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Bacterial Diversity in a Sri Lankan Geothermal Spring Assessed by Culture-Dependent and Culture-Independent Approaches

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Abstract
Hot springs harbour diverse and interesting groups of microorganisms adapted to extreme conditions. However, due to limitations in the culture-dependent approach, most of such thermophiles remain uncultured and unexplored. Hence, this study was conducted to gain a comprehensive understanding of the bacterial diversity of Mahapelessa hot spring, Sri Lanka using both culture-dependent and culture-independent approaches. The in situ temperature of the water sample was 44.5 °C and the pH was 8.14. 16S rRNA Sanger sequencing of DNA extracted from the 18 bacterial isolates revealed the presence of eight genera belonging to two phyla: Proteobacteria (84%) and Firmicutes (16%) and the most abundant genus being *Klebsiella.* A total of 23 bacterial phyla representing 80 classes, 43 orders, 123 families, 205 genera and 83 species were detected by 16S rRNA V3-V4 region by amplicon metagenome sequencing of DNA extracted from water samples, where the most abundant phylum was the Proteobacteria (57.39%), followed by Firmicutes (23.7%) and Chloroflexi (4.14%). The three phyla Actinobacteria, Planctomycetes and Bacteroidetes were also detected less than 3% in abundance while 4.48% of bacteria could not be fit into any known phylum. The most abundant genera were *Burkholderia* (14.87%), *Desulfotomaculum* (7.23%) and *Stenotrophomonas* (6.1%). Four strictly anaerobic bacteria, *Anaerosolibacter carboniphilus* (0.71%), *Bellilinea caldifistulae* (0.04%), *Salimesophilobacter vulgaris* (0.1%), *Anaerobacterium chartisolvens* (0.12%); two potential plant growth-promoting bacteria, *Azospirillum halopraeferens* (0.04%) and *Bradyrhizobium liaoningense* (0.16%) and one potential alkali tolerant and sulphate-reducing bacterium, *Desulfovibrio alkalitolerans* (0.45%) were recorded. *Pigmentiphaga* sp. was isolated from Mahapelessa hot spring and to the best of our knowledge, this is the first record of this genus from a hot spring. This study gives insight into the vast bacterial diversity present in the Mahapelessa hot spring from the culture-independent approach which could not be identified using standard culturing techniques.

Introduction

Many extreme environments, such as hot springs, deserts, saline and/or alkaline lakes and, ocean beds are too harsh for common life to exist. Natural geothermal springs, including terrestrial hot springs, are widely spread in various parts of the world. These hot springs are primarily associated with tectonically active zones in areas where the Earth's crust is relatively thin [1, 2]. These geothermal springs contain new sources of fascinating microorganisms that adapted to those extreme environments [2]. The adaptations to these harsh habitats make thermophiles and their thermostable proteins suitable for various industrial and biotechnological applications [3]. Thermophiles can be defined as organisms that are adaptable to grow optimally at higher temperatures. These thermophiles are classified into several groups: facultative thermophiles (survive below 45 °C); moderate thermophiles (optimum growth temperature between 45 °C and 60 °C); strict thermophiles (optimum growth temperature between 60 °C and 90 °C); extreme thermophiles or hyperthermophiles (grow best at temperatures greater than 90 °C) [4, 5]. Most thermophilic microorganisms are prokaryotes (Domain Bacteria and Domain Archaea) surviving in extreme heat, pH, salinity, and pressure conditions [6].

Microbial communities in hot springs are widely explored and microbial diversity in some hot springs has been studied using both culture-dependent and culture-independent approaches. In recent years, the application of the

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culture-independent approach has proved to be a promising tool to investigate the population diversity, gene content, function, and ecological significance of microbial communities living in different hot springs [7]. High-throughput sequencing methods (Next Generation Sequencing/NGS) have currently provided opportunities to study and understand the microorganisms from broader and different angles. For instance, 16S rRNA metagenomic techniques characterize microorganisms found in hot springs by the total DNA, which also includes the genetic material of microorganisms that cannot be cultured [7].

Culturing is very useful for isolating and identifying bacteria with high biotechnological potential. For instance, the outstanding discovery of thermophilic microorganisms and subsequent use of their thermo-stable enzymes for molecular techniques have significantly changed the biotechnology field [3]. Hence, researchers are continuing to isolate and identify novel microorganisms from terrestrial geothermal springs. However, most thermophilic microorganisms are unculturable because of the difficulty in providing similar environmental conditions during laboratory cultivation [8]. Therefore, culture-dependent methods provide limited information about microbial diversity. The majority of microbes in various environments, including hot springs, are still not isolated using standard culturing methods [9]. Hence, the culture-dependent microbial analysis does not give a clear idea about the different microbial communities residing in a particular environment.

Microbial communities in thermal springs have been studied in different countries, such as those in China [8], India [10–12], Jordan [13], Eritrea [14], and United States [15]. Different thermophilic environments have different microbial phenotypes due to various physical and chemical conditions, biogeography, and geological history [15]. Even though Sri Lanka is endowed with many thermal hot springs, apart from their prospects in geothermal energy, these have not yet been fully studied concerning microbial diversity and biotechnological potentials. Along the Highland Group-eastern Vijayan tectonic boundary of Sri Lanka, a 350 km long thermal spring line indicates a large geothermal system beneath. The surface temperatures of these hot water springs (Mahapelessa, Maha Oya, Wahawa, Nelumwewa, Kanniyai, Rankihiriya and Kapurella) range from 34 to 62 °C [16] (Fig. 1). Nelumwewa, Maha Oya, Marangala and Kapurella hot water springs are located in the middle part of Sri Lanka, and they are characterized by high-sulfate content in water. Mahapelessa hot spring is in the southern part of the country and is also located away from other geothermal

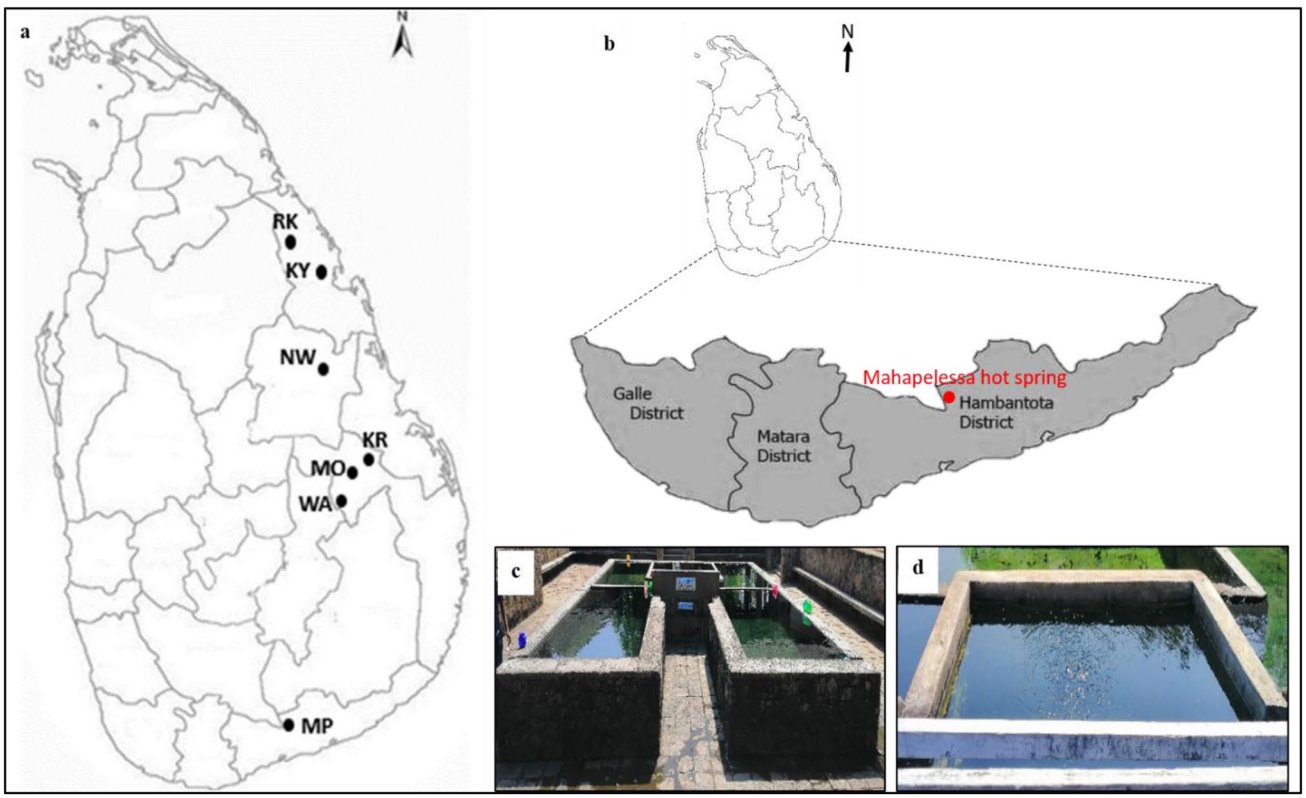

Fig. 1 **a** Map of Sri Lanka showing the distribution of hot water springs. RK-Rankihiriya (39.1 °C), KY-Kanniyai (41.7 °C), NW-Nelumwewa (62.2 °C), KR-Kapurella (58 °C), MO-Maha Oya (53.5 °C), WA-Wahawa (43.4 °C), MP-Mahapelessa (45.5 °C) [16]; **b** geographical location of the sampling site; **c** Major spring with other five adjacent tanks; **d** Major spring

springs in Sri Lanka. It is characterized by higher chloride ions (2384 mg/l), sodium ions (1128 mg/l), and electric conductivity/EC (7360 µS/cm). Moreover, water from Mahapelessa hot spring is characterized by a higher concentration of alkali and alkaline earth elements such as K, Li, Ca and Sr than the other hot springs of Sri Lanka [16].

Several researchers studied the thermophilic bacterial diversity of certain Sri Lankan hot springs using culture-dependent approaches [17–19]. Medhavi et al. (2018) [18] isolated *Bacillus thermoamylovorans*, *Meiothermus* sp. and *Bacillus schlegelii* from Maha Oya hot springs. According to Nandanee et al. (2015), [19] bacteria, isolated from Mahapelessa, Maha Oya, Wahawa and Nelumwewa hot water springs in Sri Lanka (temperatures ranging from 35 °C to 61 °C) had thermostable enzymes that could be used for industrial applications. Moreover, Magana-Arachchi and Wanigatunge, (2008) [17] revealed the cyanobacterial and archaeal diversity in Mahapelessa hot spring using morphological and molecular techniques. However, previous studies did not focus on the total bacterial diversity in hot springs in Sri Lanka. Hence, more comprehensive studies are necessary to understand the bacterial community composition and distribution in Sri Lankan hot springs.

In this research, we intended to identify the bacterial species inhabiting the Mahapelessa hot spring that have not been discovered previously, with the aid of culture-dependent and culture-independent approaches. We hypothesize that as Mahapelessa hot spring is geographically isolated, insight into the vast bacterial diversity could be achieved only through a culture-independent method.

Materials and Methods

Sample Collection

Water samples were collected into thermal flasks (500 ml) from the surface and at 4 m depth from the surface of the water column with the aid of a hand corer (Wildco® Instruments, USA) from the hot water spring of Mahapelessa, Sri Lanka in January 2019 (6° 15′ N & 80° 59′ E) (Fig. 1). Water samples were immediately transported to the research laboratory at the National Institute of Fundamental Studies, Kandy within 12 h and isolation of bacteria was initiated immediately after transferring the samples to the laboratory.

Physicochemical Parameters of Surface Waters in Hot Spring

Temperature, pH, conductivity, total dissolved solids (TDS), salinity and dissolved oxygen (DO) of the surface water was measured in situ using a multi-parameter (SyberScan PCD 650, Eutech instrument).

Isolation of Bacteria

Thermophilic bacteria were isolated by inoculating a 50 µl of water samples into nutrient agar (HiMedia), tryptone soy agar (Oxoid), MacConkey agar (HiMedia), xylose lysine decarboxylase agar (HiMedia), and M17 agar (Merck) media and incubated at 44.5 °C (similar to surface temperature) for 48 h with duplicates. Plates were constantly observed for the appearance of bacterial colonies. Single colonies with distinct morphologies were selected from each plate and they were sub-cultured on the same selective medium to obtain pure cultures. Further, water samples were inoculated into a cyano-specific BG11 medium and incubated at 44.5 °C with fluorescence light with the intensity of ~2500 lux at a 12:12 h dark: light cycle to isolate cyanobacteria. Morphological identification of cyanobacteria was made using standard cyanobacterial keys [20, 21].

Genomic DNA Extraction and PCR Amplification

Genomic DNA was extracted from bacterial pure cultures using the standard cetyltrimethylammonium bromide (CTAB) method [22]. Amplification of the 16S rRNA gene was conducted using a pair of universal primers, forward primer 27F (5′-AGAGTTTGATCCTGGCTC AG-3′) and reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) [23]. The PCR mixture (25 µl) contained 5 µl of 5 × GoTaq® Flexi buffer, 1.5 µl of 25 mM of $MgCl_2$, 2.5 µl of 1 mM dNTPs, 2 µl of 5 µM forward and reverse primers, 0.3 µl of 5 U/µl of GoTaq® DNA polymerase (Promega Inc., USA) and ~300 ng of template DNA. All PCR reactions were carried out under the following conditions: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 15 min. The amplified product (25 µl) was separated on 1.5% agarose gel prepared in 1 × TBE buffer containing 10 mg/ml ethidium bromide by gel electrophoresis at 100 V for 25 min and amplified DNA was visualized using a gel documentation system (Syngene, UK). Amplicons (~1500 bp) were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA).

16S rRNA Sequencing and Sequence Analysis

PCR products were sequenced by the Sanger sequencing method at Macrogen Inc., South Korea. The 16S rRNA Sanger sequencing data of all the isolates were aligned using Molecular Evolutionary Genetics Analysis 7 (MEGA7) software and analyzed by Basic Local Alignment Search Tool (BLAST) with the closest cultured sequences retrieved from the National Center for Biotechnology Information (NCBI) database and the type strains (https://lpsn.dsmz.de/). The phylogenetic tree was constructed using the neighbor-joining

method in MEGA7 software with representative type strains to determine the evolutionary relationship among the strains. *Meiothermus hypogaeus* (NR_113226.1) was used as an outgroup taxon for the phylogenetic tree construction. The sequence data were deposited in GenBank, under accession numbers MN631026.1- MN631030.1; MN631032.1- MN631037.1; MN631039.1- MN631041.1; MN631043.1- MN631046.1.

16S rRNA Metagenomic Analysis

The composite water sample (100 ml) from the surface and the subsurface (4 m depth from the surface) was centrifuged at 3000 rpm for 20 min and the supernatant (500 µl) and the pellet (500 µl) were taken into a microcentrifuge tube. Genomic DNA was extracted and purified from the sample using the modified Booms method [24]. The sample was prepared according to the "16S Metagenomic Sequencing Library Preparation" guide (Part # 15,044,223 Rev. B; Illumina, Macrogen Inc., South Korea). The amplicon PCR (16S V3-V4 region) was performed using the following primers: Bakt_341F: 5′-CCTACGGGNGGCWGC AG-3′ Bakt_805R 5′-GACTACHV GGGTATCTAAT CC-3′. The library was sequenced on an Illumina MiSeq platform (Illumina, USA), following the manufacturer's protocol at Macrogen Inc., South Korea. The sequence data were deposited under NCBI BioProject PRJNA609251. The raw data generated were trimmed and analyzed to identify bacteria using GAIA: Metagenomics data analysis software [25]. Operational taxonomic units (OTU) analysis was carried out to determine their differential abundance using DESeq2. Beta diversity was determined using GAIA: Metagenomics data analysis software and plotted by PAST data analysis software (https://past.en.lo4d.com/windows), through Non-metric Multidimensional Scaling plot (NMDS), using Bray–Curtis decrease distance matrices.

Results

The in situ surface temperature of the Mahapelessa hot spring was 44.5 °C and the slightly alkaline pH (8.14) was recorded. Moreover, water conductivity, TDS, salinity and DO were recorded as 6.934 mS, 7.601 ppt, 8.935 ppt, 7.1%, respectively.

Morphologically different bacterial colonies that appeared on different culture media at 44.5 °C were selected for obtaining pure cultures to be used in characterization. A total of eighteen thermophilic bacterial isolates were obtained. Eight isolates were from the surface waters and the rest were from the 4 m depth. All the isolates were in circular colony form. According to the microscopic observations, the shapes of the cells were either bacilli or coccobacilli. Though cyanobacterial isolates were not sequenced, they were identified based on the morphology [20, 21]. Morphological identification of cultured cyanobacteria revealed the presence of five different cyanobacterial genera, a single isolate each from *Gloeocapsa*, *Chlorogloeopsis, Pseudanabaena, Oscillatoria* and the other three isolates were belonging to *Leptolyngbya* which were isolated from the surface water.

According to the 16S rRNA gene sequencing and the BLAST analysis at the NCBI database, sequenced bacterial isolates were grouped into 8 different bacterial genera. BLAST search based on near-complete 16S rRNA sequences of the isolates showed that there was a high similarity (> 97%) between the test isolates and the representative strains of *Klebsiella, Bacillus, Acinetobacter, Gulbenkiania, Enterobacterales*, and *Pannonibacter.* Moreover, *Pigmentiphaga* and *Balneatrix* species showed 95% similarity with the BLAST search (Table 1).

The taxonomic position of all 8 bacterial genera belonged to 7 taxonomic orders under 4 different classes in the two bacterial phyla, Proteobacteria and Firmicutes. The relative abundance was highest for order *Enterobacteriales* (Table 2). The phylum-level distribution also showed that Proteobacteria were more dominant (15 isolates, 7 genera); the majority being the *Klebsiella* sp. Few genera with the least number of isolates were also obtained such as *Balneatrix* and *Gulbenkiania.* Isolates belonging to phylum Firmicutes were found in a low number (3 isolates) grouping to a single genus. A variation in diversity was observed in bacterial isolates when compared between the surface and at 4 m depth. According to the results, *Klebsiella* sp. and *Pannonibacter* sp. were isolated from both surface and at 4 m depth of the water column. *Acinetobacter* sp., *Pigmentiphaga* sp. and *Gulbenkiania* sp. were limited to surface water while *Balneatrix* sp., *Bacillus* sp. and *Enterobacter* sp. were only isolated from at 4 m depth.

The phylogenetic relationships of the 18 thermophilic bacterial isolates were determined in comparison to type strains with the assistance of the neighbor-joining method (Fig. 2). The generated dendrogram clades were represented by two major lineages, Proteobacteria consisting of the genera *Pannonibacter, Klebsiella*, *Acinetobacter, Balneatrix, Enterobacter, Pigmentiphaga, Gulbenkiania;* Firmicutes consisting of genus *Bacillus.*

The culture-independent method revealed a total of 23 phyla demonstrating 80 classes, 43 orders, 123 families, 205 genera, and 83 species. Based on the total number of reads, in the studied sample 118,100 OTUs represented 23 distinct phyla dominated by Proteobacteria (57.39%), Firmicutes (23.7%), Chloroflexi (4.14%), Actinobacteria (2.82%), Planctomycetes (2.52%), Bacteroidetes (2.44%) and unclassified bacteria (4.48%) (Fig. 3). It had a lower cyanobacterial diversity (0.12%) consisting of *Leptolyngbya*

Table 1 Taxonomic identification of bacterial isolates based on 16S rRNA gene sequences

Sample code	Culture medium*	Identified organism (GenBank accession No.)	Max identity based on BLAST searches (%)	Reference strains
mms 1.1[a]	TSA	*Klebsiella pneumoniae* (MN631026.1)	99	*Klebsiella pneumonia subsp. ozaenae* ATCC 11,296 AF130982[T]
mms 1.2[a]	XLD	*Klebsiella pneumoniae* (MN631027.1)	97	*Klebsiella pneumonia subsp. rhinoscleromatis* NCTC 5046 AF009169[T]
mmb 1[b]	TSA	*Klebsiella pneumoniae* (MN631041.1)	97	
mmb 5[b]	XLD	*Klebsiella pneumoniae* (MN631044.1)	98	
mmb 9[b]	XLD	*Klebsiella pneumoniae* (MN631045.1)	97	
mms 1.3[a]	TSA	*Acinetobacter baumannii* (MN631029.1)	98	*Acinetobacter baumannii* DSM 30,007 AJ247197[T]
mms 8[a]	XLD	*Acinetobacter* sp. (MN631032.1)	94	
mms 4[a]	MA	*Gulbenkiania mobilis* (MN631035.1)		*Gulbenkiania_mobilis* E4FC31 AM295491[T]
mms 5[a]	MA	*Pigmentiphaga* sp. (MN631037.1)	95	*Pigmentiphaga aceris* SAP-32 LT719155[T]
mms 6[a]	MA	*Pigmentiphaga* sp. (MN631034.1)	96	*Pigmentiphaga litoralis* JSM 061,001 EU583723[T] *Pigmentiphaga humi* IMT-318 MH667611[T] *Pigmentiphaga soli* BS12 JF806521[T] *Pigmentiphaga daeguensis* K110 EF100696[T] *Pigmentiphaga kullae* K24 AF282916[T]
mms 10[a]	M17	*Pannonibacter phragmitetus* (MN631040.1)	98	*Pannonibacter phragmitetus* C6/19 AJ400704[T]
mmb 12[b]	M17	*Pannonibacter indicus* (MN631046.1)	97	*Pannonibacter indicus* HT23 EF608175[T]
mmb 13[b]	M17	*Pannonibacter* sp. (MN631043.1)	96	
mmb 10[b]	XLD	*Enterobacter hormaechei* (MN631033.1)	97	*Enterobacter hormaechei subsp. oharae* EN-314 AJ853889[T] *Enterobacter hormaechei subsp. steigerwaltii* DSM 16,691 MT781399[Y] *Enterobacter hormaechei subsp. xiangfangensis* 10–17 HF679035[T] *Enterobacter hormaechei* DSM 12,409 KF516255[T] *Enterobacter hormaechei subsp.Nhoffmannii* DSM 14,563 CP017186[T]
mmb 2[b]	TSA	*Balneatrix* sp. (MN631030.1)	94	*Balneatrix alpica* DS 16,621 Y17112[T]
mmb 4[b]	NA	*Bacillus subtilis* (MN631039.1)	98	*Bacillus subtilis subsp. stercoris* JCM 30,051 MN536904[T] *Bacillus subtilis subsp. spizizenii* NRRL B-23049 EU138464[T] *Bacillus subtilis subsp. inaquosorum* NRRL B-23052 EU138464[T]
mmb 11[b]	NA	*Bacillus subtilis* (MN631036.1)	97	
mmb 14[b]	NA	*Bacillus subtilis* (MN631028.1)	98	

**NA* nutrient agar, *TSA* tryptone soya agar, *MA* MacConkey agar, *XLD* xylose lysine decarboxylase agar, *M17* M17 agar

[a]Bacteria isolated from surface water

[b]Bacteria isolated from 4 m depth from the surface

sp. and *Oscillatoria* sp. Out of the total number of reads, 30.32% are from the class Betaproteobacteria (Online Resource 1). The order Burkholderiales of Betaproteobacteria constitutes 22.44% of the sequence reads followed by 15.9% of the family Burkholderiaceae (Online Resource 2, 3). The most abundant bacterial genus was *Burkholderia* (14.87%) comprising of four species *Burkholderia cepacia, Burkholderia multivorans, Burkholderia* sp.*, Burkholderia thailandensis,* followed by the genera, *Desulfotomaculum* (7.23%), *Hydrogenophilus* (4.83%), *Acinetobacter* (4.09%), *Pelotomaculum* (3.12%), *Prosthecomicrobium* (1.56%), *Achromobacter* (1.01%), and *Bacillus* (1.01%). All the other detected genera were below 0.7% (Fig. 3, Online Resource 4). Figure 4 shows the species-level diversity of bacteria in the Mahapellessa hot spring. At the species level, *B. cepacia* (9.7%) was the most dominant species found in Mahapelessa hot spring, followed by *Stenotrophomonas maltophilia* (4.43%), *Desulfotomaculum reducens* (2.8%),

Table 2 Relative abundance of the cultured bacterial isolates

Phylum	Class	Order	No. of isolates	Relative abundance (%)
Proteobacteria	α- Proteobacteria	*Rhodobacterales*	3	16.67
	β- Proteobacteria	*Burkholderiales*	2	11.11
		Neisseriales	1	5.55
	γ- Proteobacteria	*Enterobacteriales*	6	33.33
		Pseudomonadales	2	11.11
		Oceanospirillales	1	5.55
Firmicutes	Bacilli	*Bacillales*	3	16.67

and *Acinetobacter* sp. (2.3%). Four strictly anaerobic bacteria, *Anaerosolibacter carboniphilus* (0.71%), *Bellilinea caldifistulae* (0.04%), *Salimesophilobacter vulgaris* (0.1%), *Anaerobacterium chartisolvens* (0.12%); two potential plant growth-promoting bacteria, *Azospirillum halopraeferens* (0.04%) and *Bradyrhizobium liaoningense* (0.16%) and one potential alkali tolerant and sulphate-reducing bacterium, *Desulfovibrio alkalitolerans* (0.45%) were recorded for the first time from a geothermal spring by culture-independent method (Online Resource 5). However, the culture-independent method revealed the presence of 73.4% unknown bacterial species in the Mahapelessa hot spring (Fig. 4a). Although there is a high percentage of unknown bacteria at the species level, most of those unknown bacteria were classified in major hierarchy levels (Online Resources 6, 7).

Mahapelessa hot spring had 23 unique bacterial species including *Bacillus licheniformis* (0.070%), *Caldanaerocella colombiensis* (0.066%), *Desulfotomaculum reducens* (2.808%), *Geosporobacter subterraneus* (0.26%), *Enterobacter asburiae* (0.19%), *Hydrogenophilus thermoluteolus* (0.11%) and *Bacillus licheniformis* (0.07%) when compared with three other hot springs namely Wahawa, Nelumwewa and Maha Oya and a natural spring located at Digana in Sri Lanka (data unpublished, Online Resource 8).

As a measure of beta diversity, NMDS plots were calculated based on the Bray–Curtis dissimilarity index to visualize the dissimilarities in bacterial community composition between Mahapelessa and other hot spring samples. Species-level beta diversity was analyzed in Mahapelessa hot spring with Wahawa, Nelumwewa and, Maha Oya hot springs of Sri Lanka (Fig. 5a). According to the results, Mahapelessa hot spring was clustered closely to the Wahawa hot spring (42.8 °C, pH = 7.17) an indication that both hot springs had more similar bacterial species composition. Also, phylum-level beta diversity was analyzed in Mahapelessa hot spring comparing with the four hot springs in other countries: Taptapani hot spring of Odisha, India (48 °C), Reshi hot spring of the Sikkim Himalaya (43 °C), Akwar hot spring in Eritrea (49.5 °C) and, Ma'in hot spring of Jordanian (48 °C) (Fig. 5b). According to the results, the Mahapelessa hot spring was clustered near to the Ma'in hot spring of Jordanian (48 °C, pH = 7.76) than the other hot springs. It was shown the composition of bacterial phyla in Ma'in could be more similar to Mahapelessa hot spring.

Discussion

Geothermal springs provide fascinating habitats for thermophilic microorganisms. The temperature and the other physicochemical factors were known to have a greater impact on microbial composition in hot spring environments. In this study, we attempted to determine the bacterial diversity in Mahapelessa hot spring, Sri Lanka being characterized as rich in chloride—sodium content [16]. Generally, with increasing temperature the microbial diversity decreases [26]. However, other factors like salt content, pH, and arsenic content can play a decisive role in the bacterial community composition in hot springs [27]. The present study revealed the presence of high bacterial diversity in the Mahapellessa hot spring using both culture-dependent and culture-independent approaches. Besides the temperature and pH, relatively high electrical conductivity (6.934 mS) and salinity (8.935 ppt) might have influenced the high bacterial diversity in Mahapelessa hot spring. Therefore, our findings also suggest that besides temperature, salinity and conductivity could play a significant role in bacterial community composition in hot springs.

The culture-based approach is much tedious and time-consuming, but it has its advantages in microbiology as these techniques provide valuable germplasm and allow preserving the microbial strains for future studies and to explore their biotechnological and industrial applications [28]. In this study, bacterial isolates were obtained from culturing of microbes in different culture media. During the culturing, media composition and laboratory conditions are critical factors for the isolation of microorganisms from the environmental samples. The use of different culture media can increase the number of different bacterial species in culture [29]. In the present study, different culture media were used

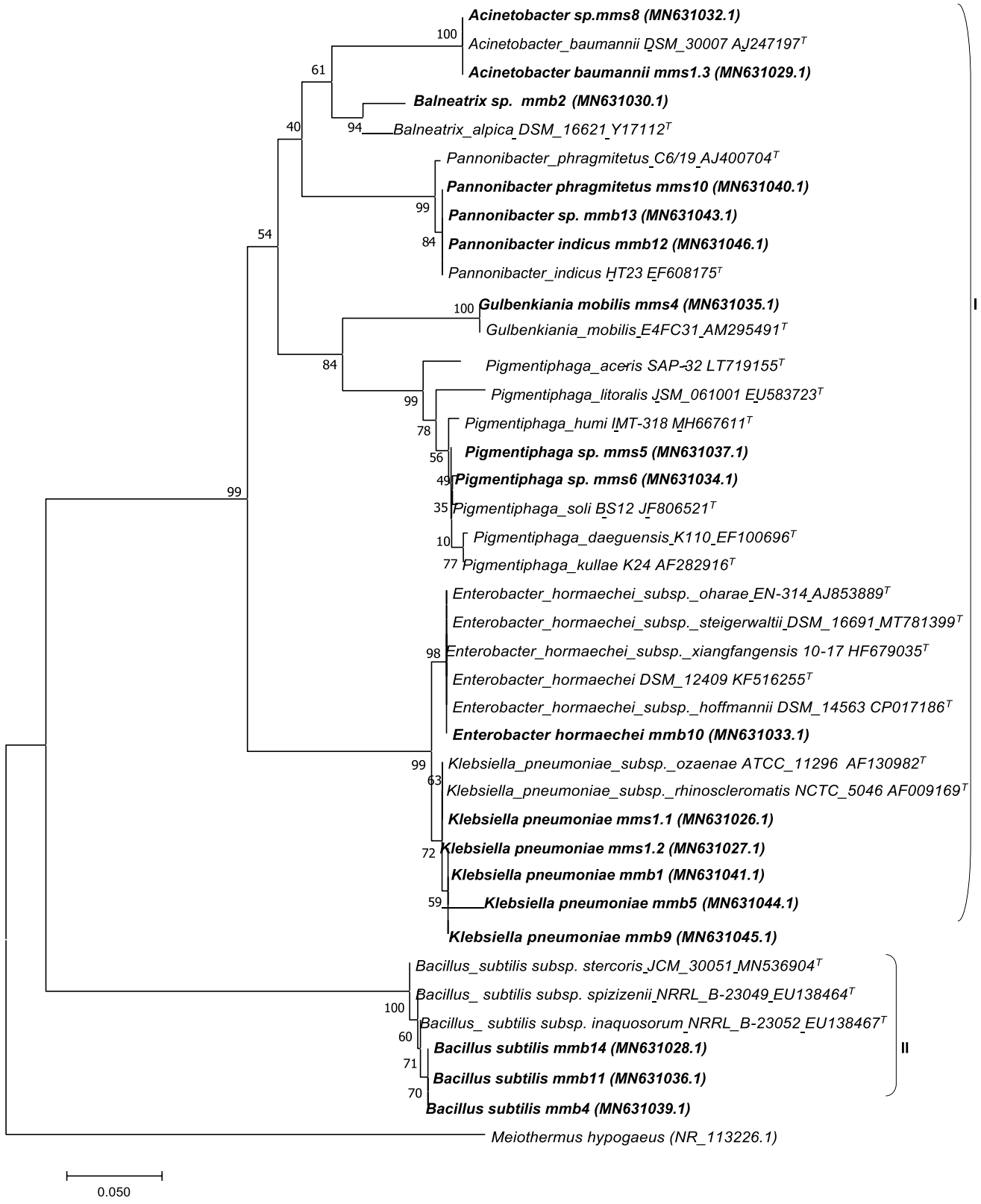

Fig. 2 The phylogenetic tree of the representative 18 bacterial isolates and their closest relatives based on the nearly complete 16S rRNA gene. Superscript T designates type strains

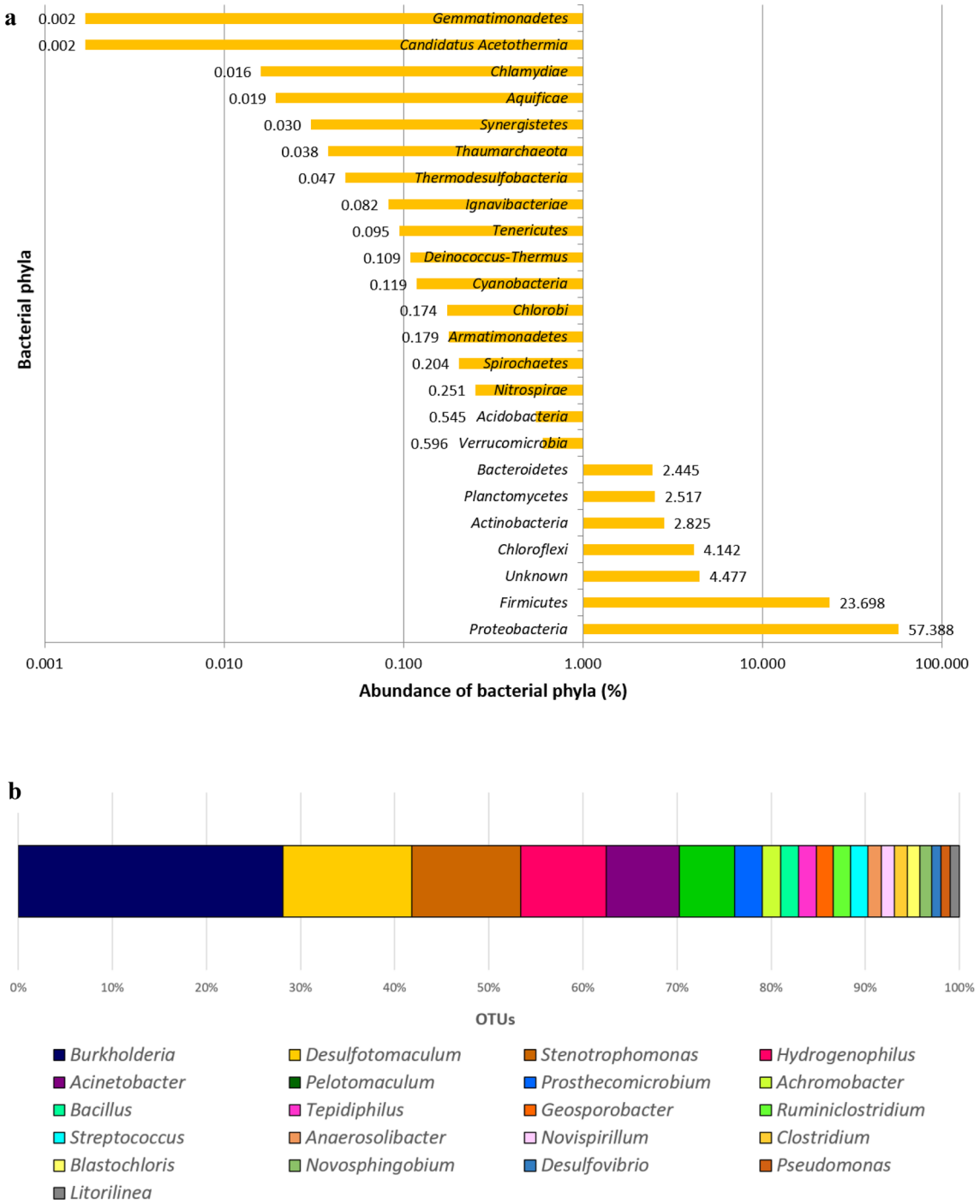

Fig. 3 Relative abundance of **a** phyla **b** most abundant genera (> 0.5%) based on the number of OTUs from metagenomic sequencing

to isolate a maximum number of bacterial isolates and incubation of bacterial cultures was performed at surface water temperature (44.5 °C). But the actual temperature of subsurface water (4 m depth from the surface) must be greater than the surface temperature as the bottom temperature of the spring is 101 °C [16] and this could be a reason for the isolation of a lesser number of different bacterial species from subsurface water than the surface water. Moreover, most thermophilic bacteria are unculturable under laboratory conditions [8].

The previous studies on Mahapelessa hot spring were mainly focused on the morphological and molecular characterization of cyanobacteria and archaea [17]. In the present study, high thermophilic cyanobacterial diversity was

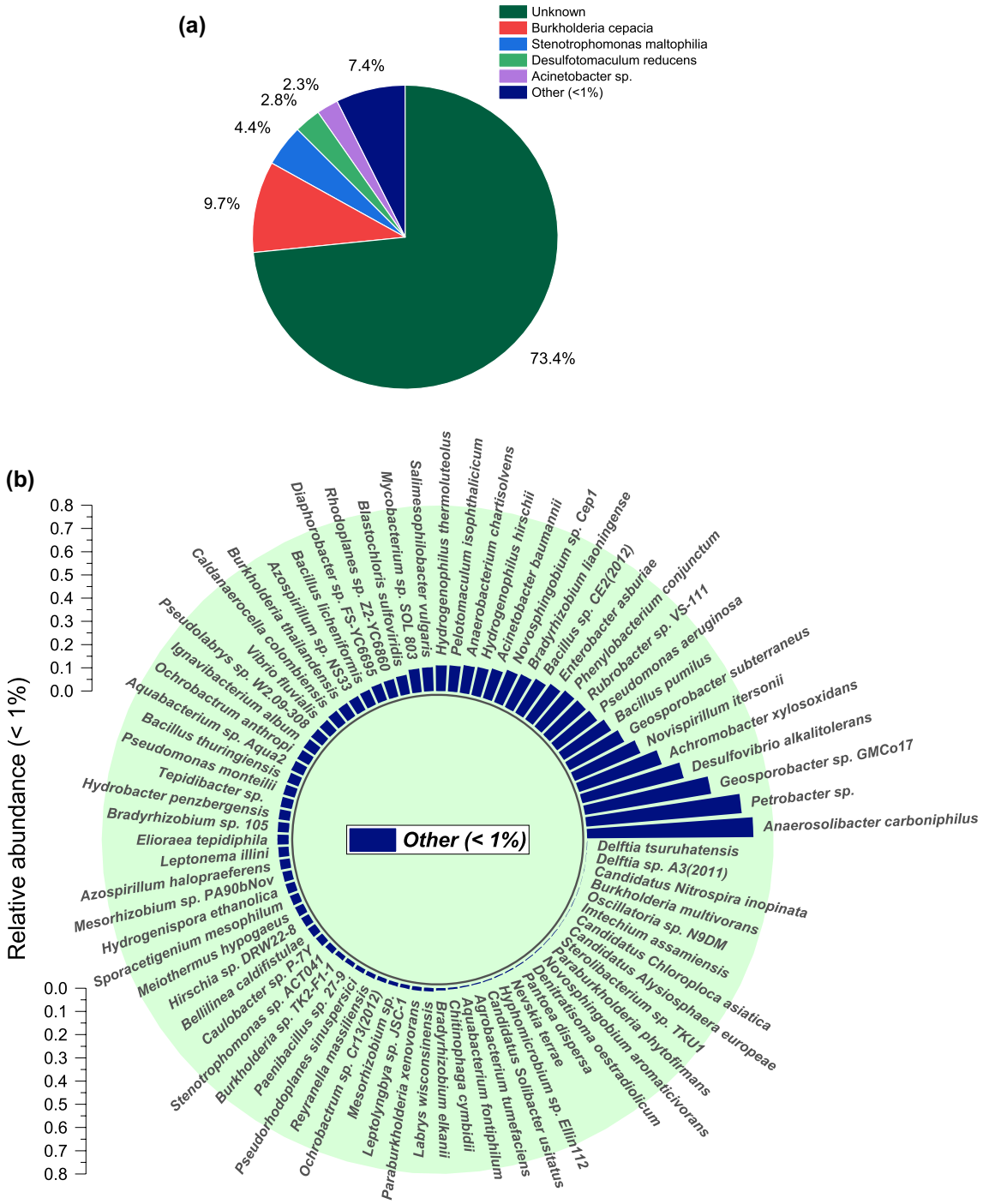

Fig. 4 Relative abundance of species **a** Relative abundance of species more than 1%, **b** Relative abundance of species less than 1%

observed and these findings are in agreement with a previous study conducted in Mahapelessa hot spring [17]. However, cyanobacterial isolates in this study should be further identified by 16S rRNA gene sequencing to confirm their identity. According to the Magana-Arachchi and Wanigatunge, 2008, 16S rRNA gene sequences together with morphological data confirmed the presence of cyanobacterial species belonging to the genus *Chlorogloeopsis*, *Leptolyngbya* and *Pseudanabaena* in Mahapelessa spring [17] and these genera were also reported in the present study. Moreover, genera *Gloeocapsa, Oscillatoria* and *Pseudanabaena* identified in this study were also reported from an Indian hot springs with temperatures ranging from 38 to 52 °C [30]. Also *Chlorogloeopsis* and *Leptolyngbya* species detected in

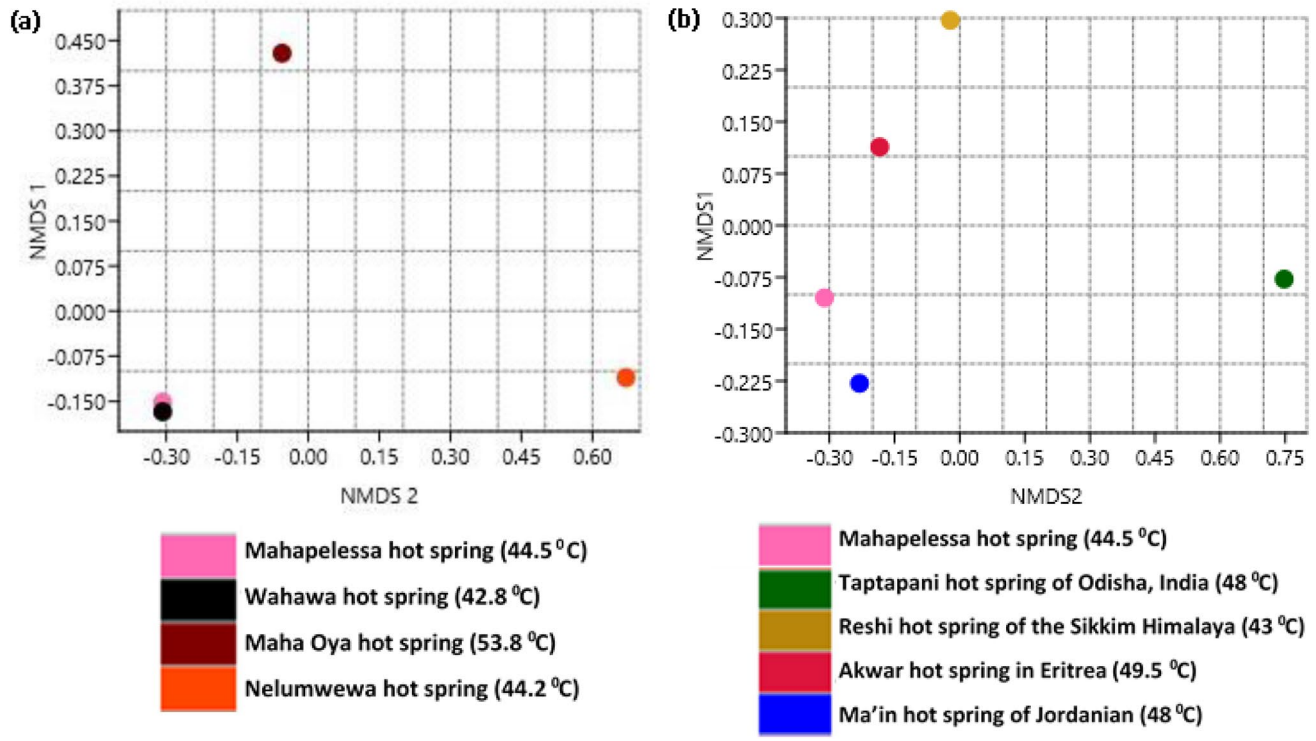

Fig. 5 Beta diversity. Bray–Curtis index calculated using amplicon reads and plotted on Non-metric Multidimensional Scaling plot (NMDS plot). **a** Species-level beta diversity of the Mahapelessa hot spring with three other hot springs of Sri Lanka; **b** phylum-level beta diversity of Mahapelessa hot spring with four hot springs in other countries (Hot springs were selected based on the temperature)

Mahapelessa hot spring were also reported from Algerian hot springs with temperature 45 °C [31].

In the present study, many thermophilic bacteria were detected from the Mahapelessa hot spring. Based on 16S rRNA sequencing, all the isolated bacteria belonged to the genera *Klebsiella, Bacillus, Acinetobacter, Balneatrix, Enterobacter, Gulbenkiania, Pigmentiphaga*, and *Pannonibacter*. In our study *Bacillus* sp. belonging to phylum Firmicute were isolated only from the water samples collected from the 4 m depth from the surface. However, the temperature used for incubation was the surface temperature of 44.5 °C recorded from the Mahapelessa hot spring. Though these *Bacillus* sp. were able to be isolated around 44.5 °C temperature. Furthermore, *Bacillus* sp. have been isolated from hot springs with temperatures ranging between 45 and 59 °C [18, 32, 33], and those springs' temperatures relatively higher than the surface temperature of the Mahapelessa hot spring. Moreover, *Bacillus* sp. generally show high resistance to environmental stress which helps them to survive in extreme environmental conditions such as hot water springs [33]. Therefore, *Bacillus* strains have been studied by different researchers across the world due to their thermophilic nature and enzyme efficacy in industrial and biotechnological applications [34]. *Balneatrix* sp. which was isolated from sub-surface water also reported from a spa therapy center and they have demonstrated that it could survive at 20–46 °C [35].

Bacteria belonging to the genus *Acinetobacter* (11%) were isolated from the present study and this genus was also isolated from other hot springs with optimal growth temperature 37–55 °C [36, 37]. Besides, *Klebsiella pneumoniae* was isolated from, both surface and at 4 m depth from the surface of the spring. Also, genus *Klebsiella* has been previously reported from a hot spring in India which had 45 °C temperature, using a culture-dependent method [33]. Moreover, genus *Klebsiella* has the capability of surviving in a wider temperature range between 38 °C and 60 °C [38]. Representatives from *Acinetobacter, Enterobacter,* and *Gulbenkiania* obtained from the present study are identified as pathogens associated with the human intestine [33]. It is important to study the presence of these pathogens in this hot spring as people use this hot spring for bathing to avoid health risks. The *Pannonibacter* species which was also isolated from this study had been also recorded from sediment samples collected from hot springs in India (43 °C and the pH was 7.4) [39]. To the best of our knowledge, this is the first record for the genus *Pigmentiphaga* from a hot spring. In previous reports, *Pigmentiphaga* species had been isolated from tidal flat sediments and soil environments and were able to grow at temperatures of 15–42 °C [40].

Currently, a greater diversity of microorganisms has been detected by the culture-independent methods [8, 10–15]. Many bacterial phyla were identified from the culture-independent method that was not previously described either in Mahapelessa hot springs or from any other hot springs in Sri Lanka. In our study, 23 phyla were observed from the culture-independent method and only three phyla were recorded from the culture-based method: phyla Cyanobacteria, Proteobacteria and Firmicutes. This result displays the importance of culture-independent methods for understanding bacterial diversity in an environment. The phylum Proteobacteria was the most abundant phylum observed in Mahapelessa hot spring. Proteobacteria has also been reported in high abundance from many studies based on culture-independent studies of hot springs with moderately high and very high temperatures (44–100 °C) at various geographical locations: hot springs in Eritrea (49–100 °C) [14]; hot springs in Central India (43.5–98 °C) [41]; hot springs of the Sikkim Himalaya (43–62 °C) [42].

In the current study, the culture-independent method was useful in recording the three thermophilic phyla, Aquificae, Deinococcus-Thermus and Cyanobacteria with an abundance of 0.01%, 0.11%, and 0.12%, respectively. According to the literature, the occurrence of Aquificae, Deinococcus-Thermus, and certain Cyanobacteria and Crenarchaeota were highly dependent on temperature [27]. The *Hydrogenophilus hirschii* (0.12%) and *Hydrogenophilus thermoluteolus* (0.11%) which were detected in this study had also been confirmed as thermophiles in previous studies [43]. In Mahapelessa hot spring the most abundant bacterial genus was *Burkholderia* (14.87%) which may have the ability to produce extracellular thermostable enzymes such as proteases and cellulases as described by Sahay et al. 2017 [44]. Genera *Desulfotomaculum, Hydrogenophilus*, and *Pelotomaculum* detected from the culture-independent method in this study had also been recorded from in previous studies with similar temperatures [45–47]. About 4.48% unknown phyla of the total OTUs obtained from our study could not be characterized and hence were considered as unclassified. When it comes to the OTUs obtained from species level, this unknown percentage becomes higher (73.4%) hinting at the presence of many undiscovered and unexplored microbiota in the Mahapelessa hot spring.

Beta diversity is the variation in community composition among the sampling sites within a geographical area of interest [13]. In the beta diversity analysis, samples were clustered based on location (Fig. 5). Mahapelessa (44.5 °C, pH = 8.14) and Wahawa (42.8 °C, pH = 7.17) hot springs were clustered together. In Wahawa hot spring 95 bacterial species were reported in metagenomics analysis and out of them, 54 species were common to both springs. The most abundant species was *S. maltophilia* (3.93%) in Wahawa followed by *Leptolyngbya* sp. (1.9%) and *B. cepacia* (1.38%) whereas those bacterial genera were reported from Mahapelessa hot spring with the abundance of 4.43%, 0.02% and 9.72%, respectively. This similar bacterial species composition may be due to more similar temperatures in both hot springs. The Maha Oya and Nelumwewa springs formed a separate cluster and did not coincide with the Mahapelessa spring. 86 bacterial species were observed in Maha Oya hot spring and among them, 40 species were unique to Maha Oya compared with Mahapelessa spring. *Dechloromonas* sp. (6.2%) and *Flectobacillus roseus* (5.88%) were the most abundant and unique species observed in Maha Oya. The highest surface water temperature was recorded in Maha Oya hot spring (53.8 °C, pH = 7.86) and this may be the reason for unique bacterial diversity in spring. Therefore, Maha Oya spring did not cluster with the Mahapelessa spring at the species-level beta diversity analysis. Although Nelumwewa hot spring had (44.2 °C, pH = 8.60) similar temperature with Mahapelesa hot spring, it was also clustered separately. 77 bacterial species were observed in Nelumwewa and among them, 46 species were the same as the Mahapelessa. *Tepidimonas ignava* (17.34%) and *Chloroflexus aurantiacus* (4.6%) were the most abundant species observed in Nelumwewa and those were not detected in Mahapelessa. The Nelumwewa geothermal spring is originated in a small piece of land within a lake [16] and this specific geographical isolation may have influenced the unique bacterial species composition in Nelumwewa spring.

The phylum-level beta diversity was analyzed with four hot springs in other countries, which had surface temperatures in close range to Mahapelessa. Accordingly, Mahapelessa hot spring clustered with Ma'in hot spring in Jordan (48 °C; pH = 7.76). Although the temperature and pH were different, higher mineralization was observed in both Mahapelessa and Ma'in hot springs [16, 48] and this could be a reason for the presence of more similar bacterial phyla in both springs. A culture-independent study conducted in Ma'in hot spring identified 26 phyla [13]. Among them, 22 phyla were also observed in our study. Ma'in hot spring had recorded a relatively high abundance of Proteobacteria (65.8%) [13] same as us. Akwar hot spring in Eritrea (49.5 °C; pH = 6.97) [14], Reshi hot spring of the Sikkim Himalaya (43 °C; pH = 7.5) [42] and Taptapani hot spring of Odisha, India (48 °C; pH = 8.56) [12] did not closely cluster with the Mahapelessa hot spring in phylum-level beta diversity analysis. Besides the temperature, other physicochemical parameters like pH, conductivity, salinity may influence the occurrence of different bacterial phyla in each hot spring. Moreover, studies on Akwar hot spring in Eritrea (49.5 °C) revealed the presence of 49 phyla and 272 families [14]. Though this study revealed more bacterial diversity than the Mahapellessa hot spring, predominant bacterial phyla: Proteobacteria (6.2–82.3%), Firmicutes (1.6–63.5%), Deinococcus-Thermus (0.0–19.2%), Planctomycetes (0.0–11.8%),

Aquificae (0.0–9.9%), Chlorobi (0.0–22.3%) and Bacteroidetes (2.7–8.4%) were also detected in the present study. Reshi hot spring (43 °C) exhibited 27 bacterial phyla and the highest abundance of bacterial phyla was Proteobacteria (59%), Firmicutes (21%), and Bacteroidetes (4.4%) and the relative abundance of phylum Proteobacteria and Firmicutes were more like ours. In Taptapani hot spring (48 °C), Cyanobacteria (29%) was the most abundant phylum followed by Proteobacteria (26%), Firmicutes (13%) and Actinobacteria (5%). In contrast, 0.1% of phylum Cyanobacteria were detected in Mahapelessa spring. This beta diversity analysis is important to visualize the differences in microbial community composition among habitats.

Both the culture-dependent and culture-independent methods capture different portions of microbial communities from the hot spring environments. However, *Klebsiella pneumoniae, Pigmentiphaga* sp., *Balneatrix* sp. and *Gulbenkiania mobilis* revealed from the culturing could not be identified from the culture-independent method. But their respective families or orders were detected through 16S rRNA metagenomic analysis. *K. pneumoniae* and *Pigmentiphaga* sp. belonging to Family Enterobacteriaceae and Alcaligenaceae were reported with 0.24132% and 5.27434% abundance, respectively. Further, *Balneatrix* sp. and *G. mobilis* belonging to order Oceanospirillales and Neisseriales were reported with 0.01693% and 0.02371% abundance, respectively. These observations could be due to the low taxonomic resolution of the 16S V3-V4 region with short-read sequencing platforms which is used for 16S rRNA metagenomic analysis when compared with the near-complete 16S rRNA gene sequences used for culture-dependent approach. Furthermore, Johnson et al. (2019) [49] showed full-length 16S rRNA gene sequence data provide high taxonomic resolution of bacterial communities at species and strain levels than 16S variable regions with short-read sequences. Additionally, the current study used specific media for bacterial isolation. *K. pneumonia* was the most dominant species found in a culture-dependent method and it was grown in XLD and TSA media. The use of selective media might have influenced the occurrence of these bacteria. In this study, total bacterial DNA was extracted manually and whether that has affected the outcome of the 16S rRNA metagenomic sequencing data are another possibility.

Hot spring microorganisms are usually important in bioprospecting for hydrolytic enzymes due to their thermostability. Therefore, different media and culturing conditions needed to be applied to obtain more isolates to find potential enzyme producers that can be used for industrial applications. Additionally, previous studies have proved that *Bacillus* sp. and *Acinetobacter* sp. have thermotolerant enzyme production ability [34, 36] and they were also isolated from our study. Further understanding of the metabolic potential of the present isolates possibly will help to find more efficient strains to be used in the industry.

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Declarations

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References

1. Rampelotto PH (2013) Extremophiles and extreme environments. Life 3:482–485. https://doi.org/10.3390/life3030482
2. Hreggvidsson GO, Petursdottir SK, Björnsdottir SH, Fridjonsson OH (2012) Microbial speciation in the geothermal ecosystem. In: Stan-Lotter H, Fendrihan S (eds) Adaption of microbial life to environmental extremes, 1st edn. Springer, Vienna, pp 37–67. https://doi.org/10.1007/978-3-211-99691-1_3
3. Turner P, Mamo G, Karlsson EN (2007) Potential and utilization of thermophiles and thermostable enzymes in biorefining. Microb Cell Fact 6:1–23. https://doi.org/10.1186/1475-2859-6-9
4. Baker GC, Gaffar S, Cowan DA, Suharto AR (2001) Bacterial community analysis of Indonesian hot springs. FEMS Microbiol Lett 200:103–109. https://doi.org/10.1016/S0378-1097(01)00207-5
5. Mathai S, Roy KR, Rajendran N (2014) Importance of marine thermophiles in biotechnological applications. Int J Pharm Sci Rev Res 27:153–160
6. Merino N, Aronson HS, Bojanova DP et al (2019) Living at the extremes: extremophiles and the limits of life in a planetary context. Front Microbiol. https://doi.org/10.3389/fmicb.2019.00780
7. López-López O, Cerdán ME, González-Siso MI (2013) Hot spring metagenomics. Life 3:308–320. https://doi.org/10.3390/life3020308
8. Tang J, Liang Y, Jiang D et al (2018) Temperature-controlled thermophilic bacterial communities in hot springs of western Sichuan, China. BMC Microbiol 18:1–14. https://doi.org/10.1186/s12866-018-1271-z

9. Streit WR, Schmitz RA (2004) Metagenomics—the key to the uncultured microbes. Curr Opin Microbiol 7:492–498. https://doi.org/10.1016/j.mib.2004.08.002
10. Mehetre GT, Paranjpe A, Dastager SG, Dharne MS (2016) Investigation of microbial diversity in geothermal hot springs in Unkeshwar, India, based on 16S rRNA amplicon metagenome sequencing. Genome Announc 4:4–5. https://doi.org/10.1128/genomeA.01766-15
11. Singh A, Subudhi E (2016) Structural insights of microbial community of Deulajhari (India) hot spring using 16S-rRNA based metagenomic sequencing. Genomics Data 7:101–102. https://doi.org/10.1016/j.gdata.2015.12.004
12. Sahoo RK, Subudhi E, Kumar M (2015) Investigation of bacterial diversity of hot springs of Odisha, India. Genomics Data 6:188–190. https://doi.org/10.1016/j.gdata.2015.09.018
13. Hussein EI, Jacob JH, Shakhatreh MAK et al (2017) Exploring the microbial diversity in Jordanian hot springs by comparative metagenomic analysis. Microbiologyopen 6:1–8. https://doi.org/10.1002/mbo3.521
14. Ghilamicael AM, Budambula NLM, Anami SE et al (2017) Evaluation of prokaryotic diversity of five hot springs in Eritrea. BMC Microbiol 17:1–13. https://doi.org/10.1186/s12866-017-1113-4
15. Inskeep WP, Jay ZJ, Tringe SG et al (2013) TheYNP metagenome project : environmental parameters responsible for microbial distribution in the Yellowstone geothermal ecosystem. Front Microbiol 4:1–15. https://doi.org/10.3389/fmicb.2013.00067
16. Chandrajith R, Barth JAC, Subasinghe ND et al (2013) Geochemical and isotope characterization of geothermal spring waters in Sri Lanka: evidence for steeper than expected geothermal gradients. J Hydrol 476:360–369. https://doi.org/10.1016/j.jhydrol.2012.11.004
17. Magana-Arachchi DN, Wanigatunge RP (2008) Molecular and morphological characterization of cyanobacteria and archaea in hot water springs Mahapelessa, Sri Lanka. Pragña XIX:51–59. http://ifs.nsf.ac.lk/handle/1/543. Accessed on 18 March 2020
18. Medhavi PIHR, Samarasinghe DGSN, Herath HM et al (2018) Isolation and identification of thermophilic bacteria and cyanobacteria in Maha Oya hot springs. Digital repository University of Kelaniya, Sri Lanka. http://repository.kln.ac.lk/handle/123456789/19096. Accessed on 17 March 2020
19. Nandanee GGW, Dassanayaka PN, Wijeyaratne SC (2015) Screening and characterization of thermophilic bacteria from hot springs of Sri Lanka for potential industrial application. University of Sri Jayewardenepura, Nugegoda. http://dr.lib.sjp.ac.lk/handle/123456789/3985?show=full-. Accessed on 17 March 2020
20. Abeywickrama BA, Abeywickrama L, Arulgnanum P, Jansen MAB (1986) The genera of the freshwater algae of Sri Lanka: part 2: Cyanophyceae (blue-green algae). UNESCO man and the biosphere national committee for Sri Lanka, Special publication 6. National science council Sri Lanka, Colombo, pp 103. http://search.lib.ou.ac.lk/cgi-bin/koha/opac-detail.pl?biblionumber=155571. Accessed on 10 June 2021
21. Holt JG, Krieg NR, Sneath PHA et al (1994) Bergey's manual of determinative bacteriology. Williams & Wilkins Co., Baltimore, USA
22. Somerville W, Thibert L, Schwartzman K, Behr MA (2005) Extraction of *Mycobacterium tuberculosis* DNA: a question of containment. J Clin Microbiol 43:2996–2997. https://doi.org/10.1128/JCM.43.6.2996-2997.2005
23. Frank JA, Reich CI, Sharma S et al (2008) Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. Appl Environ Microbiol 74:2461–2470. https://doi.org/10.1128/AEM.02272-07
24. Boom R, Sol CJA, Salimans MMM et al (1990) Rapid and simple method for purification of nucleic acids. J Clin Microbiol 28:495–503. https://doi.org/10.1128/jcm.28.3.495-503.1990
25. Paytuví A, Battista E, Scippacercola F et al (2019) GAIA: An integrated metagenomics suite. bioRxiv. https://doi.org/10.1101/804690
26. Wang S, Hou W, Dong H et al (2013) Control of temperature on microbial community structure in hot springs of the Tibetan Plateau. PLoS ONE. https://doi.org/10.1371/journal.pone.0062901
27. Prieto-Barajas CM, Alfaro-Cuevas R, Valencia-Cantero E, Santoyo G (2017) Effect of seasonality and physicochemical parameters on bacterial communities in two hot spring microbial mats from Araró, Mexico. Rev Mex Biodivers 88:616–624. https://doi.org/10.1016/j.rmb.2017.07.010
28. Sharma SK, Kumar R, Vaishnav A et al (2017) Microbial cultures: maintenance, preservation and registration. In: Varma A, Sharma A (eds) Modern tools and techniques to understand microbes. Springer, Cham, pp 335–367. https://doi.org/10.1007/978-3-319-49197-4_22
29. Sipkema D, Schippers K, Maalcke WJ et al (2011) Multiple approaches to enhance the cultivability of bacteria associated with the marine sponge *Haliclona* (*gellius*) sp. Appl Environ Microbiol 77:2130–2140. https://doi.org/10.1128/AEM.01203-10
30. Debnath M, Mandal NC, Ray S (2009) The study of cyanobacterial flora from geothermal springs of Bakreswar, West Bengal, India. Algae 24:185–193. https://doi.org/10.4490/algae.2009.24.4.185
31. Amarouche-Yala S, Benouadah A, El Ouahab BA, López-García P (2014) Morphological and phylogenetic diversity of thermophilic cyanobacteria in Algerian hot springs. Extremophiles 18:1035–1047. https://doi.org/10.1007/s00792-014-0680-7
32. Jaffer YD, Vinothkumar R, Irfan AB et al (2019) Isolation and characterization of thermophilic bacteria from Maharashtra hot springs: *Bacillus* sp. and *Staphylococcus* sp. J Entomol Zool Stud 7:691–695
33. Kumar M, Yadav AN, Tiwari R et al (2014) Evaluating the diversity of culturable thermotolerant bacteria from four hot springs of India. J Biodivers Bioprospecting Dev 01:1–9. https://doi.org/10.4172/2376-0214.1000127
34. Baysal Z, Uyar F, Aytekin Ç, (2003) Solid-state fermentation for production of α-amylase by a thermotolerant *Bacillus subtilis* from hot-spring water. Process Biochem 38:1665–1668. https://doi.org/10.1016/S0032-9592(02)00150-4
35. Dauga C, Gillis M, Ageron E et al (1993) Balneatrix alpica gen. nov., sp. nov., a bacterium associated with pneumonia and meningitis in a spa therapy center. Res Microbiol 1070:35–46. https://doi.org/10.1016/0923-2508(93)90213-l
36. Liu L, Salam N, Jiao JY et al (2016) Diversity of culturable thermophilic *Actinobacteria* in hot springs in Tengchong, China and studies of their biosynthetic gene profiles. Microb Ecol 72:150–162. https://doi.org/10.1007/s00248-016-0756-2
37. Narsing Rao MP, Dong ZY, Luo ZH et al (2021) Physicochemical and microbial diversity analyses of Indian hot springs. Front Microbiol 12:1–10. https://doi.org/10.3389/fmicb.2021.627200
38. Mukherjee T, Banik A, Mukhopadhyay SK (2020) Plant growth-promoting traits of a thermophilic strain of the *Klebsiella* group with its effect on rice plant growth. Curr Microbiol 77:2613–2622. https://doi.org/10.1007/s00284-020-02032-0
39. Bandyopadhyay S, Schumann P, Das SK (2013) *Pannonibacter indica* sp. nov., a highly arsenate-tolerant bacterium isolated from a hot spring in India. Arch Microbiol 195:1–8. https://doi.org/10.1007/s00203-012-0840-z
40. Lee JJ, Srinivasan S, Kim MK (2011) *Pigmentiphaga soli* sp. nov., a bacterium isolated from soil. J Microbiol 49:857–861. https://doi.org/10.1007/s12275-011-1375-8
41. Saxena R, Dhakan DB, Mittal P et al (2017) Metagenomic analysis of hot springs in central india reveals hydrocarbon-degrading thermophiles and pathways essential for survival in extreme

environments. Front Microbiol. https://doi.org/10.3389/fmicb.2016.02123
42. Sharma N, Kumar J, Abedin MM et al (2020) Metagenomics revealing molecular profiling of community structure and metabolic pathways in natural hot springs of the Sikkim Himalaya. BMC Microbiol 20:1. https://doi.org/10.1186/s12866-020-01923-3
43. Stöhr R, Waberski A, Liesack W et al (2001) *Hydrogenophilus hirschii* sp. nov., a novel thermophilic hydrogen-oxidizing β-proteobacterium isolated from Yellowstone National Park. Int J Syst Evol Microbiol 51:481–488. https://doi.org/10.1099/00207713-51-2-481
44. Sahay H, Yadav AN, Singh AK et al (2017) Hot springs of Indian Himalayas: potential sources of microbial diversity and thermostable hydrolytic enzymes. 3 Biotech. https://doi.org/10.1007/s13205-017-0762-1
45. Haouari O, Fardeau ML, Cayol JL et al (2008) *Desulfotomaculum hydrothermale* sp. nov., a thermophilic sulfate-reducing bacterium isolated from a terrestrial Tunisian hot spring. Int J Syst Evol Microbiol 58:2529–2535. https://doi.org/10.1099/ijs.0.65339-0
46. Vésteinsdóttir H, Reynisdóttir DB, Örlygsson J (2011) *Hydrogenophilus islandicus* sp. nov., a thermophilic hydrogen-oxidizing bacterium isolated from an Icelandic hot spring. Int J Syst Evol Microbiol 61:290–294. https://doi.org/10.1099/ijs.0.023572-0
47. Imachi H, Sekiguchi Y, Kamagata Y et al (2002) *Pelotomaculum thermopropionicum* gen. nov., sp. nov., an anaerobic, thermophilic, syntrophic propionate-oxidizing bacterium. Int J Syst Evol Microbiol 52:1729–1735. https://doi.org/10.1099/ijs.0.02212-0
48. Schäffer R, Sass I (2014) The thermal springs of Jordan. Environ Earth Sci 72:171–187. https://doi.org/10.1007/s12665-013-2944-4
49. Johnson JS, Spakowicz DJ, Hong BY et al (2019) Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nat Commun 10:1–11. https://doi.org/10.1038/s41467-019-13036-1103