Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2019; 7(1): 691-695

Received: 13-11-2018
Accepted: 16-12-2018

Jaffer YD
Division of Irrigation and Drainage Engineering, ICAR-Central Soil Salinity Institute, Karnal, Haryana, India

R Vinothkumar
ICAR-Central Marine Fisheries Institute, Kerala, India

Irfan AB
College of Fisheries Science, Birsa Agricultural University, Ranchi, Jharkhand, India

Ishfaq NM
College of Fisheries Science, Birsa Agricultural University, Ranchi, Jharkhand, India

Parvaiz Ahmad Ganie
ICAR-Directorate of Cold Water Fisheries Research, Bhimtal, Uttarakhand, India

Raja Adil Hussain Bhat
ICAR-Directorate of Cold Water Fisheries Research, Bhimtal, Uttarakhand, India

A Vennila
Division of Crop Production, ICAR-Sugarcane Breeding Institute, Coimbatore, Tamil Nadu, India

Correspondence
Jaffer YD
Division of Irrigation and Drainage Engineering, ICAR-Central Soil Salinity Institute, Karnal, Haryana, India

Isolation and characterization of thermophilic bacteria from Maharashtra hot springs: *Bacillus* sp. and *Staphylococcus* sp

Jaffer YD, R Vinothkumar, Irfan AB, Ishfaq NM, Parvaiz Ahmad Ganie, Raja Adil Hussain Bhat and A Vennila

Abstract
In the present study the microbes from the thermal springs of Maharashtra were characterized to reveal the different bacterial groups. Screening and isolation, and their identification by biochemical and molecular methods (16S rRNA sequencing) were the major exercises of this study. The water samples from the three thermal springs Sativali, Nimbavali and Ganeshpuri of Thane, Maharashtra were collected for isolation of microbes and physico-chemical characteristics. The presence of two strains of *Bacillus* and one of *Staphylococcus* belonging to the same OTU (Operational Taxonomic Unit) *Firmicutes* were found in the thermal springs by Sequencing of the 16S rRNA of the isolates followed by BLAST search. This investigation indicates that the thermal springs of Maharashtra, India is a rich source of many thermophilic bacteria. Furthermore, these microbes have potential for producing the extracellular thermostable hydrolytic enzymes having a great commercial prospect in various industrial, medical and agriculture applications.

Keywords: *Thermal springs, Bacillus, Staphylococcus, Firmicutes*, 16S rRNA

1. Introduction
A hot spring or thermal spring is produced by the emergence of geo-thermally heated ground water from earth's crust. With respect to geographical, physical, environmental and chemical characteristics, hot springs are unique sites for extremophilic microorganisms [7]. This variety in growth parameters results in enormous genetic and metabolic diversity present in hot springs [2]. Terrestrial geothermal hot springs have been present since the early evolution of the earth, and harbour a diversity of microorganisms [17].

A variety of lithotrophic and heterotrophic micro-organisms have been isolated so far from hot-spring communities. The primary production, the conversion of carbon dioxide into biomass forms basis of life in the hot-springs [18]. At lower temperatures cyanobacteria and eukaryotic algae are common and are the main primary producers. At higher temperatures the species composition changes [9], and above 70 °C photosynthesis is not known to occur [18].

For the last decades or ever since thermophilic bacteria were first discovered in the 1960's, detection and isolation of new thermophilic microorganisms has increased [4]. This is mainly because of progress in molecular methods, such as amplification of the 16S rRNA gene, which allows detection of DNA sequences of many different organisms at a time and is now commonly performed in ecological studies. Despite these great progresses only a small fraction of all microorganisms have been isolated and characterized [15].

From different environments bacteria have been isolated through standard cultivation [14]. These cultivation techniques have served the basis for studying the physiology and biochemistry of microbes. Culture-based methods of analyzing microbes have been the mainstay of microbiology since their origin in the pioneering works of Robert Koch and Louis Pasteur. However, culture-based techniques have many limitations due to differential approaches needed to culture different groups of microbes. For example, aerobic and anaerobic organisms cannot be cultured together; fastidious organisms will often not grow, because essential nutrients for growth or optimal environmental conditions such as temperature, pH, essential mixtures of gases may not be present [13]. Despite these limitations, culture-based techniques have proved successful to generate the germplasm of microbes. Working with strains isolated from hot springs offers the major advantage of preserving those strains for

future studies and exploring them in due course for potential biotechnological applications [1].

Numerous hot springs dwell over a stretch of 300 km along the West Coast of India grouped under West Coast geothermal province [10, 16]. People usually take bathe in these springs as the water contains dissolved minerals like sulphur which have positive therapeutic effect on skin disease, asthma, neuralgia, arteriosclerosis, rheumatism and shoulder, neck and wrist pains. They also have a detoxifying and mucolytic effect due to enzymes released by beneficial microbes. Albeit numerous studies have been carried over geological, geophysical, hydrological and geothermal properties, meager knowledge is known about the microbial diversity of thermal springs of India. The objectives of this study were to investigate the diversity of thermophilic bacteria from environmental samples by culture dependent approach based on molecular phylogenetic analysis.

2. Material and Methods

2.1. Description of the study sites

The sampling for present study was carried out in selected thermal springs in Thane district of Maharashtra. The thermal springs occur in eight different localities in the district and mainly concentrated in two separate groups namely Sativali-Haloli-Koknere and Akloli-Nimbavali-Ganeshpuri (Vajreshwari group). The Sativali-Haloli-Koknere group is traversed by Surya and Vaitarna Rivers, whereas Vajreshwari group is associated with Tansa River (Fig. 1). Water samples were collected from three springs each at Sativali, Nimbavali and a spring at Ganeshpuri.

2.1.1. Sativali spring

It is situated at geographical coordinates of 19º 37.85" N and 72º 54.54" E on the banks of Vandri stream. It is a group of six thermal springs located over a 500 m stretch. Two springs located between paddy fields and one in front of a temple which showed profuse gas emission was selected for the study. These springs are protected from human activity by concrete tank around each spring. In the temple premise, overflow from main tanks is diverted to a secondary tank for bathing purpose.

2.1.2. Nimbavali spring

It is situated at geographical coordinates of 19º 30.434‟‟ N and 73º 0.918‟‟ E on bank of Tansa river. Among three hot springs selected for the study, one spring namely Agnikund sprouts at the middle of river. The other two springs are located away from the river bed.

2.1.3. Ganeshpuri

It is situated at geographical coordinates of 19º 30.150‟‟ N and 73º 0.841‟‟ E. The spring is located within the premise of temple, and water is discharged into different concrete tanks for bathing purpose.

2.2. Sample collection

The coordinates of the sampling points were recorded using GPS (eTrex Venture HC, Garmin USA). Samples were collected in sterile bottles (Sterile Uricol TMPW 016) supplied by Hi- Media Laboratories, Mumbai. Water samples were collected from a depth of around 50 cm water column. The samples were immediately stored in thermo flasks and processed within six hours.

2.3. Analysis of physicochemical parameters of water

Temperature, pH and salinity of water samples were measured at the site itself using a mercury thermometer calibrated up to 0.1 °C, portable digital pH meter (Eutech Instruments, Malaysia) and (Eutech Instruments, Malaysia), respectively. The water samples were collected separately for the estimation of dissolved oxygen, total sulphide and total sulphate in respective bottles by following the APHA (2005) guidelines for sample collection and preservation. The estimation of dissolved oxygen and total sulphide was carried out by Winkler's titrimetric method and iodometric method, respectively. Sulphate was analysed by turbidimetric method, and the turbidity was measured at 420 nm in a UV-Visible spectrophotometer (UV 1 model, Thermo Scientfic, USA). The chloride was estimated by argentometric titration (APHA, 2005).

2.4. Media and other chemicals

The bacteriological grade media and chemicals used for the microbiological work were procured from Merck (Mumbai) and Hi-media Laboratories (Mumbai). NucleoSpin Tissue DNA Extraction Kit (Macherey-Nagel, Germany Cat. No. 740 780.50), was used as per the manufacturer's instruction. PCR reagents and DNA molecular markers were procured from MBI Fermentas Life Sciences, Lithuania. Ethidium bromide, 6x gel loading dye, agarose, PCR buffer and other molecular biology grade chemicals were procured from Bangalore GeNei, India.

2.5. Sterilization

All the chemicals, plastic ware and glassware except the heat labile ones were sterilized by autoclaving at 121 °C for 15 minutes. All the glasswares used were washed using chromic acid wash solution and rinsed with deionized water.

2.6. Enrichment and isolation

Water samples were used immediately for enrichment in LB broth at 40±2 °C. Five-day enrichment culture was streaked on LB agar (HiMedia, Mumbai) to obtain separate colonies. Well differentiated colonies were selected and streaked over LB agar (Miller). Re-inoculation of all the colonies was performed to attain the purity of isolates.

2.7. Morphological and Molecular Characterization

A light microscope (Carl Zeiss, India) was used to study the morphology, gram staining, cell shape and motility of bacterial cells. Genomic DNA from pure strains was extracted using Nucleo Spin DNA extraction kit (Macherey-Nagel, Germany Cat. No. 740 780.50) DNA concentrations were measured by using Nanodrop Spectrophotometer (Thermo Scientific, USA). The DNA was visualized using 1.5% agarose gel pre-stained with ethidium bromide under DNr Bio-Imaging System (Jerusalem, Israel). The 16S rDNA gene of the bacterial isolates was amplified using primers 27F (5'AGAGTTTGATCCTGGCT3') and 1492R (5'GGTTACCTGTTACGACTT3') (Bioserve Biotechnologies, Hyderabad). The amplification program consisted of initial denaturation for three minutes at 94 °C, followed by 35 cycles with each cycle consisting of 1.5 min. at 94 °C, two minutes at 54 °C and two minutes at 72 °C. The reaction was completed by final extension step at 72 °C for 12 minutes. The DNA sequencing was carried out at Eurofins Genomics Pvt. Ltd. Sequencing services using ABI BigDye Terminator method. The 16S rRNA sequence was searched

against Gen Bank Database (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) using the BLAST algorithm.

3. Results and Discussion

Thermal springs represent extreme niches whose pristine quality is maintained over a period of time. The terrestrial hot springs that exist on earth [19] represent hot spots for unusual forms of life, genes, and metabolites. Ever since Thomas Brock discovered the presence of *Thermus aquaticus* in the thermal vents of Yellowstone National Park, a number of researchers have investigated similar environments all over the world. The earth we exist on is filled with variety of microorganisms that researchers are still far away from being able to complete their identification and isolation, this lead to intensive and extended researches to be carried out in order to fully investigate such promising microorganisms. Thermal springs have been used for bathing and washing purposes as human beings believe in the therapeutic effect of spring waters, and most of the thermal springs have been a tourist attraction. The healing effect of these waters is mainly due to dissolved minerals like sulphur, calcium, magnesium, lithium, etc. and also due to geothermal energy of underground water. The thermal springs of Sativali, Nimbavali and Ganeshpuri are also used by the people.

Temperature of the water discharged from the sampling sites varied from 52 to 59 °C, whereas pH ranged between 8.8 and 9.0 DO (5.87 mg l^{-1}) was found to be maximum in Nimbavali. Sulphate content of Ganeshpuri spring was found to be the lowest. Sulphide content ranged from 9.73 to 15.87 mg l^{-1}. Chloride ranged from 352.28±7.35 to 634.63±9.47mg l^{-1} (Table 1). Sarolkar [16] also observed similar temperature range (56.3 – 59.3 °C) in Sativali and in Ganeshpuri thermal spring (52 °C). The temperature recorded in Unkeshwar thermal spring (Nanded district of Maharashtra) [12], Bakreshwer hot spring of West Bengal [8] and Tattapani thermal spring in Himachal Pradesh (Chandrasekharam and Antu, 1995) was 62 °C, 37– 44 °C and 60 – 80 °C, respectively. Dissolved oxygen content of thermal spring water was below 5.0 °C in all the springs except at Nimbavali. In higher temperatures, solubility of gases decreases and hence the low dissolved oxygen content is expected. However, in Unkeshwar hot spring (Nanded district of Maharashtra) with the temperature of 62 °C, Pathak and Rekadwad [12] reported a high dissolved oxygen content (10.43 mg l^{-1}) and the reason is proposed that the mixing of water from the cold spring existing in the vicinity of hot spring or catalase kind of activity. The total sulphide content varied from 9.73 – 15.87 mg l^{-1}. The concentration of sulphate (96.01 - 165.05 mg l^{-1}) and chloride (316.10 - 635.35 mg l^{-1}) was very high indicating the leaching of minerals. Sarolkar [16] also reported similar concentrationof sulphate (144 mg l^{-1}, Akloli nearer to Nimbavali) and chloride (875 mg l^{-1}, Sativali) in thermal spring water. The pH of thermal springs was alkaline (8.8 – 9). The pH of waters of hot springs could be acidic, moderately alkaline or alkaline depending on the major ions present. Although, the sulphate content is high in these springs, the thermal waters of these springs are alkali-chloride type [16].

From each thermal spring source only one colony was isolated. The morphological characterization of the thermophilic isolated microbes is given in Table 2. The DNA extraction process yielded 70–114 ng µl-1 with the purity (260:280 nm) of 1.63–1.88. The PCR amplification of 16S rRNA yielded around1.5 kb product on an Agarose gel (Fig. 1). The DNA sequences were subjected BLAST search for identifying the closest match in the GenBank database of NCBI. Taxonomic affinity and percentage homology of bacteria isolated from thermal springs is shown in Table 3. The 16S rDNA sequences were submitted to NCBI database and the accession numbers obtained are KP238591, KP238590 and KP238589. All the bacteria found in thermal springs belong to *Firmicutes*. *Bacillus spp.* were earlier found by Panda *et al.*, [11] from hot spring of Tarabalo, Odisha and Amjad and Khalil [3] from thermal ponds in Jordan. Staphylococcus includes at least 40 species. Of these, nine have two subspecies, one has three subspecies, and one has four subspecies [6]. Most are harmless and reside normally on the skin and mucous membranes of humans and other organisms. Thus it is predicted that this microbe might be having the source from the humans using the thermal springs for bathing purposes.

Table 1: Physicochemical characteristics of thermal springs of Maharashtra

Spring	Temp. °C	pH	DO mg l^{-1} (Mean±SD)	Sulphide mg l^{-1} (Mean±SD)	Sulphate mg l^{-1} (Mean±SD)	Chloride mg l^{-1} (Mean±SD)
Sativali	59	9.0	2.67±0.82	9.73±0.08	160.88±9.28	634.63±9.47
Nimbavali	59	8.8	5.87±0.73	14.99±1.35	165.52±10.95	366.62±3.27
Ganeshpuri	52	9.0	4.27±0.78	15.87±0.56	96.01±5.68	352.28±7.35

Table 2: Morphological characterization of thermophilic bacteria from thermal springs

Strain	Source	Gram staining	Cell shape	Motility
Bacillus sp. S1(2014c)	Sativali	+	Rod	-
Staphylococcus sp. N3(2014)	Nimbavali	+	Spherical	-
Bacillus sp. G(2014)	Ganeshpuri	+	Rod	-

Table 3: Taxonomic affinity and percentage homology of bacteria isolated from thermal springs of Thane district of Maharashtra, India

Phylum	Order	Family	Genus	Strain	Closest relatives and 16s r RNA identities (%)	GenBank accession number
Firmicutes	Bacillales	Bacillaceae	*Bacillus*	*Bacillus* sp. S1(2014c)	*Bacillus licheniformis* strain UN1 (100%)	KP238591
Firmicutes	Bacillales	Bacillaceae	*Bacillus*	*Bacillus* sp. G(2014)	*Bacillus licheniformis* strain UN1 (100%)	KP238589
Firmicutes	Bacillales	Staphylococcaceae	*Staphylococcus*	*Staphylococcus* sp. N3(2014)	*Staphylococcus sciuri* strain 3-20 (100%)	KP238590

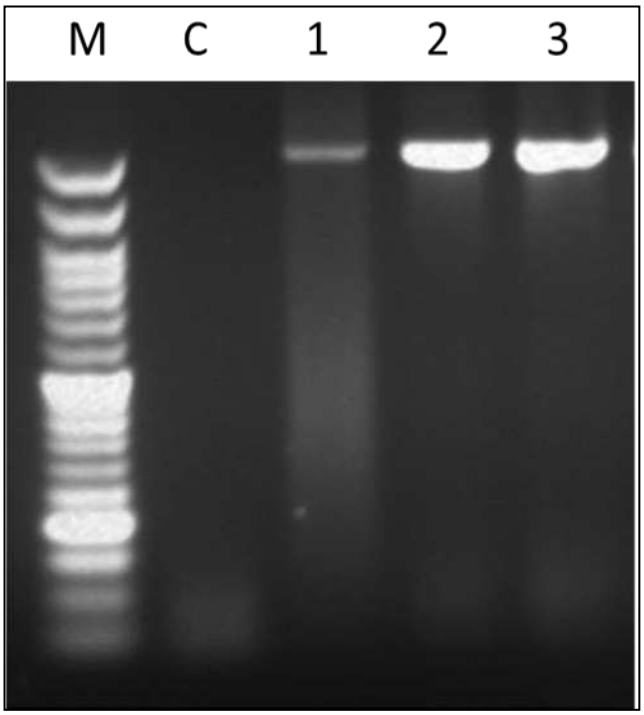

Fig 1: PCR amplification of 16S rRNA gene of bacterial isolates Lane M: Molecular weight marker (50 bp) Lane C: Control Lane1-3: Amplified isolates

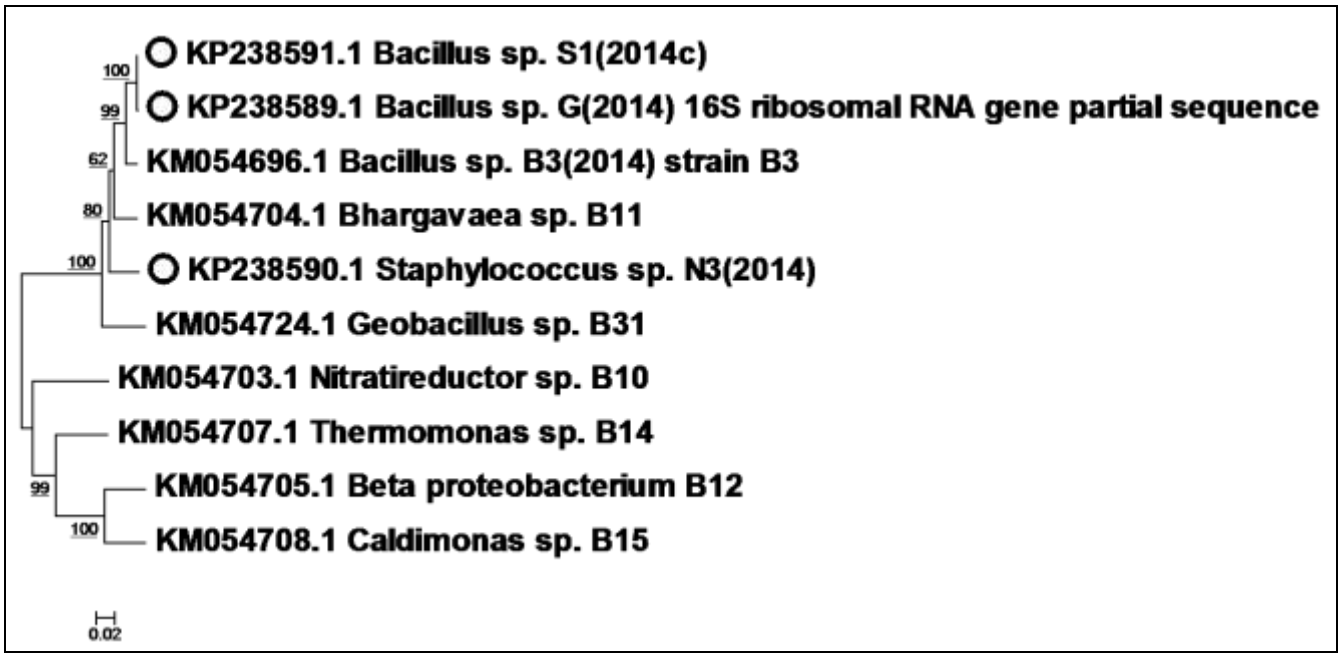

Fig 2: Phylogenetic tree based on the 16S rRNA gene sequences of the SOB from thermal springs. The evolutionary history was inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 849 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

4. Conclusion

The isolated microbes have provided the insight into the broader spectrum of the thermophilic microbes. The bacterial isolates can be excellent candidates for the future development of alternative biotechnological processes for the treatment of hazardous wastes containing. Furthermore, due to paucity of information on the diversity of microbes in the thermal springs of Maharashtra, there is a tremendous scope to characterize the microbial diversity and isolate organisms with novel activities.

5. Acknowledgment

The authors are indebted to, Director ICAR-Central Institute of Fisheries Education, Mumbai, India, for providing support and necessary facilities for carrying out this experiment.

7. References

1. Akanbi TO, Kamaruzaman AL, Abu-Bakar F, Hamid SAN, Radu S, Abdul-Manap MY *et al*. Highly thermostable extracellular lipase-producing Bacillus strain isolated from a Malaysian hot spring and identified

using 16S rRNA gene sequencing. International Food Research Journal. 2010; 17:45–53.
2. Amend JP, Shock EL. Energetics of overall metabolic reactions of thermophilic and hyperthennophilic Archaea and Bacteria. FEMS Microbiology Reviews. 2001; 25:175-243.
3. Amjad B, Khali. Isolation and characterization of thermophilic *Bacillus* species from thermal ponds in Jordan. Pakistan Journal of Biological Sciences. 2002; 5:1272-1273.
4. Brock TD. Thermophilic microorganisms and life at high temperatures. Springer-Verlag, New York, 1978, 465.
5. Chandrasekharam D, Antu MC. Geochemistry of Tattapani thermal springs, Madhya Pradesh, India – field and experimental investigations. Geothermics. 1995; 24:553-559.
6. Harris LG, Foster SJ, Richards RG. An introduction to Staphylococcus aureus, and techniques for identifying and quantifying *S. aureus adhesins* in relation to adhesion to biomaterials: review. European Cells and Materials, 2002; 4(3).
7. Hugenholtz P, Piulle C, Hershberger KL, Pace NR. 1998. Novel division level bacterial diversity in a Yellowstone hot spring. Journal of Bacteriology. 1998; 180:366-376.
8. Jana BB, Sarkar HL. The limnology of Swetganga, a thermal spring of Bakreshwar, West Bengal, India. Hydrobiologia, 1971; 72:113-123.
9. Kristjansson JK, Hreggvidsson GO, Alfredsson GA. Isolation of halo tolerant *Thermus* spp. from submarine hot springs in Iceland. Appied Environmental. Microbiology, 1986; 52:1313-1316.
10. Padhi RN, Pitale UL. Potential geothermal fields in the context of national scenario on non-conventional energy resources development programme in India. Procee. WGC. 1995, 525-530.
11. Panda MK, Sahu MK, Tayung K. Isolation and characterization of a thermophilic *Bacillus* sp. with protease activity isolated from hot spring of Tarabalo, Odisha, India. Iranian Journal of Microbiology. 2013; 5:59-165.
12. Pathak AP, Rekadwad BN. High dissolved oxygen content of Indian hot water spring a novel report. International Journal of Current Research. 2012; 2:01-01.
13. Piterina AV, Pembroke JT. Preparation and analysis of environmental DNA: optimisation of techniques for phylogenetic analysis of ATAD sludge. In: Mendez-Vilaz A (ed) Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, Formatex, 2010, 1533-1538
14. Ranjard L, Poly F, Nazaret S. Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. Research in Microbiology. 2000; 151:167-177.
15. Reysenbach AL, Shock E. Merging genomes with geochemistry in hydrothermal ecosystems. Science. 2002; 296:1077-1082.
16. Sarolkar PB. Geochemical characters of hot springs of west coast, Maharashtra state, India. In: Proceedings World Geothermal Congress Antalya, Turkey, 2005, 24-29.
17. Satyanarayana T, Raghukumar C, Shivaji S. Extremophilic microbes: Diversity and perspectives. Current Science. 2005; 89:78-90.
18. Spear JR, Walker JJ, Race NR. Hydrogen and bioenergetics in the Yellowstone geothermal ecosystem. Proceedings of the National Academy of Sciences. 2005; 102:2555-60.
19. Tobler DJ, Benning LG. Bacterial diversity in five Icelandic geothermal waters: Temperature and sinter growth rate effects. Extremophiles. 2011; 15:473-485.