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Research Article

Exploring the Bacterial Diversity of the Armutlu Geothermal Spring (Türkiye) through a Metagenomic Sequenced Based Approach

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Abstract

The geothermal or hot springs are home to a diverse microbial population. Identification of the thermophilic microbial world of these niches can direct us to the exploration of novel economically valuable biocatalysts, antibiotics, and a variety of other biomolecules. Due to the tectonic features within its borders, Türkiye has many thermal springs in different regions. In this study, a thermal site, the Armutlu geothermal spring in the eastern Marmara region, was investigated to identify the bacterial taxonomic composition and diversity through a metagenomic sequence-based approach which is the first study to the best of our knowledge. During the period of sampling, the water temperature and pH at the surface were 74.9 °C and 7.1, respectively. According to 16S rRNA sequencing results, Bacteria (98.36%) are the dominant taxa compared to Archaea (1.64%). Based on the diversity findings, the bacterial community is primarily dominated by *Proteobacteria* (42.49%), which, together with *Chloroflexi*, *Acidobacteria*, *Actinobacteria*, *Firmicutes*, *Nitrospirae*, and *Bacteroidetes,* constitute ~87% of the overall bacterial population. Furthermore, the microbial community was also investigated for the activity of hydrolase enzymes such as protease, amylase, and amylopullulanase through a culture-dependent approach for possible future applications. As a conclusion, the microbiota of the Armutlu geothermal spring was open to investigate the novel species and hydrolytic biocatalysts.

Keywords: Bacterial community, Armutlu geothermal spring, Thermophile, 16S rRNA sequencing, Hydrolases

1. INTRODUCTION

The geothermal or hot springs and hydrothermal vents are examples of extreme environments that inhabit a very diverse of microorganisms [1]. These microorganisms that include bacterial and archaeal species can resist high temperatures in their habitat due to their stable cell structure, metabolism, and biomolecules [2].

Thermozymes are biomolecules that can function at high temperatures and have the potential to be used in new ways in biocatalytic processes [3]. These different biocatalysts can be utilized in different areas of industry including agriculture, bioremediation, pharmacy, and medicine [4].

The geothermal resources of Türkiye are extensive since it is located in an active volcanic/tectonic geography [5]. According to data from the General Directorate of Mineral Research and Exploration (MTA), the official institution responsible for the exploration of Türkiye's underground resources, there are approximately 1500 geothermal resources within Türkiye's borders, some of which have water temperatures reaching 287.5 °C [6].

Because of the abundance of thermal areas in Türkiye, many studies on microbial diversity have been encouraged [7,8]. Some of them have focused on not only the identification of novel species [9,10] but also the purification and characterization of various thermozymes such as phytase [11], amylase [12], and cellulase from different thermal sources [13].

Considering that only 1% of microorganisms in nature can be cultured, the identification of organisms that thrive in extreme conditions such as thermal springs is a very challenging task [14]. However, a "metagenome" containing the DNA of all

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microorganisms in the environment will enable the identification of all species in the target area, whether they can be cultured or not. Moreover, it is possible to obtain detailed information that conventional PCR-based 16S rRNA analysis cannot provide. In addition to obtaining sequence-based information, functional analysis can be performed with designed metagenome libraries to identify enzymes with different features [15].

Recently, with the development in the sequencing methodology, the utilization of next-generation sequencing (NGS) has placed itself as an effective tool for the aim of unraveling the biodiversity of complex systems and habitats. Among its wide-range applications, 16S rRNA amplicon sequencing from metagenomic samples, has been extensively used for the investigation of the microbial taxa in different areas such as hot springs [16], pediatric gut [17], human urine [18], and cornea [19].

The geothermal sources in Türkiye are widely used in multiple fields, including but not limited to tourism, heating, and agriculture [5]. Nonetheless, current knowledge regarding the microbial world of these extreme regions is quite limited. From an evolutionary perspective, the identification of the organisms that survive in these areas and their metabolisms may shed light on how life on Earth began. Furthermore, the biomolecules produced by these organisms to survive in these extreme conditions can be identified and applied to biotechnology for the benefit of humanity [15]. In making these discoveries, time-consuming and laborious conventional identification methods including culturing, single colony isolation, Denaturing Gradient Gel Electrophoresis (DGGE), and Sanger sequencing were initially used [20]. With the usage of next-generation sequencing, 16S rRNA amplicon sequencing, and metagenomic approaches, this workload is no longer necessary and even the presence of non-culturable organisms in the target site can be detected and identified. In this study, by utilizing this powerful technology, it was aimed to explore the microbial community of the Armutlu geothermal spring, which is one of the several thermal sites in the Marmara region. In our previous work, this area was used for the isolation of a thermophile bacterium, *Geobacillus thermoleovorans* ARTRW1 [21]. To the best of our knowledge, this is the first work for unrevealing the whole bacterial community of the Armutlu geothermal spring located in the eastern Marmara region through a metagenomic sequence-based approach. Moreover, the activity of some industrially important enzymes, amylase, protease, and amylopullulanase, were screened via culture-based methods to understand the hydrolytic capability of the microbial community in this geothermal spring.

2. MATERIAL AND METHODS

2.1 Sampling Site and Collection of Samples

The Armutlu geothermal spring is a well-known thermal tourism site in Yalova, Türkiye. There are many hotels which are located in Armutlu and thermal water is supplied to these hotels through several vents. The sampling site was visited on June 2017. During sampling, to eliminate possible contamination, samples were taken from coordinates 40°32'44.54"N, 28°50'30.12"E, where the human impact was assessed to be the lowest. Sterilized tubes were used to collect sediment samples at a depth of 10-20 cm, and the samples were promptly transferred to the laboratory at 4 °C under aseptic conditions. These samples were kept at a temperature of -20 °C for further steps.

2.2 Culture Preparation of Sediment Samples

The thermostable microbes of the microbial community were cultured and preserved in Nutrient Broth (NB) as follows: One gram of sediment was mixed with 100 ml of NB medium (0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 0.5% (w/v) sodium chloride) and was incubated at 50 °C and 200 rpm for 24 h. After the incubation, the grown culture was stored at -80 °C in fresh NB medium containing 15% glycerol for further studies.

2.3 Metagenomic DNA Extraction

For the extraction of the total genomic DNA (gDNA) from the environmental samples, the QIAamp PowerFecal Pro DNA Kit (QIAGEN, Hilden, Germany) was used following the protocol provided by the manufacturer. The purity and quantity of the total gDNA were measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

2.4 16S rRNA Amplicon Sequencing and Bioinformatic Analyses

Using bacteria specific primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') that target the V3-V4 hypervariable regions of the 16S rRNA gene, amplicon sequencing libraries were constructed from extracted environmental DNA [22]. Purified libraries were pooled and quantified before sequencing on the Illumina MiSeq instrument (Illumina, USA) with 300 bp paired-end sequencing chemistry. CASAVA data processing software was used to demultiplex and trim adapter sequences from raw sequences (Illumina, USA). Any fragments with barcode or primer mismatches were discarded. To remove the PCR primers from the sequences, cutadapt plugin in QIIME2 (v2020.2) was used [23,24]. The paired-end reads were merged (vsearch join-pairs) and the quality was checked (quality-filter q-score-joined). Following that, deblur (deblur denoise-16S) was used to denoise the sequences [25]. The 'feature-classifier classify-sklearn' plugin was used against the pre-trained Naive Bayes classifier (classifier_silva_132_99_16S_V3.V4_341F_805R.qza) to assign taxonomy to each amplicon sequence variation (ASV).

2.5 Screening of Thermostable Hydrolytic Enzyme Activity

The activities regarding different hydrolytic enzymes of the microbial community were assessed using NB agar plates (1.5% (w/v)). The prepared culture with NB medium was diluted and spread on agar plates supplemented with enzyme-specific substrates including starch (0.5% (w/v)), red pullulan (0.5% (w/v)), and skim milk (1% (w/v)) for amylase, amylopullulanase, and protease, respectively. These plates were on hold aerobically at 60 °C for 18 h. The clear zones surrounding the colonies were indicators of enzyme activity.

3. RESULTS AND DISCUSSION

3.1 Assessment of the Sampling Site

The Armutlu Peninsula, located in the Marmara Region of Türkiye, exhibits a notable degree of thermal activity owing to its complex tectonic configuration [26]. The peninsula's northern and western regions are home to many geothermal sites with surface temperatures that can attain levels reach 70 °C [27]. Before sampling, the water temperature and pH at the surface was determined as 74.9 °C and 7.1, respectively. Although there have been many studies focusing on the geological situation of this site, to the best of our knowledge, no prior study has been conducted to unravel the microbial diversity of the Armutlu geothermal spring.

3.2 The Metagenomic Data and Microbial Taxonomic Composition

The microbial community of the Armutlu geothermal spring was evaluated according to the Illumina shotgun sequencing of V3-V4 hypervariable regions of the 16S rRNA gene. After the trimming, the sequencing process has resulted with 12368 reads, which were clustered into a total number of 1367 observed ASVs.

Due to the capability of Domain Archaea to withstand various environmental factors, archaeal diversity is generally predicted to predominate microbial diversity at high temperatures in hot springs [28]. On the contrary, based on the taxonomic profiling of the results of 16S rRNA amplicon sequencing, the Armutlu geothermal spring exhibits a different composition in the microbial community: the Domain Bacteria constituted 98.36% of the prokaryotes, whereas the Domain Archaea contributed only 1.64%. Thermal springs in various countries, including Algeria and India, exhibit a similar taxonomic pattern [29,30]. In the literature, with a temperature of 90 °C in the Zharkent geothermal spring, Kazakhstan, the bacterial community reached 99.97% of the total microbial biodiversity [31].

From this point of view, it was suggested that the structure of the microbial consortium has been affected not only by the physicochemical effectors such as temperature and pH but also by the geographical location and the chemical dynamics within its microenvironment [32,33].

3.3 The Taxonomic Diversity of Bacterial Communities in the Armutlu Geothermal Spring

According to the detailed bioinformatics analysis of the resulting amplicon sequences, the bacterial population could be classified into 35 phyla, 100 class, 261 order, 395 family, 604 genus and 731 species.

At the phylum level, the bacterial composition of the Armutlu geothermal spring was predominantly composed of Gram-negative bacteria (~%80). Owing to the observed total ASVs of the sample, *Proteobacteria* (42.49%) was the most dominant phylum, followed by *Chloroflexi* (11.23%), *Acidobacteria* (9.46%), *Actinobacteria* (8.22%), *Firmicutes* (6.64%), *Nitrospirae* (4.66%), *Bacteroidetes* (4.25%), *Rokubacteria* (2.46%), *Gemmatimonadetes* (1.60%), *Planctomycetes* (1.39%), *Spirochaetes* (1.15%), and a candidate phylum, GAL15 (1.11%). The phyla that contributed to the bacterial population with less than 1% in relative abundance were represented by 5.34% (Figure 1).

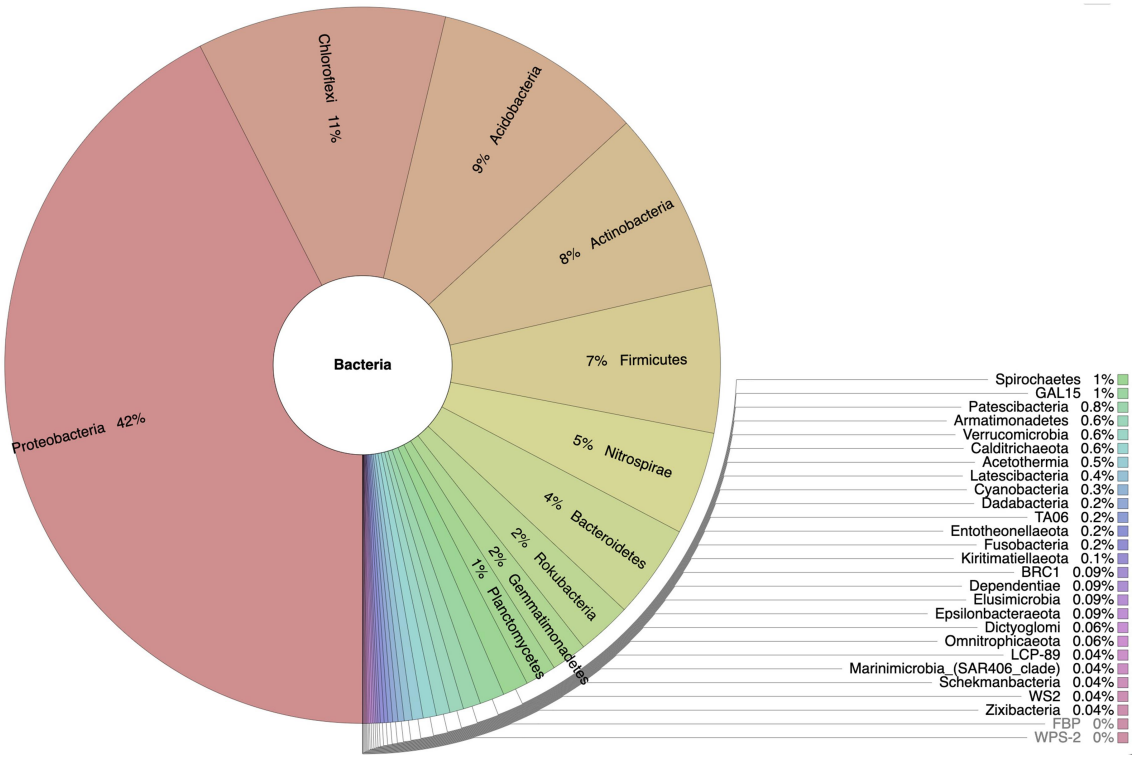

Figure 1. The graphical representation of the distribution of the bacterial consortium found in the Armutlu geothermal spring. The Krona graph is designed to exhibit the relative abundance at phylum-level

It was observed that there was a significant dominance of *Proteobacteria* phylum in the bacterial community. This pattern was also observed in different hot springs of Sikkim (50-77 °C, pH 5-8) [32], and in Pakistan (60 °C, pH 8.4) [34]. This paradigm is explained as *Proteobacterium* prefers to survive in temperatures <75 °C [35]. Since the phototrophic bacteria phylum, *Chloroflexi,* is also a moderate temperature microorganism, it took second place in the bacterial abundance, as expected [34].

Two different studies from India which had the similar temperature (76.3 °C and 71 °C) and pH values (7.52 and 6.8) to our study demonstrated that *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, *Bacteroidetes*, and *Nitrospirae* were the top ten bacterial phyla at the phylum level [32,36]. These observations demonstrated consistency with our results. Moreover, it is understood that although the predominancy of the phylum might change due to the physical conditions, these phyla members could be estimated in the bacterial community of thermal springs at the indicated temperature and pH.

Among the observed community members with low abundance, some of them participate in different ecological pathways. *Bacillus funiculus* is a soil bacterium and takes part in the denitrification process [37]. *Dehalococcoidia* and *Acidothiobacillus* species are considered in sulfur cycling [38,39].

3.4 Screening of Thermostable Hydrolytic Enzyme Activities of Microbial Community

The majority of enzymes used in different fields of biotechnology and industry are hydrolase class enzymes. Since these enzymes are generally purified from mesophilic microorganisms, the structure of the enzymes denatures, and becomes dysfunctional in chemical

processes that require high temperatures. For this reason, obtaining enzymes from microorganisms that can survive at extreme temperatures has increased the interest in thermophilic organisms and therefore, thermozymes [4,40]. The temperature-resistant nature of thermozymes has made them frequently preferred in industrial areas such as the starch industry, which requires high temperatures. In this study, the microbiota was also observed against specific substrates to reveal the potential hydrolase activity. The presence of the production of amylase, amylopullulanase, and protease was screened with supplemented agar plates. These target enzymes are used in many areas such as detergent, food, textile, paper, and pharmaceutical industry, and are therefore very essential members of hydrolases. According to the observations on agar plates, it was determined that the microbiota of the Armutlu geothermal consists of members that have these screened hydrolases (Figure 2).

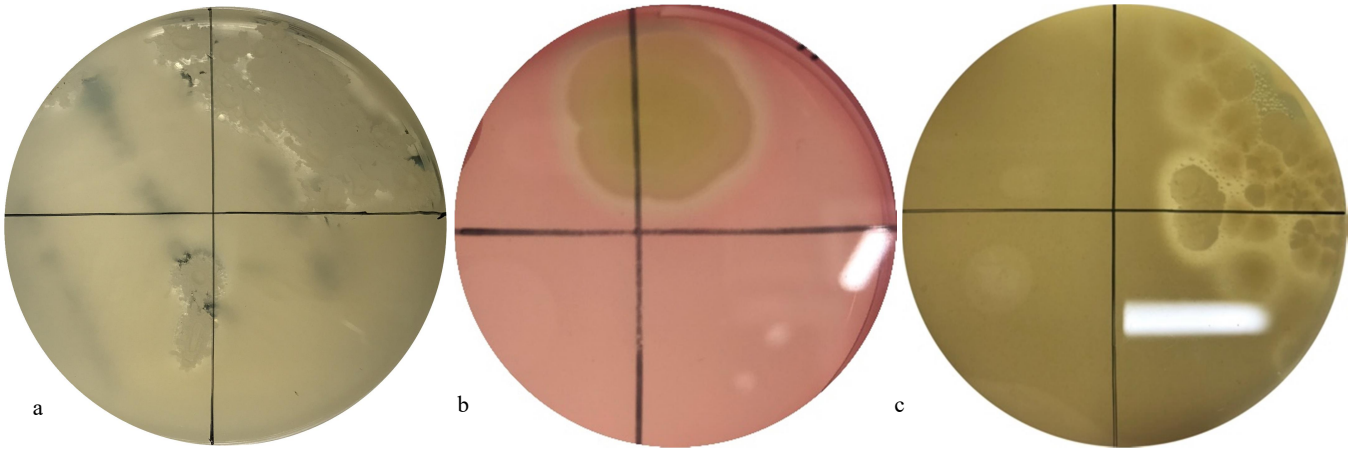

Figure 2. The view of agar plate-based screening for a. protease, b. amylopullulanase and, c. amylase. The holo around the colonies demonstrate the hydrolytic activity against the specific substrate

4. CONCLUSION

The investigation of microbial diversity inhabiting extreme environments, such as geothermal or hot springs, in different locations around the world has been the point of a large number of different studies. The use of next-generation sequencing methodologies in combination with a metagenomic approach has enabled researchers to study these extreme environments without being culture-dependent. In this sense, this present work shows a significant potential to provide the discovery of novel biological molecules in these extreme environments.

To the best of our knowledge, this is the first report of the microbial diversity of the Armutlu geothermal spring via utilizing the metagenomics approach. The bacterial community's ability to produce hydrolase class enzymes including amylase, amylopullulanase, and protease, has been demonstrated through culture-dependent techniques. In addition, the 16S rRNA sequences obtained in the present study were compared with species for which this data has been previously submitted in the associated databases, but the majority of the sequences matched mostly with uncultured species. This could be a proof of the microbial variation in such extreme areas. Moreover, among microbial species that were identified in this present work, such as denitrifying bacteria like *Bacillus funiculus*, which can be used for bioremediation, *Dehalococcoidia* species, which are involved in the sulfur cycle, and *Acidothiobacillus* species, which could be utilized in the biohydrometallurgical area. Therefore, new genomic, metaproteomic, and metatranscriptomic methods should also be applied to elucidate the properties of microbial communities in these regions and to discover new biomolecules from these communities.

The microbial identification of these extreme environments will provide information about not only the evolution of the microorganisms from “the beginning” but also the estimation of life in new environments and habitats that human has not reached and discovered, yet. Moreover, from the industrial perspective, the discovery of novel enzymes with high stability and activity under high temperatures will contribute a high impact on the industrial yield where these features are needed.

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AUTHOR'S CONTRIBUTIONS

AC: Designing and performing the study, writing and editing the manuscript. MO: Sampling, performing the study. HM: Sampling, performing the study. NGK: Conceptualization and methodology, supervision, writing and reviewing the manuscript, funding.

CONFLICTS OF INTEREST

All authors declared that there is no conflict of interest.

RESEARCH AND PUBLICATION ETHICS

All authors declare that this study complies with Research and Publication Ethics.

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