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
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

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 Çıtğöl 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 CO₂, SO₂, HCl and H₂S 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 dH₂O, 5x FirePol. Master Mix (Solis Biodyne, Estonia). 27F (5'-AGA GTT TGA TCA TGG CTC AG-3') [18] and 1492R (5' GGT TAC CTT GTC AGT 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., Izmir, 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$_2$O 0.10, K$_2$HPO$_4$ 0.30, KH$_2$PO$_4$ 0.40, MgSO$_4$.7H$_2$O 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.

| 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 (-)        |

Table 1. The microscopic and Gram properties of isolated microorganisms
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.

![ARDRA analysis of isolates](image)

**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).

| 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.
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 3.** Phylogenetic analysis of isolates

**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](image)

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 Yılmaz Cankılıç [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, Yılmaz Cankılıç and colleagues reported thermophilic bacteria isolated from Balıkesir (Sindirgi, Gure, and Havran) and Kütahya (Eynal). *Geobacillus 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 *Paenibacillus 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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REFERENCES

1. Nural Yaman B, Mutlu M B, Aytar Çelik P, Çabuk A, Metagenomics (16S amplicon sequencing) and DGGE analysis of bacterial diversity of acid mine drainage, J Microbiol Biotechnol Food Sci 2020; 9: 932-936.

2. Nural Yaman B, Aytar Çelik P, Mutlu M B, Cabuk A. A combinational analysis of acidophilic bacterial diversity of iron-rich environment, Geomicrobiol J 2020; DOI: 10.1080/01490451.2020.1795320

3. Aytar P, Kay C M, Mutlu M B, Cabuk A, Coal desulfurization with *Acidithiobacillus ferrivorans*, from Balya acidic mine drainage, Energ Fuel 2013; 27: 3090-3098.
4. Nural Yaman B, Deniz Sonmez G, Aytar Celik P, Korkmaz F, Mutlu M B, Cabuk A, Culture-dependent diversity of boron-tolerant bacteria from boron mine tailings pond and solid wastes, Water Environ J 2019; 33: 574-581.

5. Gedikli S, Aytar Çelik P, Demirbilek M, Mutlu M B, Denkbaş E B, Çabuk A, Experimental exploration of thermostable poly(β-hydroxybutyrylates) by Geobacillus kaustophilus using Box-Behnken design, J Polym Environ 2019; 27: 245-255.

6. Wilkins LGE, Ettinger C L, Jospin G & Eisen J A, Metagenome-assembled genomes provide new insight into the microbial diversity of two thermal pools in Kamchatka, Russia, Sci Rep 2019; 9:1-15.

7. Coman C, Drugă B, Hegeduś A, Sicora C & Dragos N. Archaeal and bacterial diversity in two hot spring microbial mats from a geothermal region in Romania. Extremophiles 2013; 17:523-534.

8. Özçelik B, Aytar P, Gedikli S, Yardımcı E, Çalıkkan F & Çabuk A, Production of alkaline protease using Bacillus pumilus D3 without inactivation by SDS, its characterization and purification, J Enzyme Inhib Med Chem 2014; 29:388-396.

9. Yiğit Şat E, Nural Yaman B, Gedikli S, Aytar Çelik P, Çabuk A, Heat and pH stable protease produced by a bacterium isolated from fields with high boron, J Microbiol Biotechnol Food Sci 2020; 9: 1047-1052.

10. Yardımcı Akkır E, Şahin Y B, Gedikli S, Çelik P A & Çabuk A, Extremely thermostable, EDTA-resistant alkaline protease from a thermophilic Geobacillus subterraneus C2-1 isolate. J Microbiol Biotechnol Food Sci 2017; 7:50-56.

11. Gemici Ü & Tarcan G, Hydrogeochemistry of the Simav geothermal field, western Anatolia, Turkey. J Volcanol Geotherm Res 2002; 116:215-233.

12. Bello O, Özgür N & Çalışkan T A, Hydrogeological, hydrogeochemical and isotope geochemical features of Geothermal waters, in Simav and environs, Western Anatolia, Turkey, Procedia Earth Planet Sci 2017; 17:29-32.

13. Amann R, Ludwig W & Schleifer K H, Phylogenetic identification of individual microbial cells without cultivation, Microbiol Rev 1995; 59:143-169.

14. Mutlu M B & Güven K, Detection of prokaryotic microbial communities of Çamaltı Saltern, Turkey, by fluorescein in situ hybridization and real-time PCR. Turkish J Biol 2011; 35:687-695.

15. Aytar P, Kay C M, Mutlu M B, Çabuk A & Johnson D B, Diversity of acidophilic prokaryotes at two acid mine drainage sites in Turkey, Environ Sci Pollut Res 2015; 22:5995-6003.

16. Castenholz RW, Thermophilic blue-green algae and the thermal environment, Bacteriol Rev 1969; 33:476-504.

17. Brock TD, Thermophilic microorganisms and life at high temperatures (Springer-Verlag, New York) 1978.

18. Lane D J, Pace B, Olsen G J, Stahl D A, Sogin M L & Pace N R, Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses, Proc Natl Acad Sci 1985; 82:6955-6959.
19. Marchesi J R, Sato T, Weightman A J, Martin T A, Fry J C, Hiom S J & Wade W G, Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA, Appl Environ Microbiol 1998; 64:795-799.

20. Saitou N & Nei M, The neighbor-joining method: A new method for reconstructing phylogenetic trees, Mol Biol Evol 1987; 4:406-425.

21. Kumar S, Stecher G, & Tamura K, MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 2016; 33:1870-1874.

22. Anson M L, The estimation of pepsin, trypsin, papain and cathepsin with haemoglobin, J Gen Physiol 1938; 22:79–89.

23. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding, Anal Biochem 1976; 72: 248-254.

24. Takami H, Akiba T & Horikoshi K, Production of extremely thermostable alkaline protease from Bacillus sp. no. AH-101, Appl Microbiol Biotechnol 1989; 30:120-124.

25. Tamura K, Nei M & Kumar S, Prospects for inferring very large phylogenies by using the neighbor-joining method, Proc Natl Acad Sci USA 2004; 101:11030-11035.

26. Akkaya S & Kızılanç M, Termofil Bakteriler Sıcak Su Kaynaklarında Yaşayan Gr (+) Basillerin İzolasyon ve İdentifikasyon Yöntemleri, AKU-J Sc. Eng 2008; 8:61-70.

27. Yılmaz Cankılıc M, Determination of Cyanobacterial Composition of Eynal (Simav) Hot Spring in Kütahya, Turkey, Appl Ecol Environ Res 2016; 14:607-622.

28. Yılmaz Cankılıc M, Celikoglu E & Celikoglu U, Screening of Hydrolytic Enzyme Production and Fatty Acid Methyl Esters (Fame) Analysis of Thermophilic Bacteria from Hot Springs, Fresen Environ Bull 2017; 26:5865-5872.

29. Chen C, Maity J P, Bundschuh J, Bhattacharya P, Baba A & Gündüz O, Occurrence of arsenic and related microbial signature of hydrothermal systems in Western, Turkey (London: CRC Press) 2012; pp. 486-488.

30. Rai S K, Roy J K & Mukherjee A K, Characterisation of a detergent-stable alkaline protease from a novel thermophilic strain Paenibacillus tezpurensis sp. nov. AS-S24-II, Appl Microbiol Biotechnol 2010; 85:1437-1450.

31. Pandey A, Dhakar K, Sharma A, Priti P, Sati P & Kumar B Thermophilic bacteria that tolerate a wide temperature and pH range colonize the Soldhar (95 °C) and Ringigad (80 °C) hot springs of Uttarakhand, India, Ann Microbiol 2015; 65: 809-816.

32. Cavallaro I, Urbia M S, Segretin A B, Giaveno A, Cavalitto S & Donati E R, Assessment of keratinase and other hydrolytic enzymes in thermophilic bacteria isolated from geothermal areas in Patagonia Argentina, Geomicrobiol J 2018; 35:156-165.

33. Panosyan H H, Thermophilic bacilli isolated from Armenian geothermal springs and their potential for production of hydrolytic enzymes. Int J Biotech Bioeng 2017; 3: 239-244.