chloride-Tris-EDTA (STE) (150 mM NaCl, 50 mM Tris, 50 mM Na_2-EDTA) buffer. And then, resuspension was carried out to lyse the cells at 75°C for 30 min in 500 μL STE buffer. After this stage, the lysate was applied with cetyltrimethylammonium bromide/NaCl mixture and finally the DNA was extracted using phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated in isopropanol (Nadaroglu et al. 2015).

Gene amplification, cloning and sequencing for phylogenetic studies

The 16S rRNA, evolutionary gene of the prokaryotic ribosome, from the total DNA of the test isolates was amplified by PCR with the forward primer UNI16S-L: (5′-ATTCTAGAGTTTGATCATGGCTCA-3′) corresponding to positions 11–26 of *Escherichia coli* 16S rRNA, and the reverse primer UNI16S-R: (5′-ATGGTACCGTGTGACGGGCGG TGTGTA-3′) corresponding to the complement of the positions 1411–1393 of *E. coli* 16S rRNA (Beffa et al. 1996). The amplified fragments were cloned to a vector system (pGEM-T, Promega, the UK) and the clones were sequenced (Macrogen, Amsterdam, the Netherlands). The results of 16S rRNA gene sequencing were analyzed using the GenBank and EzTaxon (http://blast.ncbi.nlm.nih.gov/blast.cgi and http://www.eztaxon.org) server (Altschul et al. 1990; Yanmis et al. 2015). Considering the results of the study, a phylogenetic tree was formed via the neighbor joining method using the software package MEGA 4.0 (Benson et al. 1999; Yanmis et al. 2015).

PCR-based genomic fingerprinting

Test isolates were subjected to rep-PCR with the special primer of GTG5 elements to obtain genomic fingerprinting (Gevers et al. 2001; Adiguzel et al. 2012a). Immediately after the completion of the PCR, the results were converted to a binary matrix (where "1" stands for the presence and "0" stands for the absence of a determined band at a specified position). The data were evaluated by SPSS version 11.0, Windows and Jaccard's coefficient of similarity (1908) was calculated (Adiguzel et al. 2012b).

To obtain the PCR products, 50 ng of purified DNA was used as the template in 30 μl reaction mixture. Twenty-seven microliters of the reaction cocktail was prepared as follows: Specific Gitschier Buffer 5 μl, dimethyl sulfoxide 2.5 μl (100%, 20X), dNTPs (10 mM) 1.25 μl, bovine serum albumin 1.25 μl (20 mg/ml), primers (5 μM) 3.0 μl, Taq polymerase (250 U) 0.3 μl and water 13.7 μl. A negative control (no DNA) was included in each PCR assay. PCR reactions were performed with a 3 × 32-well ProFlex^{TM} PCR System, using the following conditions: an initial denaturation at 94°C for 7 min; 30 cycles consisting of 94°C for 30 s and annealing at 45°C for 1 min with GTG5 primary, extension at 65°C for 8 min; and a final polymerization at 65°C for 15 min before cooling at 4°C.

The PCR products (27 μl) were mixed with 3 μl gel loading buffer (6X) and subjected to agarose (1.5% w/v) gel electrophoresis in Tris–Acetate–EDTA (TAE) buffer at 100 V for 110 min. After separation of the amplification products by the gel, the fragments were stained with ethidium bromide solution (2 μl Etbr/100 ml 1× TAE buffer). The amplified DNA product

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was monitored using the Quantum Vilber Lourmat Gel Documentation System (Australia).

Preliminary enzyme assays

Amylase assay

Amylase assay was performed at 55°C for two days in a medium which contained $Na_2HPO_4.7H_2O$ (6 gL^{-1}), KH_2PO_4 (3 gL^{-1}), NaCl (0.5 gL^{-1}), NH_4Cl (1 gL^{-1}), $MgSO_4.7H_2O$ (0.24 gL^{-1}), $CaCl_2.2H_2O$ (0.24 gL^{-1}), peptone (3 gL^{-1}), starch (10 gL^{-1}) and agar (15 gL^{-1}). The amylase activity was screened by Lugol's solution. Clear and large zones were evaluated as positive, or as amylase production; however, no zone formation was evaluated as negative, or as no amylase production (Yanmis et al. 2015).

Cellulase assay

A medium containing carboxymethylcellulose (10 gL^{-1}), peptone (5 gL^{-1}), yeast (5 gL^{-1}), KH_2PO_4 (1 gL^{-1}), $MgSO_4.7H_2O$ (0.2 gL^{-1}), NaCl (10 gL^{-1}) and agar (15 gL^{-1}) was used for cellulase assay. Incubation was carried out at 55°C for 2 days, and after the incubation, the Petri dishes were stained by Congo-Red Dye (Yanmis et al. 2015).

Lipase assay

Test isolates were inoculated on tributyrin agar medium which contained 1% tributyrin (glycerol tributyrate) and incubated at 55°C. Formation of hydrolysis zone around the culture on Petri dish was controlled for 2 days, and the strains with transparent and the highest zone formation (lipolytic activity) were determined as lipase producers (Ertugrul et al. 2007).

Protease assay

Twenty-four hour bacterium culture in NB was propagated in Skimmed Milk Agar medium at 55°C for 24–48 h to assay proteolytic activities of the test isolates. The plates were evaluated according to the zone formations, and the observation of a halo zone indicated positive protease activity (Panda et al. 2013).

Results

Conventional analysis of the test isolates

In this study, the first results were obtained from the isolation studies. A total of 19 bacterial strains were collected and isolated from different geothermal parts of Turkey; these strains were encoded in the form of numbers between O1-O12, O14-O17 and O19-O21.

The microorganisms with different colony formations and cell morphologies were subjected to conventional tests to characterize the isolates, and the results are shown in Table 1. These tests indicated that all of the isolates were Gram-positive (except O4), rod-shaped and motile (except O4), and capable of endospore formation (except O4). They were also positive for catalase (except O2) and oxidase (except O12 and O16). The ranges of pH and temperature at which the growth of test isolates is favored, on the other hand, were determined as 5–9 and 50–65°C, while the optimum values for pH and temperature were pH 7.0 and 56°C, respectively. From the results of salt test, it was observed that all the isolates were grown between the salt concentrations of 2 and 10%.

FAMEs analysis

In this study, 19 bacterial strains were investigated and 29 different fatty acids were detected (Table 2). Six different fatty acids (*15:0 iso, 15:0 anteiso, 16:0 iso, 16:0, 17:0 iso* and *17:0 anteiso*) were found in all the isolates; however, *15:0 iso* was the major fatty acid at a concentration of 21.77–71.08% (except O5, O6 and O11); and the fatty acid *16:0 iso* was also found, which is present in all other remaining strains. Four different fatty acids *14:0* (except O9 and O17), *16:1w7c* (except O6, O9, O11 and O16); *16:1w11c* (except O3, O9, O16 and O20) and *17:1 iso w10c* (except O8, O9, O12, O16 and O20) were mostly present in all isolates. Strains except O1, O2, O4-O6, O8, O11, O12 and O20 exhibited the same FAME profile (*15:0 iso, 15:0 anteiso, 17:0 iso* and *17:0 anteiso*) with the concentration between 2.61 and 59.83%. Fatty acid profile of O4 strain was also 1*5:0 iso* (21.91%), *16:0 iso* (19.98%), *17:0 iso* (11.76%) and

Table 1. Conventional analysis results of the test isolates.

Isolate code	pH range	Salt tolerance (%)	Catalase	Oxidase	Gram staining	Endospore formation	Motility
O1	5–8	2–8	+	+	+	+	+
O2	5–8	2–8	−	+	+	+	+
O3	6–8	2–8	+	+	+	+	+
O4	6–8	2–8	+	+	−	−	−
O5	6–8	2–6	+	+	+	+	+
O6	6–8	2–8	+	+	+	+	+
O7	6–8	2–6	+	+	+	+	+
O8	6–8	2–8	+	+	+	+	+
O9	6–8	2–8	+	+	+	+	+
O10	6–8	2–6	+	+	+	+	+
O11	6–8	2–6	+	+	+	+	+
O12	6–8	2–8	+	−	+	+	+
O14	5–8	2–8	+	+	+	+	+
O15	6–8	2–6	+	+	+	+	+
O16	6–8	2–10	+	−	+	+	+
O17	6–8	2–6	+	+	+	+	+
O19	6–8	2–6	+	+	+	+	+
O20	6–9	2–4	+	+	+	+	+
O21	6–8	2–6	+	+	+	+	+

Table 2. Cellular fatty acid compositions of the test strains (%w/w).

Fatty acids	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	O11	O12	O14	O15	O16	O17	O19	O20	O21
9:0	-	-	-	-	0,63	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10:0 iso	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0,65	0,45	-	-	2,12
11:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0,53	0,72
11:0 iso	-	-	-	-	-	-	-	-	-	-	-	-	-	0,19	-	-	-	-	-
12:0 iso	-	-	-	-	-	-	-	-	-	-	-	-	-	0,77	-	-	-	0.61	0,98
11:0 iso 3OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1,89
12:0 Aldehyde	-	-	-	-	-	-	-	-	-	0,22	-	-	-	0,61	-	-	-	-	-
12:0 2OH	-	-	-	-	-	-	-	-	-	-	56,97	-	-	-	-	-	-	-	-
13:0 iso	-	-	0,48	-	-	-	0,37	-	-	0,13	-	-	0,44	0,25	-	-	0,57	0.39	-
13:0 anteiso	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0,39
14:0 iso	0,56	0,80	1,17	-	0,95	-	1,19	-	0,44	2,69	-	0,52	1,25	2,83	0,38	-	1,05	0.81	2,65
14:0	3,17	1,14	0,94	3,22	3,21	3,49	0,77	1,35	-	1,24	1,57	1,83	0,78	1,29	0,25	-	1,19	1.52	1,41
14:1w5c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.26	-
15:0 iso	21,77	54,35	58,93	21,91	19,71	21,66	58,69	71,08	50,54	30,24	11,48	69,31	58,28	29,50	51,18	59,30	59,83	62.42	28,17
15:0 anteiso	6,64	14,65	19,80	8,71	6,29	5,97	19,91	2,81	16,59	24,30	2,59	3,41	20,77	24,92	15,57	21,74	20,08	2.78	24,01
16:1 w7c	0,69	0,52	0,79	1,80	1,67	-	0,90	2,99	-	3,51	-	2,67	0,63	3,85	-	0,66	0,46	3.29	3,61
16:0 iso	11,88	3,90	2,27	11,64	10,79	11,75	2,41	1,95	3,15	7,50	5,09	2,99	2,64	7,05	3,06	2,40	2,23	2.43	6,51
16:1 w11c	2,64	0,51	-	2,91	3,15	3,87	0,61	2,84	-	1,15	1,21	0.80	0,39	1,89	-	0,65	0,41	-	1,51
16:0	21,48	5,76	2,49	19,98	22,63	21,98	2,30	4.77	2,66	4,05	8,87	4,17	2.20	3,67	2,95	2,65	2,32	4.68	3,64
17:1 iso w10c	1,39	1,67	2,09	1,39	1,51	1,92	1,97	-	-	2,04	0,44	-	1,44	2,41	-	1,47	1,42	-	2,18
17:1 iso w5c	-	-	-	-	-	-	-	4,22	-	-	-	2,32	-	-	-	-	-	3.14	0,38
17:1 isol/Antei	0,65	-	0,61	1,01	2,33		0,61	1,05	-	1,36	0,23	-	-	1,52	-	0,39	-	0.95	1,47
17:0 iso	12,88	12,05	7,82	11,76	11,01	13,26	7,66	9,43	18,38	10,76	4,72	10,25	8,25	9,78	18,40	7,59	7,76	11.60	9,36
17:0 anteiso	16,25	4,63	2,62	15,66	14,43	16,10	2,61	2,27	8,24	9,37	6,84	2,54	2,93	9,23	7,56	2,70	2,67	3.0	8,59
17:0 cyclo	-	-	-	-	-	-	-	-	-	0,36	-	-	-	-	-	-	-	-	-
17:0	-	-	-	-	-	-	-	-	-	0,38	-	-	-	-	-	-	-	-	-
18:0 iso	-	-	-	-	-	-	-	-	-	0,32	-	-	-	-	-	-	-	-	-
18:0	-	-	-	-	-	-	-	-	-	0,38	-	-	-	0,43	-	-	-	-	0,40
18:1 w7c	-	-	-	-	0,90	-	-	-	-	-	-	-	-	-	-	-	-	-	-

17:0 anteiso (15.66%). Finally, *15:0 iso, 15:0 anteiso* and *17:0 iso* were the major fatty acids of O10, 015 and O21.

16S rRNA sequencing

16S rRNA gene amplification was performed selectively after extracting the total DNA from the test isolates, and all the isolates contained 1421–1449 nucleotides. Later, the nucleotides were analyzed using BLAST, GenBank and EzTaxon; the results are summarized in Table 3. Five of the isolates (O1, O3, O5, O6 and O11) were detected to be the strains belonging to *Aeribacillus pallidus* at a similarity rate of 99%. Further, six of the isolates (O2, O7, O8, O14, O17 and O19) were related to *Bacillus pumilus* (with 99% similarity ratio), two isolates (O12 and O16) to *Bacillus licheniformis* with 99% ratio, three isolates (O10, O15 and O21) to *Bacillus thermoamylovorans* (≥99%), one of them (O20) to *Anoxybacillus kaynarcensis* (97% ratio of similarity), one of them (O9) to *Anoxybacillus gonensis* with a similarity rate of 99%, and in addition, one of the isolates (O4) belonged to *Thermomonas hydrothermalis* at a ratio of 99% (Figure 1).

Table 3. Comparison of 16S rRNA gene sequences of the obtained isolates with those in GenBank.

Isolate code	GenBank accession number	Closest phylogenetic relative	Number of nucleotides*	Percent identity**
O1	KM668038	*Aeribacillus pallidus*	1427 bp	99%
O2	KM596787	*Bacillus pumilus*	1449 bp	99%
O3	KM596788	*Aeribacillus pallidus*	1427 bp	99%
O4	KM596789	*Thermomonas hydrothermalis*	1421 bp	99%
O5	KM596790	*Aeribacillus pallidus*	1427 bp	99%
O6	KM596791	*Aeribacillus pallidus*	1427 bp	99%
O7	KM596792	*Bacillus pumilus*	1424 bp	99%
O8	KM596793	*Bacillus pumilus*	1431 bp	99%
O9	KM596794	*Anoxybacillus gonensis*	1429 bp	99%
O10	KM596795	*Bacillus thermoamylovorans*	1426 bp	99%
O11	KM596796	*Aeribacillus pallidus*	1427 bp	99%
O12	KM596797	*Bacillus licheniformis*	1426 bp	99%
O14	KM596799	*Bacillus pumilus*	1423 bp	99%
O15	KM596800	*Bacillus thermoamylovorans*	1425 bp	99%
O16	KM596801	*Bacillus licheniformis*	1425 bp	99%
O17	KM596802	*Bacillus pumilus*	1423 bp	99%
O19	KM596803	*Bacillus pumilus*	1423 bp	99%
O20	KM668039	*Anoxybacillus kaynarcensis*	1429 bp	97%
O21	KT364469	*Bacillus thermoamylovorans*	1426 bp	99%

*The number of 16S rRNA nucleotides used for the alignment.
**The percentage identity with the 16S rRNA sequence of the closest phylogenetic relative.

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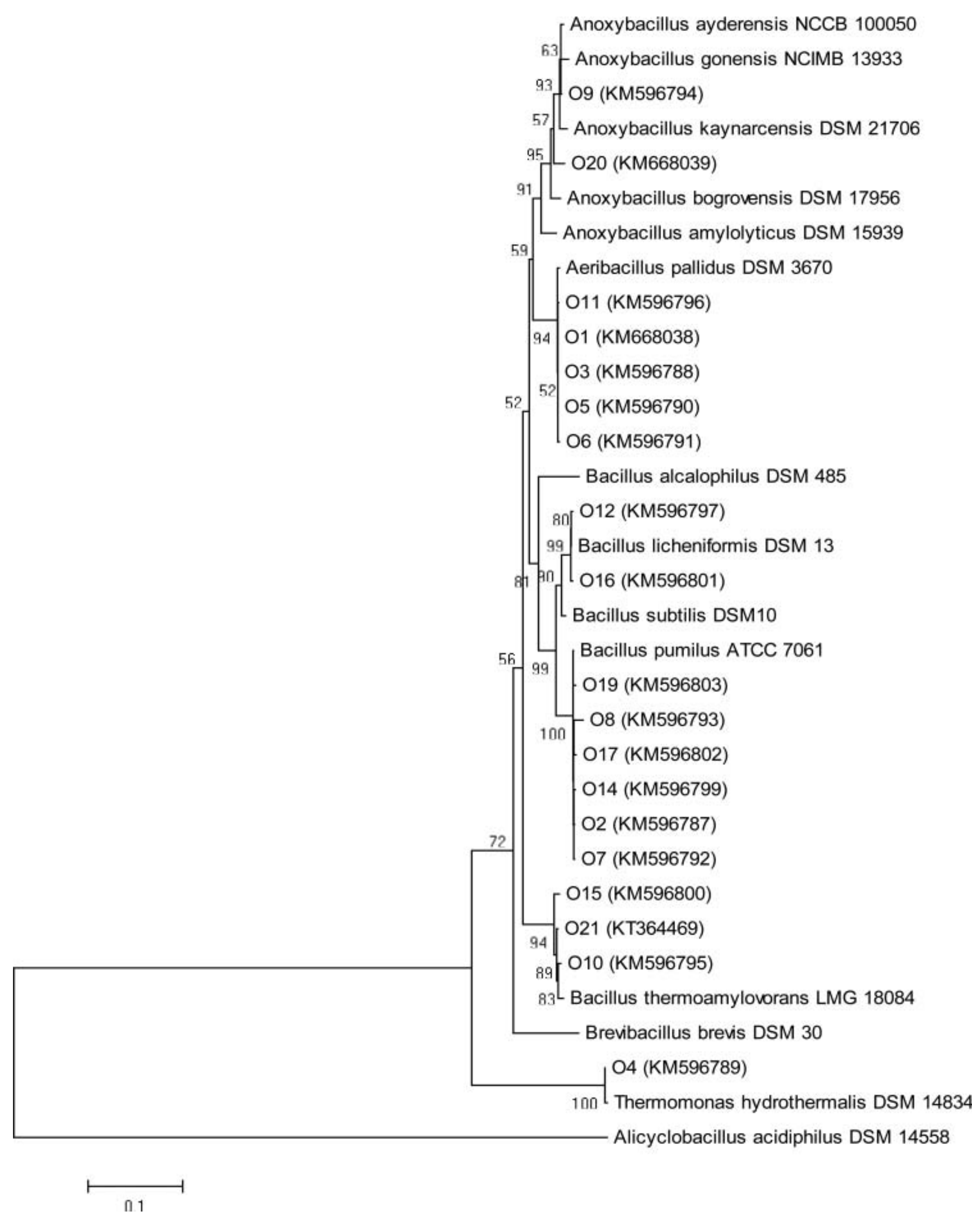

Figure 1. Neighbor joining phylogenetic tree on the basis of 16S rRNA gene sequence data of the thermophilic bacteria isolated from various hot springs in Turkey. *Alicyclobacillus acidiphilus* was used as out-group. Bootstrap values based on 1000 replications are listed as percentages at branching points. The accession numbers are given in parentheses. Only bootstrap values >50% are shown at nodes. The scale bar represented 1% divergence.

Genomic fingerprinting of the isolates

As a result of GTG5-PCR, thermophilic bacterial strains exhibited 5–17 fragments between 200 and 6000 bp (Figures 2 and 3) with the GTG5 primer. According to the cluster analysis that was taken into consideration after the GTG5-PCR, the test isolates were classified into two main clusters and one of the main cluster had three sub-clusters (Figure 4). The dendrogram showed the clusters level by level. *Anoxybacillus bogrovensis* DSM 17956 separately formed the first main cluster, while the first sub-cluster was composed of *Bacillus licheniformis* (O12 and O16) and *Bacillus licheniformis* DSM 13. The third cluster was composed of *A. gonensis* (O9), *A. kaynarcensis* (O20) and *A. gonensis* NCIMB 13933, while the second sub-cluster was comprised of the remaining strains.

Enzyme productions

Among the thermophilic isolates, eleven, eight, fourteen and eight of the isolates in this study were identified as producers of lipase, protease, amylase and cellulase, respectively (Table 4). Producers of protease were *Aeribacillus pallidus* (O3), *Bacillus pumilus* (O2, O7, O8, O14 and O19), *Bacillus thermoamylovorans* (O15) and *Bacillus licheniformis* (O16), while cellulase-positive strains were *Aeribacillus pallidus* (O3), *Bacillus pumilus* (O7, O8, O14, O17 and O19), *Anoxybacillus gonensis* (O9) and *Bacillus licheniformis* (O16). On the other hand, *Aeribacillus pallidus* (O3), *Bacillus pumilus* (O7), *Bacillus thermoamylovorans* (O10 and O21) and *Anoxybacillus kaynarcensis* (O20) were not producers of amylase and *A. pallidus* (O1, O5, O6 and O11), *A. gonensis* (O9), *B. licheniformis* (O12), *B. pumilus* (O14) and *B. thermoamylovorans* (O15) were not producers of lipase. In

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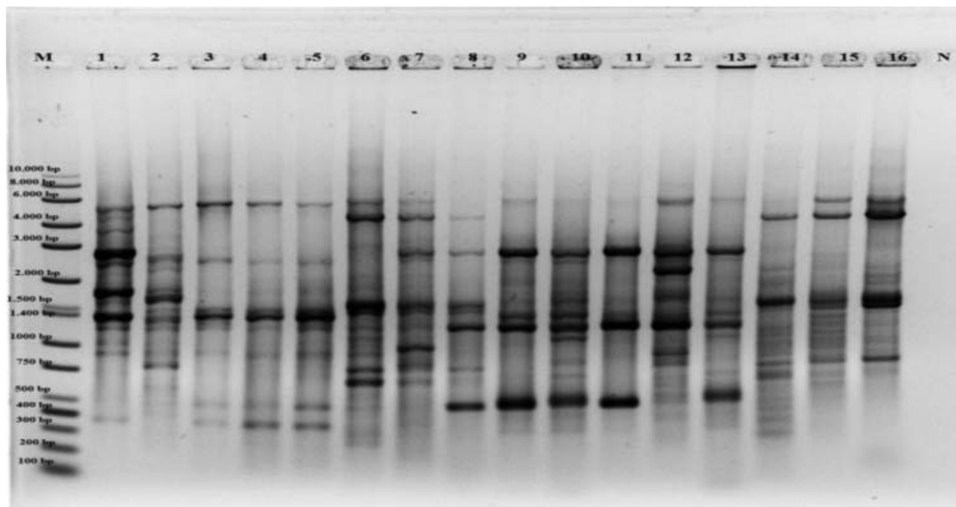

Figure 2. GTG5 profile generated with the GTG5 primer. Lanes: 1) *Aeribacillus pallidus* DSM 3670; 2) O1; 3) O5; 4) O6; 5) O11; 6) O8; 7) *Bacillus pumilus* ATCC 7061; 8) O3; 9) O2; 10) O7; 11) O14; 12) O17; 13) O19; 14) *Bacillus licheniformis* DSM 13; 15) O16; 16) O12; N) Negative Control; M) Molecular Marker (10 kb).

addition, *B. pumilus* (O8 and O19) and *B. licheniformis* (O16) had the production capacity for all four enzymes.

Discussion

The thermophilic test isolates from water and sludge samples of Turkey's different geothermal areas were isolated and then characterized by several methods. According to conventional studies that were carried out, the optimum growth temperatures of the isolates were higher than 50°C, and this observation was compatible with the studies conducted about thermophilic microorganisms (Adiguzel et al. 2011; Slobodkin and Slobodkina 2014; Yanmis and Adiguzel 2014; Hedlund et al. 2015). The Gram staining of the O4 strain was different from the remaining isolates, i.e. Gram-negative, which is compatible with the literature data (Alves et al. 2003).

FAME analysis was conducted to test the isolates in this study; *15:0 anteiso* (except O1, O4, O5, O6, O8, O11, O12 and O20), *16:0* (only present in O1, O2, O4, O5, O6 and O11), *17:0 iso* (except O11), *17:0 anteiso* (only present in O1, O4, O5, O6, O9 and O10) and *15:0 iso* were the major fatty acids. As a result, these findings were found to be related to the FAME profiles of *Anoxybacillus*, *Bacillus* and *Aeribacillus* that have been reported in the literature (Nazina et al. 2005; Zaliha et al. 2007; Adiguzel et al. 2009; Yanmis and Adiguzel 2014; Yanmis et al. 2015). One of the points of attention in the results is that Belduz et al. (2003) and Dulger et al. (2004) defined *15:0 iso* as the main fatty acid in *Anoxybacillus* species with 62–68% content, and in this study *A. kaynarcensis* (O20) had *15:0 iso* at a concentration of 62.42%. Our results showed that the fatty acid profile of the O4 strain was also remarkable. *T. hydrothermalis* (O4) had major fatty acids as *15:0 iso* (21.91%), *16:0 iso* (19.98%), *17:0 iso* (11.76%) and *17:0 anteiso* (15.66%), and these results were in agreement with the literature studies (Alves et al. 2003; Mergaert et al. 2003). The other point of attention in the results was that, in their study, Coorevits et al. (2011) determined the FAME profiles of *B. thermoamylovorans* as being comprised of *15:0 iso, 15:0 anteiso* and *17:0 iso* as it was the same in our results for O10, O15 and O21. As a result of the analysis, the major fatty acid content of the isolates was well-matched with similar researches in the literature. However, 16S rRNA sequencing and GTG5-PCR were performed to confirm the types of test isolates. To summarize the results, the microbial identification system libraries showed insufficiencies to identify thermophilic bacteria *Aeribacillus, Anoxybacillus, Bacillus* and *Thermomonas* at the species level. Therefore,

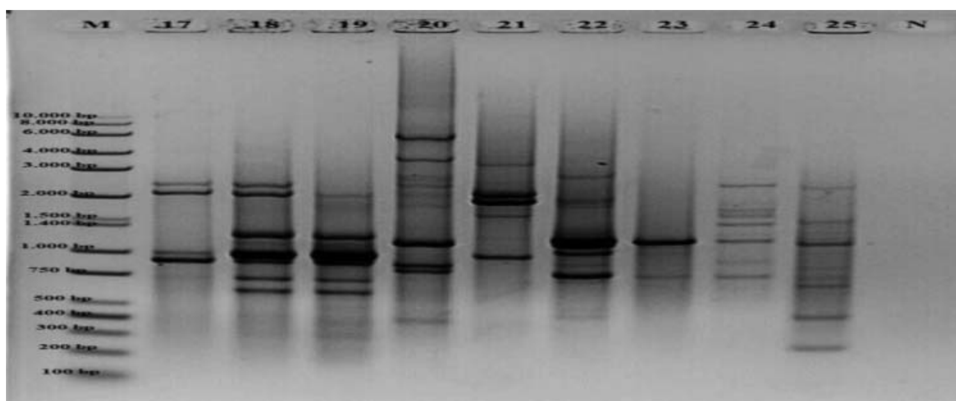

Figure 3. GTG5 profile generated with the GTG5 primer. Lanes: 17) *Anoxybacillus gonensis* NCIMB 13933; 18) O9; 19) O20; 20) *A. bogrovensis* DSM 17956; 21) *A. kaynarcensis* DSM 21706; 22) O21; 23) O10; 24) O4; 25) O15; N) Negative Control; M) Molecular Marker (10 kb).

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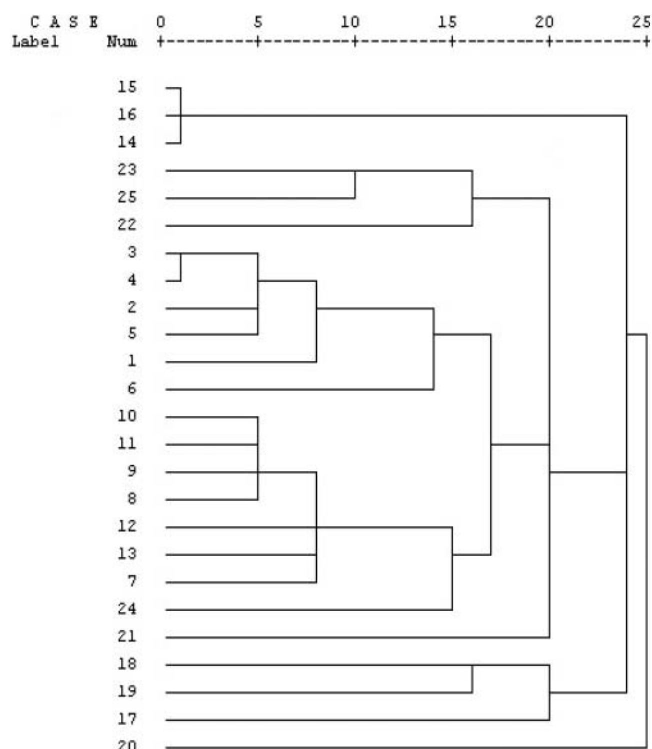

Figure 4. GTG5-PCR cluster analysis: 1) *Aeribacillus pallidus* DSM 3670; 2) O1; 3) O5; 4) O6; 5) O11; 6) O8; 7) *Bacillus pumilus* ATCC 7061*;* 8) O3; 9) O2; 10) O7; 11) O14; 12) O17; 13) O19; 14) *Bacillus licheniformis* DSM 13; 15) O16; 16) O12; 17) *Anoxybacillus gonensis* NCIMB 13933*;* 18) O9; 19) O20; 20) *Anoxybacillus bogrovensis* DSM 17956; 21) *Anoxybacillus kaynarcensis* DSM 21706; 22) O21; 23) O10; 24) O4; 25) O15.

phylogenetic relationship of the test isolates with the known types was determined by 16S rRNA sequencing and with GTG5 genomic characterization methods, respectively. The sequencing data were examined with BLAST (http://blast.ncbi.nlm.nih.gov/blast.cgi and http://www.eztaxon.org) to discover the evolutionary relationships of the test isolates. The similarity ratio (≥99%) of O1, O3, O5, O6 and O11 to *A. pallidus* in this study also demonstrated compliance with the results by Yasawong et al. (2011) and Yanmis and Adiguzel (2014). O2, O7, O8, O14, O17 and O19 belonged to *B. pumilus,* and Cihan et al. (2012) isolated *B. pumilus* at the same similarity ratio with our results. As resulted in our study, Chebbi et al. (2014) also isolated *B. licheniformis* in their study at a rate of ≥99%. Our data for O20 showed that this isolate matched with *A. kaynarcensis*; Inan et al. (2013) also isolated *A. kaynarcensis* from a hot water resource with ≥97% similarity; in addition, O9 isolate matched with *A. gonensis* (Belduz et al. 2003) ≥99%. The evolutionary study detected 19 bacterial strains and 14 related species by neighbor joining method. The nucleotide difference and the p-distance were used to draw the tree by scaling with branch lengths of the same units. A total of 1582 positions were obtained in the final data without gaps and missing data.

Table 4. Extracellular enzyme profiles of the test isolates from some geothermal regions of Turkey.

Isolate code	Lipase	Protease	Amylase	Cellulase
O1	−	−	+	−
O2	+	+	+	−
O3	+	+	−	+
O4	+	−	+	−
O5	−	−	+	−
O6	−	−	+	−
O7	+	+	−	+
O8	+	+	+	+
O9	−	−	+	+
O10	+	−	−	−
O11	−	−	+	−
O12	−	−	+	−
O14	−	+	+	+
O15	−	+	+	−
O16	+	+	+	+
O17	+	−	+	+
O19	+	+	+	+
O20	+	−	−	−
O21	+	−	−	−

16S rRNA sequencing was inadequate to differentiate species in sub-species (Vandamme et al. 1996), therefore rep-PCR (GTG5-PCR) was carried out together with 16S rRNA sequencing and GTG5-PCR showed its advantages in the aspect of determining genomic differences between the strains. As a result of 16S rRNA sequencing analysis, O9 isolate showed high compatibility with A. *gonensis* at a power rate of ≥99, and also showed higher similarity to O20 isolate more than *A. gonensis* NCIMB 13933 standard strain according to GTG5-PCR results. Considering all these data, 16S rRNA results were supported with GTG5-PCR method and the reliability of our results were consolidated in accordance with the literature data (Gevers et al. 2001; Nazina et al. 2005; Zaliha et al. 2007; Adiguzel and Atasever 2009; Khiyami et al. 2012; Cihan 2013; Adiguzel and Yurekli 2015).

In addition to determination of the basic and general requirements of the test isolates, the enzyme Petri dish assays were carried out to control the enzyme production capacity, because the importance of the enzyme production ability has been a supply for industrial applications and this importance has been increased when the growth requirements of the microorganism were not very sensitive. In this study, among the isolates, *Aeribacillus pallidus* (Aanniz et al. 2015) was the producer of protease and *Anoxybacillus gonensis* (Genc et al. 2015) was the producer of cellulase. Some of *Bacillus sp.* isolates were both protease and cellulase producers (Kamran et al. 2015; Ladeira et al. 2015). Some of our isolates, which were members of the genus *Bacillus* (Bora and Bora 2012; Nathan and Nair 2013), *Aeribacillus pallidus* (Stathopoulou et al. 2013; Aanniz et al. 2015), *Anoxybacillus gonensis* (Faiz et al. 2007; Baltas et al. 2012), *Anoxybacillus kaynarcensis* and *Thermomonas hydrothermalis* were lipase and amylase producers. Some of the isolates, *B. pumilus* (O8 and O19) and *B. licheniformis* (O16), were producers of all four enzymes (Popović et al. 2014). However, there was no publication about the cellulase enzyme production of *Anoxybacillus kaynarcensis, Thermomonas hydrothermalis* and *Aeribacillus pallidus.*

Protease, lipase, amylase and cellulase are important enzymes in terms of industrial value; and among these, lipases have a wide area of usage in detergent, food, bioresolution of pharmaceuticals, agrochemicals, bioremediation, cosmetics and perfumery industry (Hasan et al. 2006). Proteases, which constitute 60% of the global enzyme market, have a wide area of usage in various sectors like detergents, leather, food, cosmetics, medicine and medical diagnosis (Gupta et al. 2002; Laxman et al. 2005). In addition to these enzymes, amylases, which decompose the starch, are used in areas like manufacturing

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high fructose containing syrups, fermentation of starch to ethanol, and treatment of starch processing wastewater (Aiyer 2005). On the other hand, cellulases are used in areas like conversion of cellulosic material to valuable products such as ethanol, improved malting and clarification in fermentation industry, improvement of yields in maceration, extracting, texture and pressing (Kuhad et al. 2011). When all this information is taken into account, it is clear that this is a preliminary study in terms of gaining thermophilic strains, which synthesize biotechnologically important enzymes, to literature.

Moreover, bacterial isolates in this study showed wide spectrums of pH with high temperature tolerance. Therefore, this range of pH and temperature makes the process resistant to sudden acidic and alkaline changes with high temperatures in these industrial applications of enzymes (Dhiman et al. 2011; Ghareib et al. 2014; Wang et al. 2014; Genc et al. 2015).

Conclusion

The strains that have been isolated from different geothermal areas of Turkey have the ability to produce industrial enzymes besides their thermophilic advantages. According to the enzyme assays using Petri dish, a large number of strains have the capability of producing more than one enzyme. Therefore, these strains may constitute important reservoirs for biotechnological researches, and it has revealed the originality of this research. Also, according to the literature scan, this study is the first report about the lipase, protease, amylase and cellulase productions of *Anoxybacillus kaynarcensis* and *Thermomonas hydrothermalis,* and cellulase production of *Aeribacillus pallidus*.

According to 16S rRNA sequencing results, O20 strain exhibited 97% similarity to *A. kaynarcensis*. Meier-Kolthoff et al. (2013) reported that there was no need for DNA–DNA hybridization analysis between the strains having a similarity level below 98.2%. Based on these data, it has been concluded that the O20 isolate could be a new species with a high probability. Finally, O9 strain exhibited 99% similarity to *A. gonensis*; however, it is similar to O20 strain according to GTG5-PCR. Consequently, 16S rRNA and GTG5-PCR results revealed that O9 can be a new strain belonging to O20.

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