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Microbial ecology of two hot springs of Sikkim: Predominate population and geochemistry

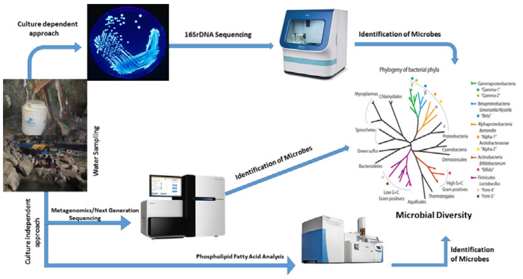
Ishfaq Nabi Najar, Mingma Thundu Sherpa, Sayak Das, Saurav Das, Nagendra Thakur *

Department of Microbiology, School of Life Sciences, Sikkim University, 6th Mile, Samdur, Tadong, Gangtok 737102, Sikkim, India

HIGHLIGHTS

- This study describes the geochemistry and microbial ecology of the hot springs of Sikkim, India.
- This study revealed the dominance of proteobacteria and bacteroidetes in the two hot spring.
- The microbial ecology of the two hot springs are depended on the geochemistry of the springs.
- The culture dependent technique was correlative with PLFA studies showing the dominance of gram positve bacteria over gram negative.

GRAPHICAL ABSTRACT



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ABSTRACT

Northeastern regions of India are known for their floral and faunal biodiversity. Especially the state of Sikkim lies in the eastern Himalayan ecological hotspot region. The state harbors many sulfur rich hot springs which have therapeutic and spiritual values. However, these hot springs are yet to be explored for their microbial ecology. The development of neo generation techniques such as metagenomics has provided an opportunity for inclusive study of microbial community of different environment. The present study describes the microbial diversity in two hot springs of Sikkim that is Polok and Borong with the assist of culture dependent and culture independent approaches. The culture independent techniques used in this study were next generation sequencing (NGS) and Phospholipid Fatty Acid Analysis (PLFA). Having relatively distinct geochemistry both the hot springs are thermophilic environments with the temperature range of 50–77 °C and pH range of 5–8. Metagenomic data revealed the dominance of bacteria over archaea. The most abundant phyla were *Proteobacteria* and *Bacteroidetes* although other phyla were also present such as *Acidobacteria*, *Nitrospirae*, *Firmicutes*, *Proteobacteria*, *Parcubacteria* and *Spirochaetes*. The PLFA studies have shown the abundance of Gram Positive bacteria followed by Gram negative bacteria. The culture dependent technique was correlative with PLFA studies. Most abundant bacteria as isolated and identified were Gram-positive genus *Geobacillus* and *Anoxybacillus*. The genus *Geobacillus* has been reported for the first time in North-Eastern states of India. The *Geobacillus* species obtained from the concerned hot springs were *Geobacillus toebii*, *Geobacillus lituanicus*, *Geobacillus Kaustophillus* and the *Anoxybacillus* species includes *Anoxybacillus gonensis* and *Anoxybacillus Caldiproteolyticus*. The distribution of major genera and their statistical correlation analyses with the geochemistry of the springs predicted that the temperature, pH, alkalinity, Ca^{2+}, Mg^{2+}, Cl^{2+}, and sulfur were main environmental variables influencing the microbial community composition and diversity. Also the piper diagram suggested that the water of both the hot springs are Ca-HCO^{3-} type and can be predicted as shallow fresh ground waters. This study has provided an insight into the ecological interaction of the diverse microbial communities and associated physicochemical parameters, which will help in determining the future studies on different biogeochemical pathways in these hot springs.

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* Corresponding author.
E-mail address: nthakur@cus.as.in (N. Thakur).

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1. Introduction

Thermal springs are natural geological phenomena that occur on all the continents. Every thermal spring has its distinctive features which depend on the several promising amalgamations of heat sources, water sources, subsurface rock types, flow paths and chemical reactions (Heasler et al., 2009). Various dissolved minerals such as megnecium, calcium, sodium, chloride, sulfates or silica are generally present in the geothermal water of an area as compared to non-geothermal groundwater (Zangana, 2015). Higher mineralogical compositions support different micro and macro floral community in and on the surrounding environment. Since XIX century, the exploration of hot springs has been in to progress and the physicochemical properties and geological features were the primary areas of study for researchers. However, the isolation and investigation of their thermophilic microbial community did not start until the 1950's (Marsh and Larsen, 1953). In recent scenario hot springs are the hotspots of research in the field of microbial ecology. Microbial community profiling of hot springs, focusing mainly on bacteria and archaea showing thermophilic and hyperthermophilic nature has attributed to different industrial enzymes and proteins of the modern era (López-López et al., 2015).

The thermophilic bacteria are recognized by their metabolic thermostability which are buoyed by their thermophilic protein. The thermostability of the thermophilic enzymes has been established as valuable biocatalysts for various biotechnological and industrial purposes. A prototype is *Taq*-polymerase from *Thermus aquaticus* that led to the advancement of the polymerase chain reaction (PCR) technique (Chien et al., 1976). To understand the geochemistry, geomicrobiology, bioenergetics and biotechnological potential of geothermal systems, various studies have been performed worldwide (Amin et al., 2017) (Liu et al., 2016). However, the conventional classical culture-dependent approach was the primary and sole technique to determine the geomicrobiology of the hot springs before the development of neo molecular techniques. Inability to culture the vast majority of microorganisms with the culture-dependent method has questioned the technique for inclusive profiling of different environment (Amann et al., 1995). To overcome this limitation, different culture-independent techniques including DGGE, PLFA, and Metagenomic studies have revealed a subsequent increase in microbial molecular ecology studies. The first approach to understand the true diversity of distinct environments was provided by a combined approach of PCR amplification of the 16S rRNA genes and their pattern analysis on denaturing gradient gel electrophoresis (DGGE). However, the products spawned during PCR-DGGE of the mixed communities often encumber the application of this technique in quantitative community profiling (Neilson et al., 2013). More recent development of Metagenomic approach has considerably increased the information related to microbial diversity, functional genomics, and transcriptomics (López-López et al., 2015) (DeCastro et al., 2016). This method is precise for gaging the structure of an environmental microbial community since it does not cover any selection and reduces technical biases, particularly the ones presented by amplification of the 16S rRNA gene (Lewin et al., 2013). Besides the above-mentioned techniques, an interesting non-culturable technique, i.e., Phospholipid Fatty Acid Analysis or PLFA is available and used since two decades to characterize microbial communities (Willers et al., 2015). The PLFA was first used to assess the microbial biomass from marine and estuarine sediments in 1979 (White et al., 1979). PLFA can be used to measure the viable microbial biomass and to identify the biomarkers for taxonomic evaluation from an environment (Jenkinson and Ladd, 1981).

Aiming the geomicrobiological features, microbial community structure of different geothermal springs has determined worldwide such as Tengchong thermal springs of China (Hou et al., 2013), Nakabusa hot springs of Japan (Kubo et al., 2011), Siloam hot water springs of South Africa (Memory Tekere, 2012), Andean Mountain hot water springs of Colombia (Bohorquez et al., 2012), Solfataric Fields of Iceland (Kvist et al., 2007), Great Basin hot springs (Costa et al., 2009), and Yellowstone National Park (USA) (Spear et al., 2005). In India geological survey has identified about 400 hot springs located in seven geothermal provinces distributed across India (Chandrasekharam, 2005). Of the 400 hot springs, only 28 springs have been explored microbiologically and 12 hot springs have been curtained with cutting-edge metagenomic approaches (Poddar and Das, 2017). Metagenomic studies of hot springs have conferred the microbial diversity and their functional and metabolic framework.

Nestling in the Himalayan mountains, the state of Sikkim is characterized by mountainous topography. Sikkim lies in ecological hotspot zone of the lower eastern Himalayan region. The state hosts several hot springs which are known for their medicinal and therapeutic values. The springs also reportedly have high sulfur content and few known to emit hydrogen (Choudhury, 2006). However, these hot springs are poorly studied for their microbial community structure. Microbial ecology studies could enhance the understanding of different metabolic framework in sulfurous hot springs of the state. The present study was aimed to investigate the unexplored microbial diversity of two hot springs of Sikkim with both the culture-dependent and culture-independent approaches and thus might provide novel insights into the ecological interactions among taxa in these communities, which in turn will also help in defining future study courses in these sites.

2. Materials and methods

2.1. Sampling

2.1.1. Description of a sampling site

The Polok and Borong hot springs selected for the current study were located in South district of Sikkim. Both the hot springs are on the banks of river Rangit which is a tributary of the Teesta River. These two springs are treated as a sacred place with medicinal properties which opened a door to tourism and people from different regions visit the place. The geographical position of coordinates and elevation range of Polok and Borong Tatopani were measured with the help of GPSMAP 78S (Garmin, India). The map of the hot spring site was prepared with the help of Google Earth software (Fig. 1).

2.1.2. Sampling and physiochemical analysis

The water samples were collected aseptically in 1 L sterile thermal flasks in triplicates from both the sampling sites. The samples were divided into three groups based on the experiments to be carried out, such as one group was kept for culture dependent bacterial isolation, the second group for chemical analysis through ICPMS (Inductive Coupled Plasma Mass Spectroscopy) and the third group for PLFA (Phospholipid Fatty Acid Analysis) studies and metagenomic studies. The samples were then immediately transferred to the laboratory and kept at 4 °C. Preliminary physicochemical parameters including temperature, pH, dO_2, TDS, electroconductivity were measured at the sampling site using portable water quality checker (Horiba, Japan; U-50 Series). Elemental analysis was done using ICP-MS (Perkin Elmer, USA).

2.2. Culture-dependent analysis

2.2.1. Isolation of bacterial strains

For culture dependent microbial diversity studies, the samples were enriched immediately after the collection. The bacteria were isolated using ten different media such as Nutrient Agar (NA), Thermus Agar (TA), Luria-Bertani Agar (LBA), Modified Luria-Bertani (mLB), YTP-2 medium, TR medium, R2A, BP medium, GYT and Actinomycetes agar. The composition of the various media used is given in a Supplementary material Table S1. The isolation was done by the standard spread and streak plate methods. The culture plates were incubated at 60 ± 2 °C for 24–72 h. After the incubation, different colonies were selected on the basis of their morphological characteristics and pure culture was obtained by subsequent sub-culturing. Isolated and purified bacterial strains were stored in 50% Glycerol stock at −80 °C till further use.