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RESEARCH ARTICLE

THERMOPHILIC PROKARYOTIC DIVERSITY OF EYNAL GEOTHERMAL SPRING AND PROTEASE PRODUCTION POTENTIALS OF ISOLATES

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ABSTRACT

Thermophilic microorganisms are quite attractive for the study of biodiversity and evolutionary process as well as biotechnological applications. These organisms provide significant advantages for industrial and biotechnological processes occurring fast and efficiently at high temperatures. Possible potential also is getting increase thanks to isolation of new strains, determination of new metabolites and their pathway.

Within the scope of this study, thermophilic bacterial community was investigated with a combination of classical microbiology and molecular biology approaches including fluorescent in situ hybridization, amplified ribosomal DNA restriction analysis, and polymerase chain reaction of 16S rRNA gene. Archaea and Bacteria domain were screened by Fluorescence in situ Hybridization technique. At the end of culture-dependent methodology, *Paenibacillus lactis* E3.1 (MK573857), *Brevibacillus borstelensis* E3.2 (MK573871), *Paenibacillus naphthalenovorans* E2.2 (MK573627), *Paenibacillus* sp. E3.5 (MK573870) were obtained. Furthermore, these isolates were screened with regards to protease production capabilities. At the end of screening studies, the highest protease activity (300 U/mg) was observed for *Paenibacillus lactis* E3.1.

Keywords: Eynal, Thermal Spring, Thermophilic, Protease

1. INTRODUCTION

Nowadays, as well as determining the underground treasures of the countries, defining and registering the biological wealth have become an important and necessary situation. The investigation of microbial diversity is important in terms of both the enrichment of the biodiversity inventory of the world and the potential of use in many fields due to the metabolic diversity of the microorganisms to be obtained [1-4]. Specifically, it will provide superiority with regard to potential industrial use to determine microbial diversity of extreme environments such as terrestrial thermal springs.

Thermal aquatic systems have a great interest to researchers such as geochemists, astrobiologists and microbiologists owing to their extreme and unique conditions as well as chemical and biological features. These harsh conditions provide with harboring extremophilic microbes. Drivers of microbial community living in these springs contain temperature, geochemistry, pH, redox potential, dissolved oxygen concentration, metal concentration etc. [5, 6]. Among these abiotic factors, temperature is one of main dynamics in defining the dominating microbial composition [7].

Thermophilic adaptations and entropic stabilization of thermophilic bacteria may be responsible for their thermostable extremozymes such as proteases which have been characterized recently. Eco-friendly

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proteases have greatest market share in commercial enzyme family. Enzymes obtained from thermophiles and hyperthermophiles can catalyse biochemical reactions that must take place at high temperatures, and these enzymes are more resistant than enzymes obtained from mesophiles. Therefore, such enzyme preparations have a longer shelf life. The high thermostability of products obtained from thermophilic microorganisms increases the durability and usage rate of these products in industrial environments. With this reason, researchers have been working to determine alternative protease producers isolated from thermal fields in recent years [8-10].

The primary objective of the present study is to determine the diversity of thermophilic bacteria harboring in Eynal thermal spring waters. Culture-dependent techniques and fluorescent in situ hybridization (FISH) were used to determine this prokaryotic diversity. Isolated microorganisms were grouped by restriction profiles according to Amplified Ribosomal DNA Restriction DNA analysis (ARDRA) method. Molecular identifications of these reduced isolates were carried out and screening of protease production capabilities of representative isolates was performed.

2. MATERIALS AND METHODS

2.1. Chemicals Reagents

In this study, all of chemical reagents for microbiological experiments were provided from Sigma Aldrich (Germany), Fluka (Switzerland) and Merck (Germany). UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories, USA) was used in genomic DNA isolation. PCR Master Mix (Solis Biodyne, Estonia) was used for 16S rRNA gene amplification. Gel loading dye and DNA marker were provided by New England Biolabs (UK).

2.2. Eynal hot Springs and Sampling Collection

Water sample was collected from hot spring being located in Eynal, Kütahya, Turkey on April, 2014, (Figure 1). In addition to Çitgöl and Naşa, Eynal thermal water is also part of Simav geothermal field and it is located in the southern part of the Simav graben in the western region of Turkey. Calcite deposition is detected around springs. Eynal thermal spring has a high geothermal potential with measured well bottom temperatures reaching 163 °C (reservoir temperature) and a 72 L/s artesian discharge rate [11]. The CO_2, SO_2, HCl and H_2S that escaped from the magma have extended the geothermal water reservoir. The equilibrium is reached between altered rocks and fluids in this reservoir. Therefore, the thermal waters escape into tectonic zone of weakness, namely the Simav fault, as hot springs to the surface [12].

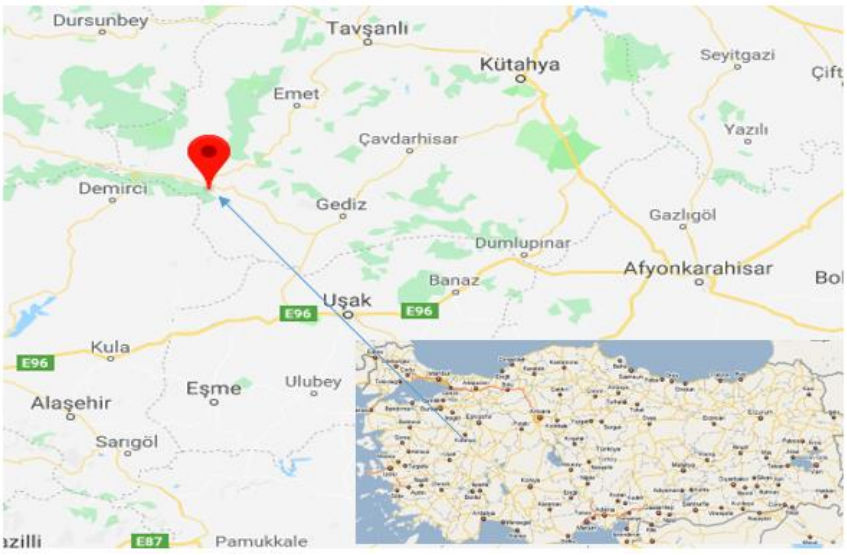

Figure 1. Map of the sampling site

GPS location of Eynal is N39°4' E28°58'. pH of water sample was measured as 8.4. Amount of dissolved oxygen was determined as 6.73 mg/L.

2.3. Diamidino-2-phenylindole (DAPI) staining and Fluorescence in situ Hybridization (FISH) analysis

The fixation of sample (5 mL of water sample) was practiced by using 37% (v/v) formaldehyde and the sample was filtered (0.22 μm filters GTTP, Millipore, MA, USA) to collect the cells. DAPI staining was performed to observe the total cells. The CY3 labelled probes including specific oligonucleotides for Archaea and Bacteria domains used for in situ hybridization were ARC915 (5' GTG CTC CCC CGC CAA TTC T 3') and EUB338 (5' GCT CCC TCC CGT AGG AGT 3'), respectively [13]. After hybridization and staining, visualization of cell was performed by using Epifluorescence microscopy (Leica 6600) [14, 15].

2.4. Isolation of Microorganisms and 16S Ribosomal RNA (rRNA) Gene Amplification

The water sample was diluted serially to isolate of thermophilic bacteria. Dilution range was adjusted from 10^{-1} to 10^{-4}. The water and its dilutions were seeded onto agar medium. The three different media were practiced in thermophilic bacteria isolation: Nutrient Agar (g/L); beef extract 5, peptone 3, agar 30. Thermus Medium (g/L) contained tryptone 3, yeast extract 1, Castenholz 10X basal salt solution [16] 100 mL and 697 Thermus Medium (g/L) included peptone 8, yeast extract 4, NaCl 2, agar 30 [17]. The plates were incubated at 50 °C for 2 days.

For molecular identification, the DNAs of pure isolated were extracted according to protocol described by UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories, USA). 16S PCR products were amplified using microbial DNA as a template in reaction mix which including dH_2O, 5x FirePol. Master Mix (Solis Biodyne, Estonia). 27F (5′-AGA GTT TGA TCA TGG CTC AG-3′) [18] and 1492R (5' GGT TAC CTT GTT ACG ACT T 3') [19] pairs were used respectively as forward and reverse primers for Bacteria domain in amplification. PCR was implemented in 3 min at 94 °C (pre-denaturation), 30 sec at 94 °C, 1 min at 50 °C, 1 min at 72 °C for 30 cycles and 10 min at 72 °C (Applied Biosystems Veriti 96 Fast Thermal Cycler, USA). PCR products were run into 1% agarose gel electrophoresis and imaged by Gel DocTM XR+ System with Image LabTM Software (BIO-RAD). 16S PCR products cut from agarose gel were cleaned up using GeneJET™ Gel Extraction Kit (Fermentas).

2.5. Amplified Ribosomal DNA Restriction Analysis (ARDRA) and Sequencing

The amplified 16S rRNA gene products of isolates were digested with HaeIII at 37 °C for 2 hours. The restriction products were separated by 2.5% agarose gel electrophoresis in 1xTBE (Tris Borate EDTA) for 150 min at 60 V and DNAs were visualized by Gel DocTM XR+ System with Image LabTM Software (BIO-RAD). Different patterns were categorized and isolates belonged to these patterns were selected for sequencing analysis.

The 16S rRNA genes of representative isolates according to ARDRA analysis were sent to commercial sequencing service (Ligand Biotechnology Lab. Suppl. and Inds. Co. Ltd., İzmir, Turkey) to analyze with Sanger Method- Dideoxynucleotide Chain Termination. Chromatograms were viewed by 4Peaks. The sequences were aligned in 16S ribosomal RNA sequence database in BLAST program.

The phylogenetic tree was constructed with sequences of four members of all isolates by Neighbor Joining algorithm in MEGA7 [20, 21].

2.6. Enzyme Production and Protease Activity

The enzyme was produced in basal medium (g/L) including peptone 5, glucose 10, NaCl 0.50, $CaCl_2.2H_2O$ 0.10, K_2HPO_4 0.30, KH_2PO_4 0.40, $MgSO_4.7H_2O$ 0.10, yeast extract 5. Isolates were inoculated as 10% to basal medium. The cultures were incubated for 4 days at 55 °C.

Protease activity assay was carried out by protocol described previously [22], which was optimized temperature being suitable for thermophilic bacteria in our previous studies. The cultures of isolates were centrifuged for 15 min at 14.000 rpm and supernatant was used as a crude enzyme. The reaction medium consists of 50 mM NaOH-glycine-NaCl buffer and 0.6% casein as a substrate. The mixture was incubated for 20 min at 50 °C then it was stopped with TCA (trichloroacetic acid) and incubation was continued at 50 °C for 30 min. Sodium carbonate was added to supernatant obtained after centrifugation at 9000 rpm. Folin-Ciocalteau was added to this mixture, and it was incubated at room temperature for 30 min. The amount of tyrosine was measured as absorbance at 660nm. Protein concentration was measured by Bradford Assay [23]. For each isolate, enzyme activity measurements were made triplicate.

One unit of enzyme activity (U) was defined as the amount of enzyme catalyzed the reaction of 1 µg of tyrosine per minute at 50 °C [24].

3. RESULTS

3.1. Isolation of Microorganisms

Cell counts were made using culture-dependent techniques. Accordingly, no colonies developed in the yeast extract medium. The numbers of colonies growing in Nutrient Agar medium, Thermus medium and 697 Thermus medium were $7.4x10^3$ cfu/mL, $8.6x10^3$ cfu/mL, and $5.3x10^7$ cfu/mL, respectively.

Fourteen microorganisms were isolated from three different media. Furthermore, medium grown and the microscopic properties of isolated microorganisms were defined in Table 1. Four microorganisms were grown in Nutrient agar; however other ten microorganisms were grown in Thermus and 697 Thermus media.

Table 1. The microscopic and Gram properties of isolated microorganisms

Isolate code	Medium	Morphology	Gram Properties
E2.1	Nutrient Agar	Small rod	Gram (-)
E2.2	Nutrient Agar	Small rod	Gram (-)
E2.3	Nutrient Agar	Small rod	Gram (-)
E2.4	Nutrient Agar	Small rod	Gram (-)
E3.1	Thermus	Small rod	Gram (-)
E3.2	Thermus	Flagellate cocci	Gram (+)
E3.3	Thermus	Small rod	Gram (-)
E3.4	Thermus	Flagellate cocci	Gram (+)
E3.5	Thermus	Small rod	Gram (-)
E4.1	697 Thermus	Cocci	Gram (-)
E4.2	697 Thermus	Small rod	Gram (+)
E4.3	697 Thermus	Small rod	Gram (-)
E4.4	697 Thermus	Small rod	Gram (+)
E4.5	697 Thermus	Small rod	Gram (-)

3.2. ARDRA

Microorganisms were digested with HaeIII. According to restriction analysis, four patterns were observed. Figure 2 shows the all pattern with signs in different colour and numbered groups. Group 1 indicated as light blue sign, including four isolates coding as #E2.1, #E2.2, #E2.3, #E2.4. Group 2 labelled purple had seven isolates coding as #E3.1, #3.4, #E4.1, #E4.2, #E4.3, #E4.4, #E4.5. Group 3 labelled yellow, included only one isolate coding as #E3.2. Group 4 indicated as green sign, including two isolates coding as #E3.3 and #E3.5.

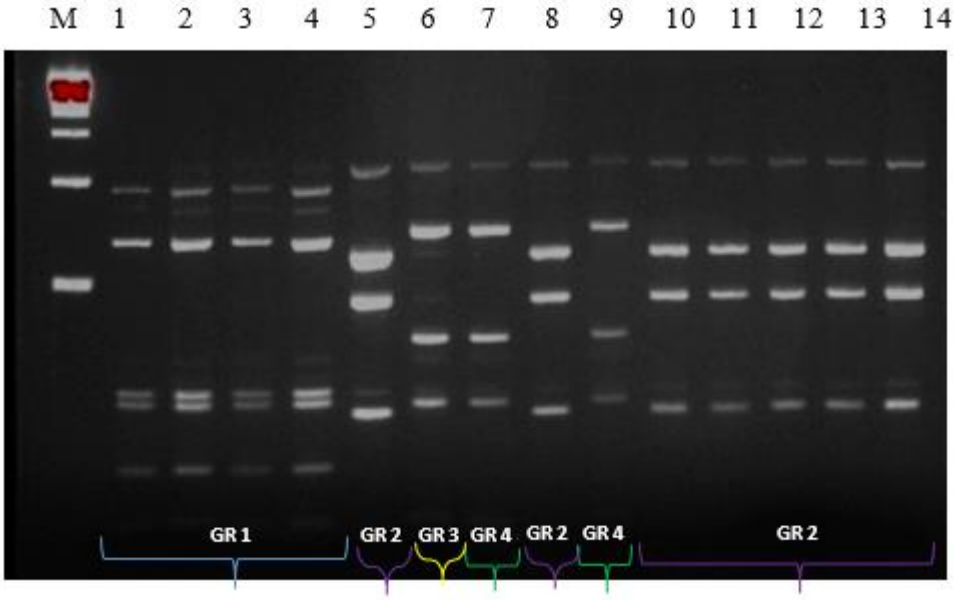

Figure 2. ARDRA analysis of isolates. M: DNA Ladder; 1:E2.1; 2:E2.2; 3:E2.3; 4:E2.4; 5:E3.1; 6:E3.2; 7:E3.3; 8:E3.4; 9:E3.5; 10:E4.1; 11:E4.2; 12:E4.3; 13:E4.4; 14:E4.5. Note: 1-4 (Group 1), 5, 8, 10-14 (Group 2), 6 (Group 3), 7 and 9 (Group 4)

3.3. Identification of Isolates

According to ARDRA analysis, four isolates (E2.2, E3.1, E3.5 and E3.2) were chosen and their 16S rRNA genes were sequenced with forward primer. #E2.2, #E3.1, #E3.5 and #E3.2 isolates were identified as *Paenibacillus naphthalenovorans, Paenibacillus lactis, Paenibacillus* sp., *Brevibacillus borstelensis,* respectively. Highly matched species were determined and these sequences were submitted to GeneBank to take the Accession number (Table 2).

Table 2. Accession numbers for 16S rRNA sequences of thermophilic isolates

Isolate	Accession Number	Highly Matched Bacteria (NCBI)	Number of Sequencing Base (bp)
E2.2	MK573627	*Paenibacillus naphthalenovorans*	1287
E3.1	MK573857	*Paenibacillus lactis*	1260
E3.5	MK573870	*Paenibacillus* sp	1199
E3.2	MK573871	*Brevibacillus borstelensis*	1293

The phylogenetic tree (Figure 3) with the sum of branch length = 0.40100133 is shown. 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 Maximum Composite Likelihood method [25]. The analysis involved 5 nucleotide sequences. *E.coli* (NR 024570.1) strain was used as extern species during the construction of phylogenetic tree. All positions containing gaps and missing data were eliminated.

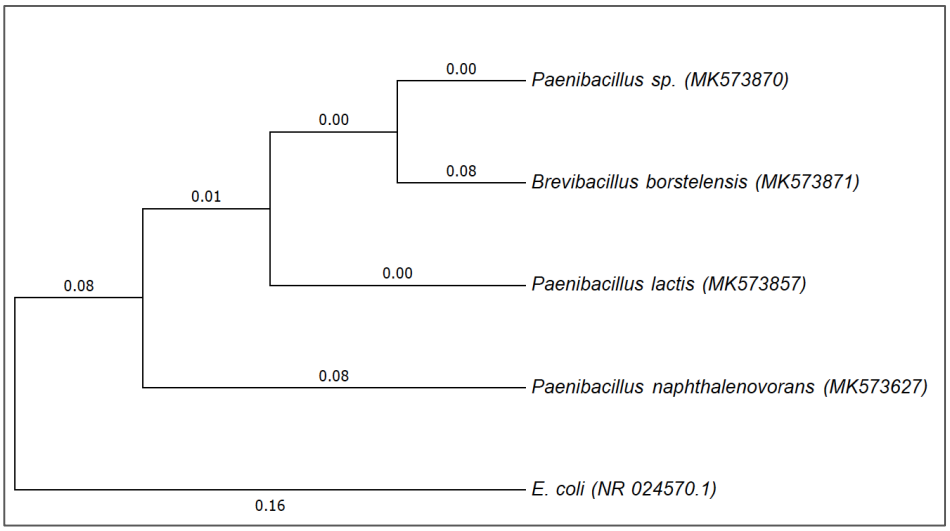

Figure 3. Phylogenetic analysis of isolates

3.4. DAPI and FISH Analysis

Cell density was screened by FISH analysis to use the CY3 labelled probes. The view of Epifluorescence microscopy was presented in the Figure 4. Figure 4a and 4b were shown respectively DAPI and FISH analysis for Archaea domain. Figure 4c and 4d were shown respectively DAPI and FISH analysis for Bacteria domain. According to these results, bacterial density was found higher than archaeal density.

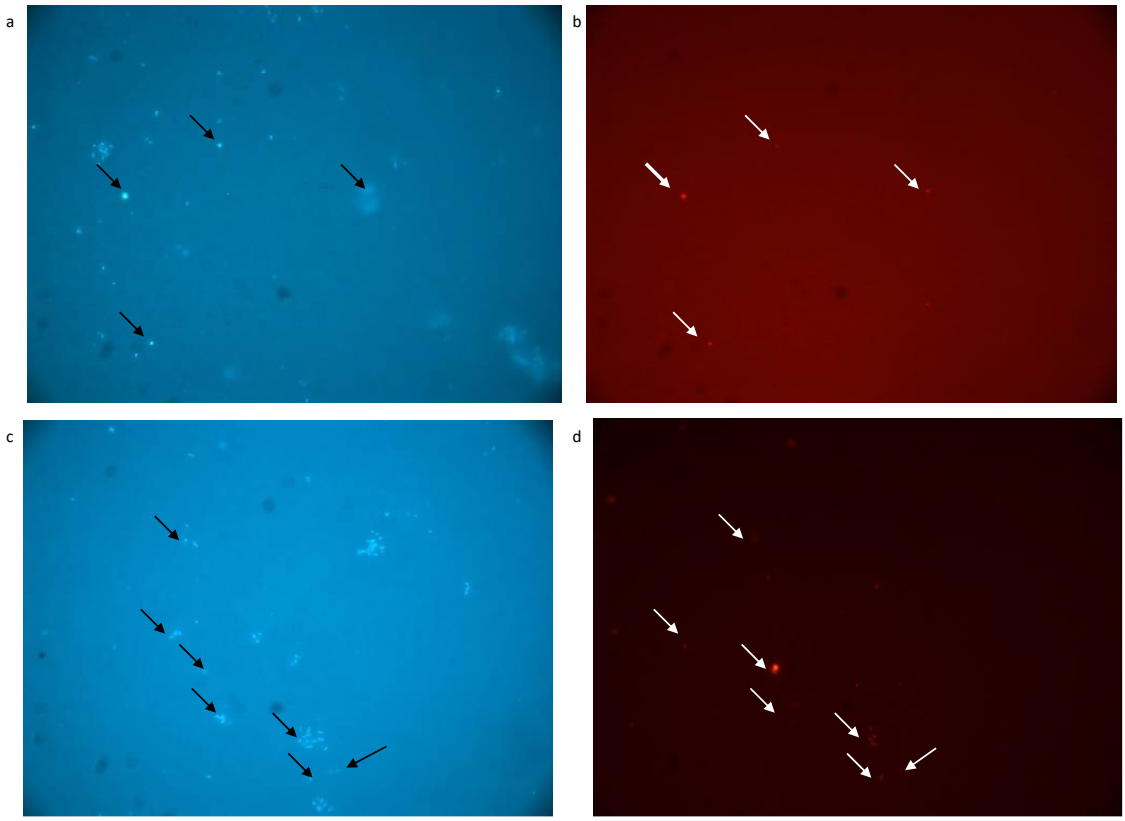

Figure 4: DAPI staining (left) and FISH analysis (right) of environmental sample. a-b: Archaea domain c-d: Bacteria domain.

3.5. The Ability of Protease Production

After fourteen isolates were reduced according to ARDRA analysis, four representative thermophilic bacteria were used to screen of ability of protease production. Specific activity was calculated by the ratio of enzyme activity and protein amount. The crude enzyme was used at screening of production ability. The isolate of *Paenibacillus lactis* E3.1 had the highest specific activity being as 300 U/mg while the protease specific activities for E2.2, E3.5 and E3.2 isolates were 212, 225 and 205 U/mg, respectively (Figure 5).

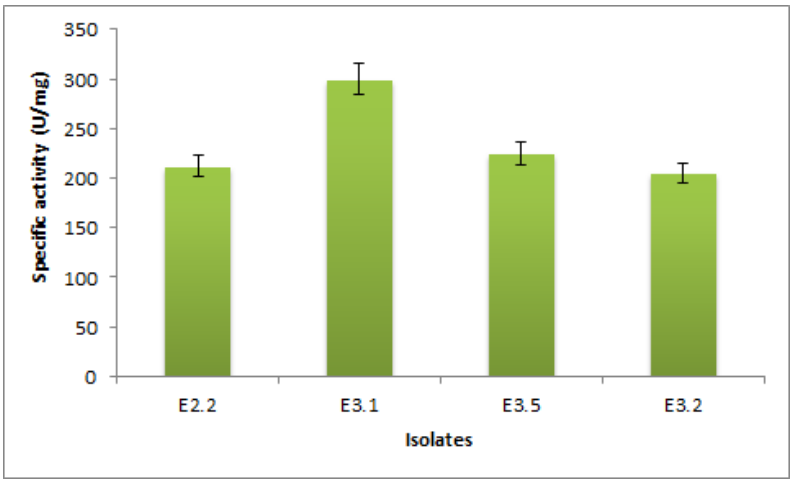

Figure 5: The screening of ability of protease production of isolates

4. DISCUSSION

In this study, isolations of thermophilic bacteria have been performed. ARDRA analysis was performed and the isolates giving different restriction profiles were chosen to identify isolates and screen of protease activity. The identified microorganisms were *Paenibacillus naphthalenovorans, Paenibacillus lactis, Paenibacillus* sp., *Brevibacillus borstelensis.* The end of the screening of protease activity, highest specific activity belonging to #E3.1 isolate was obtained.

There are a few studies about thermophilic prokaryotic diversity of Eynal in the literature. Akkaya & Kıvanç [26] have reported gram positive bacilli included in thermophilic bacteria from different thermal springs as well as Eynal.

In other study, cyanobacterial composition of hot spring in Eynal was reported by Yilmaz Cankilic [27]. *Synechococcus*, *Geitlerinema*, *Phormidium*, and filamentous thermophilic cyanobacterium were identified in the study. Besides, clone libraries and DGGE (Denaturing Gradient Gel Electrophoresis) analysis were performed to show the microbial diversity [27]. Apart from this study, Yilmaz Cankilic and colleagues reported thermophilic bacteria isolated from Balıkesir (Sindirgi, Gure, and Havran) and Kütahya (Eynal). G*eobacillus thermoleovorans, Geobacillus kaustophilus, Bacillus sonorensis, Bacillus licheniformis, Geobacillus* sp., *Aeribacillus pallidus* and *Geobacillus thermoparaffinivorans* were dominantly isolated from Eynal. They screened protease, amylase and lipase activity of all thermophilic isolates. Activities of these three enzymes had been only determined on agar plates qualitatively [28]. On the other hand, the isolates were screened in terms of protease production potential by Anton's protease activity assay quantitatively in our study.

In other different study, Chen and colleagues studied on arsenic concentrations and related microbial diversity from Western in Turkey. They investigated microorganisms related to only arsenic concentration in geothermal water [29].

There are very much study about protease production and characterization of protease enzyme in literature. Moreover, protease activities of *Paenibacillus* species were screened in this study. However, there are a few studies about protease of *Paenibacillus* isolated from thermophilic area [30-33]. Besides, there is no study about protease of *Paenibacillus lactis.*

Cavello and colleagues reported hydrolytic enzymes activities of thermophilic bacteria isolated from Patagonia, Argentina. Production abilities of hydrolytic enzymes including keratinases, proteases, esterases, amylases, cellulases, inulinases, pectinases and xylanases from thermophilic isolates were investigated in study. 83% of screened isolates containing *Paenibacillus dendritiformis* was reported to have protease activity [32]. Panosyan investigated production abilities of hydrolytic enzymes from thermophilic bacilli including *Paenibacillus* genus. Proteolytic activity was detected qualitatively according to casein hydrolysis on agar plates [33]. Besides, Rai and co-workers studied alkaline protease producing bacterial strain isolated from Assam. They produced protease of *Paenibacillıs tezpurensis* and characterized this enzyme [30]. Pandey and co-workers isolated twenty-eight microorganisms including *Paenibacillus ehimensis* members from hot spring in Uttarakhand. They determined tolerance to wide temperature and pH range of isolates and their productions of enzymes such as lipase, protease, amylase, cellulase, xylanase [31].

5. CONCLUSION

Isolated thermophilic strains can be used to produce thermostable/thermotolerant enzymes and other metabolites for biotechnological process. In this study, archaeal cell presence was observed according to FISH analysis. This finding has been firstly reported for microbial studies from Eynal thermal spring in Turkey. The strains of *Paenibacillus lactis* E3.1 (accession number: MK573857), *Brevibacillus borstelensis* E3.2 (accession number: MK573871), *Paenibacillus naphthalenovorans* E2.2 (accession number: MK573627), *Paenibacillus* sp. E3.5 (accession number: MK573870) were isolated first from Eynal spring. Besides, among these isolates, *Paenibacillus lactis* E3.1 isolate had the highest potential to produce protease with 300 U/mg of specific activity. Protease production from *Paenibacillus lactis* strain was first reported in this study. Further studies will be carried out relating to enzyme purification and characterization.

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