Bacterial diversity and Antibiotic resistance profile of four Hot Springs of Sikkim

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By

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Dedicated
To My
Parents

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ℑ Abbreviations

ℑ **BC**	:	Before Christ
ℑ **APHA**	:	American public health association
ℑ **WHO**	:	World health organization
ℑ **ºC**	:	Degree Celsius
ℑ **Taq**	:	*Thermus aquaticus*
ℑ **DGGE**	:	Denaturing gradient gel electrophoresis
ℑ **PLFA**	:	Phospholipid fatty acid analysis
ℑ **DNA**	:	Deoxyribonucleic acid
ℑ **RNA**	:	Ribonucleic acid
ℑ **USA**	:	united States of America
ℑ **rRNA**	:	Ribosomal ribonucleic acid
ℑ **FAME**	:	Fatty acid methyl ester
ℑ **Km**	:	Kilometer
ℑ **CO_2**	:	Carbon dioxide
ℑ **H_2S**	:	Hydrogen sulfide
ℑ **NH_4**	:	Ammonium
ℑ **CH_4**	:	Methane
ℑ **CO**	:	Carbon mono-oxide
ℑ **m**	:	Meter
ℑ **Sp.**	:	Species
ℑ **G+C**	:	Gaunine + Cytocine
ℑ **A+T**	:	Adenine + Thiamine
ℑ **PCR**	:	Polymerase chain reaction
ℑ **WGA**	:	Whole genome amplification
ℑ **SNP**	:	Single nucleotide polymorphism
ℑ **Pfu**	:	*Pyrococcus furiosus*
ℑ **AMD**	:	Acid mine drainage
ℑ **OD**	:	Optical density
ℑ **EDTA**	:	Ethylenediaminetetraacetic acid

- **MRG** : Metal resistance gene
- **ARG** : Antibiotic resistance gene
- **NGS** : Next generation sequencing
- **PCA** : Principal component analysis
- **MIC** : Minimum inhibitory concentration
- **ICPMS** : Inductively coupled plasma mass spectroscopy
- **RAST** : Rapid Annotation using Subsystem Technology
- **BLAST** : Basic Local Alignment Search Tool
- **NCBI** : National Center for Biotechnology Information
- **GPS** : Global positioning system
- **SEM** : Scanning Electron Microscope
- **FESEM** : Field Emission Scanning Electron Microscope
- **OTU** : Operational taxonomic unit
- **COG** : Cluster of orthologous group
- **KEGG** : Kyoto Encyclopedia of Genes and Genomes
- **ARDB** : Antibiotic Resistance Genes Database
- **PAST** : Paleontological Statistics Software
- **ATP** : Adenosine triphosphate
- **µL** : Micro liter
- **mM** : Milli-molar
- **GC** : Gas chromatography
- **EC** : Effective concentration
- **PBP** : Penicillin binding protein
- **SRA** : Sequence archive reads
- **ANOVA** : Analysis of variance

1. INTRODUCTION

Environment around us possess various ecological niches and these niches hold various ecological ranges controlled by requirement for liquid water and the physico-chemical stability limits of biomolecules. However, there are some extreme environments where only extreme state loving organisms survive. These organisms, mainly prokaryote have developed adaptations that enable them to tolerate extraordinary gamut of habitats (Knoll and Bauld 1989). These extreme niches are ubiquitous in nature (Aanniz *et al.* 2015). There are many such extreme places exist on earth such as hot springs, hydrothermal vents, marine trenches, hay composts etc. (Brumm, Land and Mead 2015). Among all these, hot springs are the subject of reverence since ages and have been used since the ancient times for medicinal, religious and social purposes. Thermal springs have been being used for religious as well as curative purposes previously 2000 BC in India (Virk, H.S, Sharma, A.K and Kumar 1998) and for hundreds of years in Crete, Egypt, China, Japan, Turkey and many European and Middle-Eastern countries as shown by Archaeological marks (Hoole 2001). Many thermal springs developed into thriving centers of religion, culture and health, such as those at Bath in England, Vichy in France and Baden-Baden in Germany (Hoole 2001; Olivier, Venter and Jonker 2011).

Although, medical sciences have reached its apex, still many people and ailed patients believe in using these hot springs for their medical treatment. Also some reports have shown that the use of hot spring water is beneficial for arthritis, muscular strains, joint pains and also for inflammatory skin diseases (Fioravanti *et al.* 2010; Kamioka *et al.* 2010; Ap *et al.* 2015). The dissolved minerals like sulphur, carbonates and other trace minerals are effective against various ailments such as rheumatiods, dermatosis, osteosis,

inflammatory bowel diseases etc. (Kamioka *et al.* 2010; Boekstein 2014; Stanhope, Weinstein and Cook 2015). Apart from medicinal, religious and social purposes, thermal springs are being used to generate electricity, provide heating and hot water for domestic and other uses, and drive industrial processes such as drying and concentrating (Lund, Freestone and Boyd 2011)

Since XIX century, the exploration of hot springs has been in progress and the physicochemical properties and geological features were the primary areas of study for researchers (López-López, Cerdán and González-Siso 2013). The water of hot springs is usually clear, but it is rich in mineral salts dissolved from the rocks passes through on its way to the surface (Olivier, Venter and Jonker 2011). In the geothermal water of an area various dissolved minerals such as magnesium, calcium, sodium, chloride, sulfates or silica are generally present in the higher concentration as compared to non-geothermal groundwater (Zangana 2015). One of the aspects to study chemical composition of hot springs is that the dissolve minerals in hot springs have considerable effect on various diseases as mentioned earlier, but at the same time when some elements present in higher concentrations above their threshold values can be harmful, e.g. chloride, fluoride, sulphate, nitrates, and phenolic compounds may cause diseases when present in higher concentrations in accordance with APHA and WHO standards (Gichuki and Gichumbi 2012; Sherpa, Das and Thakur 2013). Thus the presence of dissolved minerals in hot spring water are important to study as people are using it for various purposes such as drinking, bathing etc. On the other hand, higher mineralogical compositions support different micro and macro floral community in and on the surrounding environment (Mesa *et al.* 2017). Also under different concentrations of heavy metals the microbes may attain tolerance to

the later (Kim 1985). This tolerance to heavy metals by various microbes can be exploited in bioremediation (Valls and De Lorenzo 2002). The use of microbial biomass in heavy metal remediation has gained important credibility during recent years (Özdemir *et al.* 2009). To survive under extreme conditions, thermophilic bacteria require many adaptations for its optimal functioning. In general, it has been known that the phospholipid composition of bacteria changes with the growth temperature (De Vrij, Bulthuis and Konings 1988). Thus, they may possess different metal adsorption mechanisms as compared to the mesophilic species. It was thus interesting to study their tolerance to trace metals and use it in the improvement of bioassay and bioremediation strategies.

The other most important characteristic feature of hot springs which has gained the attention of researchers in recent decades is the microbial diversity possessed by these hot springs. In recent scenario hot springs are the hotspots of research in the field of microbial ecology (López-López, Cerdán and González-Siso 2013). The microorganisms present in such hot springs can thrive under extreme temperatures. These microorganisms are known as thermophiles, which can grow above 45ºC (Stetter 1999). The ideal growth temperature of the thermophiles varies from 45ºC to above 100ºC (Andrade, Jr and Antranikian 1999). The isolation and investigation of their thermophilic microbial community did not start until the 1950's (Marsh and Larsen, 1953). Some extremophiles have been known for more than 40 years, but the detection and isolation of new thermophilic microorganisms have increased in the last few decades or ever since thermophilic bacteria were first discovered in the 1960´s (Rampelotto 2013). Many bacteria and archaea have been isolated from hot springs. *Aquificales* are the dominant group of thermophilic bacteria. Also, *Thermotoga, Thermus (T. thermophilus), Bacillus, Clostridium, Synechococcus, Chloroflexus* etc., have

also been identified from many hot springs. In environments with a temperature above 90°C, archaeal communities are dominating (Reysenbach and Shock 2002). Archaea such as *Methanopyrus, Pyrodictium, Thermoproteus, Methanothermus, Archaeoglobus, Thermofilum, Thermococcus, Sulfolobus* etc. have been isolated from many such springs (Stetter 1999; Rampelotto 2013).

The thermophilic microorganisms are perceived by their metabolic thermo-security which are floated by their thermophilic proteins. The thermostability of the thermophilic catalysts has been built up as important biocatalysts for different biotechnological and modern purposes (Niehaus et al., 1999). A model is Taq-polymerase from *Thermus aquaticus* that prompted the foundation of the polymerase chain reaction (PCR) procedure (Chien, Edgar and Trela 1976). From the biotechnological standpoint the thermophilic microorganisms are the most attractive organisms due to their ability to produce enzymes proficient to catalyze industrial relevant process at higher temperature than corresponding enzymes from mesophiles. Extracellular-polymer-degrading enzymes and DNA-modifying enzymes have several applications in food, chemical and pharmaceutical industries and environmental biotechnology (Ladenstein and Antranikian 1998; Synowiecki 2010)

Various culture dependent and culture independent approaches has been followed by different researchers to determine the microbial diversity of hot springs (Amin *et al.* 2017). In view of some limitations to classical culture dependent methods various neo methodologies have been taken into custom such as DGGE, PLFA and metagenomics to study the microbial diversity throughout the world (Amann *et al.* 1995; Neilson, Jordan and Maier 2013). 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, Ahring and Westermann 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 studied with cutting-edge metagenomic approaches which conferred the microbial diversity and their functional and metabolic framework (Poddar and Das 2017).

Nature and its products have the direct influence on people and people are always dependent on them since ancient periods. One of the natural products which has been commercially exploited are antibiotics. Antibiotics are the chemotherapeutic agents that kill or inhibit the microorganisms (Dafale *et al.* 2016). These have extensive applications in combating bacterial diseases. However, over exposure and ready to use drugs have contributed significantly to resistance of microorganism to antibiotics and is now a growing problem around the world due to haphazard and unsubstantiated use of antibiotics (Liu, Zhang and Gao 2004). One of the most important facet is the transfer of antibiotic resistant genes from effected populations to various habitats directly or indirectly associated with them (Bennett 2008). Over time there may be the accumulation of multiple resistance traits among bacteria which makes them resistant to several classes of antibiotics (Levy 1993). Although, the characteristics of thermophilic bacteria make their pathogenic significance vague, but it has been shown that many of these bacteria could be associated as the

etiological agents for meningitis, septicemia and endocarditis (Rabkin *et al.* 1985). As the hot springs are the spots of pilgrimage, tourism or people even bath and used "to take away" the water and drank it as a blessed potion for its alleged curative properties. Thus the chances of acquisition of antibiotic resistance is higher. It is also prominent that the thermophiles have been studied widely with respect to their applications in industries etc., however, much less is known about the antibiotic susceptibility patterns and their mechanisms of antibiotic resistance (Vásquez, González and Vicuña 1984; Niehaus *et al.* 1999). Thus it becomes important to check the antibiotic profile of water samples from these hot springs. This may give us the opportunity to concoct an extensive and inclusive antibiotic resistance gene census in thermophilic bacteria. This may be also expedited by the clout of next-generation DNA sequencing. Moreover, culture-independent metagenomic investigations are adding tremendously to the pool of known genes and their distribution outside clinical settings (McArthur *et al.* 2013).

Nestling in the Himalayan Mountains, the state of Sikkim is characterized by mountainous topography, renowned for its tourism potential and is guarded by an ancestral deity - Mount Khangchendzonga. Sikkim lies in ecological hotspot zone of the lower eastern Himalayan region. Sikkim is a land locked holy state of India, which has many deep unfathomed beliefs, traditional knowledge and many virgin areas where the nature is in its juvenile form and a refresh season greets its visitors (Das *et al.* 2012). It hosts many migratory birds, butterflies, arthropods, insects and other various species of flora and fauna. 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). In nearby dialects these Hot Springs are called as Tatopani or Tshachu. Tatopani is

a Nepali word where "Tato" signifies "Hot" and "pani" signifies "water" while Tshachu is a Tibetan word where "Tsha" signifies "Hot" and "Chu" signifies "water". Accordingly Tatopani alludes to the hot springs (Nagendra *et al.* 2013). However, these hot springs are poorly studied for their microbial community structure (Panda *et al.* 2016). 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 bacterial diversity of four hot springs of Sikkim and to study their antibiotic resistant patterns with culture-dependent techniques. However, this study was supported and accomplished with culture-independent approaches such as metagenomics and Phospholipid fatty acid analysis which thus have provided 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. Other aspects were also taken into consideration for example presence of novel species were documented and extensively studied. Apart from antibiotic resistance, tolerance to various heavy metals were also investigated. And finally the screening of various industrially important enzymes such as amylase, protease and lipase were examined.

2. RATIONAL AND SCOPE

Thermophilic and thermotolerant microorganisms are of vital financial incentive because of their capacity to deliver thermostable extracellular chemicals which have essential biotechnological applications, for example, bio-bleaching of paper mash, generation of creature feed, creation of fermentable sugars for acquiring biofuel from cellulosic squanders, organic product juice extraction and illumination, refinement of vegetable filaments, restoring of espresso, cocoa and tobacco and furthermore for squander water treatment. The benefit of the utilization of thermostable catalysts for directing biotechnological forms at lifted temperatures are: lessening the danger of contamination by mesophilic microorganisms; diminishing the viscosity of the response medium; expanding the bioavailability and dissolvability of organic substrates; expanding the dissemination coefficient of substrates and items bringing about higher response rates (Zeldes *et al.* 2015; Gomes *et al.* 2016). The habitats for these important thermophilic microorganisms varies and one of the major studied habitats of thermophiles are hot springs.

In context to Sikkim, India, the present study of bacterial diversity in hot springs is first of its kind. Microbial diversity among hot springs of Sikkim has been least studied using culture dependent and culture independent approaches. As the hot springs all over the world are known as the repository of various biotechnological and industrial strains and their enzymes or other secondary metabolites, thus to study these ecosystems is very essential specially with respect to north eastern India.

The second most important aspect is the resistance to antibiotics. Resistance is a major concern and threat to the present world which is causing inoperable infections, mortality

and mounting healthcare costs (Miller et al. 2016). Pathogenic and non-pathogenic bacteria belonging to mesophilic world has been extensively studied in terms of antibiotic resistance. However, researchers have paid very less attention towards thermophilic bacteria in this field of antibiotic resistance. There are various aspects to choose thermophiles for this study. As the antibiotic resistance is increasing at an alarming rate, the thermophiles (so called ancestors) and thermotolerant microorganisms must be explored in order to get novel insights and acumens in this field so that the ways to control the antibiotic resistance may flourish. There are various factors to study thermophilic bacteria isolated from hot springs of Sikkim in terms of antibacterial resistance. The foremost importance to study antibiotic resistance of these thermophilic bacteria is that the thermophiles are now considered as a cause of new human diseases. A study carried out by Charles Rabkin (1984), have found that the thermophilic bacteria able to grow better at 50°C were causing diseases in human population. It has been shown that these thermophilic bacteria were causing several diseases such as meningitis, endocarditis, and septicemia. Among these many of the isolated bacteria were resistant to many antibiotics such as erythromycin, tetracycline, sulfamethoxazole, tobramycin and Netilmicin (Rabkin *et al.* 1985). Other studies revealed the out-break of pathogenic *Legionnaires*' disease in the hot springs of Japan (Kuroki *et al.* 2017) and Taiwan (Lin *et al.* 2007). *Legionella* causes one of the major disease such as community acquired pneumonia. The presence of such pathogenic bacteria in hot springs is a threat to visitors and there are the possible chances of transfer of their genetic elements (antibiotic resistant genes) through horizontal gene transfer. As the hot springs of Sikkim are visited by a huge number of people for various purposes such as they trusted that the water from these hot springs can fix a few infections

and hence, utilized as social prescription, religious traditions and practices. Individuals living in the regions of these hot springs utilize these hot springs for showering, as well as utilize water for drinking and cooking reason (Das *et al.* 2012). Thus, to study the antibiotic resistance of bacteria residing in these hot springs is very essential to take account on the possible post effects and the emergence of these resistance genes.

The second basis of our study was to understand the evolutionary relation of the antibiotic resistance genes that may be linked to thermophiles. The evolution and proliferation of antibiotic resistance in pathogens is very important and is still controversial. It has been shown by various studies that environmental organisms are the repository of these resistance genes. In contrast other studies, still controversial, depicts anthropogenic use involved in the emergence of these genes (Bhullar *et al.* 2012). Galapagos, a remote environment with restricted anthropogenic exposure of both human population and antibiotics was investigated for antibiotic resistance. The studies revealed that there was no acquired antibiotic resistance in bacteria (Thaller *et al.* 2010). Similarly, another survey was done in Netherlands. The antibiotic resistance was investigated in soil samples that were taken from pre and post- antibiotic eras (1940-2008). It was found that there was a significant increase in abundance of antibiotic resistance genes for antibiotic families such as β-lactams, tetracyclines and macrolides in post antibiotic era samples than those of pre-antibiotic era samples (Knapp, Dolfing and Ehlert 2010). Thus, these results encourage the hypothesis that anthropogenic activities are a cause of widespread resistance among microorganisms. However, in contrast, it is believed that the antibiotics and their biosynthetic pathways have evolved millions of years ago (Barlow and Hall 2002; Hall and Barlow 2004; Baltz 2008). Similarly, in other study it was shown that in ancient DNA

dating from the Pleistocene (30,000 years ago) was possessing abundant and diverse antibiotic resistance (Dcosta *et al.* 2011). The other study done on Lechuguilla Cave present in USA. This cave has been isolated for 4 million years and the bacterial population in this environment has never been exposed to any modern antibiotics and antibiotic resistance genes. However, this study revealed that the bacteria from this isolated environment were highly resistant to antibiotics like other surface microbes. It was also shown that some of the bacterial strains were resistant to 14 different antibiotics (Bhullar *et al.* 2012). Accordingly, this gathering of proof backings the mounting traditions that the anti-toxin obstruction is regular, antiquated and fortified in the microbial pangenome. Keeping in view all these evidences, our study to check antibiotic resistance profile and their possible mechanisms, would have escalating scope. As the hot springs are also considered to be isolated ecosystems and the prokaryotic communities such as bacteria and archaea present in them are considered to be primitive, thus it is very interesting to investigate the resistance profile of thermophilic bacteria isolated from these hot springs. Two main research questions may be highlighted in this study is that (i) what is the resistance profile of bacteria residing in such isolated environments i.e., hot springs few of which are still pristine (ii) what will be the prevalent antibiotics resistant and prevalent antibiotic resistance genes. (iii) Will there be any difference in antibiotic resistance profile between hot springs with different geographical locations.

The third concept defending the query about the importance of studying antibiotic resistance profile of hot springs is that some of the studies have shown that increasing temperature may decrease the resistance genes (Sun *et al.* 2016; Jang *et al.* 2018). However recently studies have shown that antibiotic resistance increases with increase in

temperature (Macfadden *et al.* 2018). As the hot springs of Sikkim possess different temperatures ranging from 45-75°C, thus it was interesting to check the antibiotic resistance abundance in different hot springs having different temperatures and thus correlate the effect of temperature on antibiotic resistance retention. Functional metagenomic may add to this concept and may open new areas of research. For instance, if possibly some bacterial communities retain resistance genes at higher temperatures than the other bacterial communities in the same hot spring then this may rise two research questions such as: (i) how these bacteria retain resistance genes or are there any novel mechanisms of resistance involved and (ii) if non resistant bacteria are abundant, then are they devoid of any competition or are they able to produce any known or novel protecting mechanisms or secondary metabolites such as bacteriocins.

The fourth and last importance of this study may be the generation of data. As thermophiles are less understood in terms of antibiotic resistance and very less data is present on the resistance profile. There is also no data present on MIC ranges of antibiotics ever used in thermophiles. Thus, our study may help to create a data set regarding the resistance profile of thermophilic bacteria.

2.1. Aims and Objectives.

- Physicochemical analysis of water samples from four hot springs of Sikkim–Polok Tatopani, Borong Tatopani, Reshi Tatopani and Yumthang Tatopani.
- Isolation and characterization of thermophilic or thermo-tolerant bacteria.
- Identification of the isolates by 16S rRNA sequencing and Fatty acid analysis [FAME] of some isolates.
- To check the bacterial diversity of the hot springs.
- To check the antibiotic susceptibility of the isolates.
- To check the antibiotic resistance mechanism of some isolates.

3. REVIEW OF LITERATURE

3.1. Geothermal fields:

Geothermal fields are generally systems with continuous circulation of heat and fluid, where fluid enters the reservoir deep beneath the Earth from the recharge zones and leaves through discharge areas. Any system that transfers heat from within the Earth to its surface can be called as geothermal system e.g. hot rocks, without water, is geothermal. Whereas when the transfer of heat involves water either in liquid or vapor form (hence the "hydro") refers to hydrothermal system. Thus hydrothermal is a subset of geothermal e.g. hot springs and geysers (Barbier 1997). Three geological components are required for the formation of a hydrothermal feature: (1) water, (2) heat and (3) permeability through rocks, so that water can flow in the subsurface and rise to or near the earth surface. Rainfall, snow-melt, rivers, and lakes are the sources of water (recharge zones) that enter a geothermal system at earth's surface. This is also called as meteoric water and forms most of hydrothermal fluids (Barbier 1997; Heasler, Jaworowski and Foley 2009). If there should arise an occurrence of fountain of liquid magma related frameworks, the wellspring of warmth is magma whereas the wellspring of warmth may likewise be the warmth from ordinary temperature increment with profundity in the Earth. The permeability is created by fractures in rocks or in some cases interconnected pores or large cave systems allow fluids to flow. Seismic activity may open factures or alternatively may close them. Also, after being heated by molten or hot rocks, the hydrothermal fluids may accumulate occasionally in reservoirs known as aquifers under high pressure and temperature (up to above 300°C). These aquifers are the essential parts of most geothermal fields. Depending on the

temperature, the geothermal areas have been divided into two categories; high temperature areas and low temperature areas (Lister 1980).

3.1.1. High temperature areas:

These areas are within the active volcanic areas where the heat source is a magma chamber at the depth of 2-5 km. The temperature of fluids in these areas reaches 150-350°C at the depths of 500-3000m (Arnórsson 1995). The high temperature areas are often sulphur rich and due to high temperature, little water comes to the surface and the geothermal features are mainly in the form of fumaroles and steam holes or grey and brown mud pots (Skirnisdottir *et al.* 2001). In these areas the steam and volcanic gases are emitted at the surface. The gases mainly consist of N_2 and CO_2, but H_2S and H_2 can also make up to 10% of the total amount of gases produced. Traces of CH_4, NH_4 and CO can also be found (Ármannsson 2017). The water within high temperature areas is often scarce and often very acidic which can lead to transformation of the surrounding rocks with various precipitations (Lagat 2009). On the surface, H_2S is oxidized chemically and biologically first to sulphur and then to sulphuric acid, which acts as a buffer and keeps or stabilizes the pH often at 2-2.5 (Zhang *et al.* 2000). Some of the hot springs usually neutral to slightly alkaline sulphide rich may also concur in high temperature fields but are rarer. They usually exist on the periphery of the active volcanic zones and are generated if water is abundant at lower depths (Skirnisdottir *et al.* 2000).

3.1.2. Low temperature areas:

The low temperature areas are located outside of the active volcanic areas. In these zones extinct or deep lava flows and dead magma chambers serve as heat source and with the water temperature below 150°C at the depth of 500-3000 m (Marteinsson *et al.* 2013).

These areas mainly hold clear water pools and springs which are most often alkaline with pH between 8 and 10 and often with silica around the edges of the hot-springs. These pools and springs possess the temperature range from 20°C-100°C. The gases are also emitted and are mainly CO_2 and little H_2S. On the surface, CO_2 is blown away and the silica precipitates, resulting in an increase in pH (often stabilizing at 9-10) (J.K and Stetter 1992; Marteinsson *et al.* 2013). The most commonly known geothermal areas which have been most studied are Iceland, the Naples area in Italy, the Yellowstone National Park in USA, Japan, and New Zealand etc (Huber and Stetter 2000).

3.2. Geothermal features.

Hot Springs, Geysers, Fumaroles and Mud Pots form the geothermal features. These features possess the distinct compositions, pH and temperatures. Each of them are discussed in detailed below with examples of some historic geothermal features in the world.

3.2.1. Hot Springs:

To a layperson, hot spring is nothing but a solitary spring with a hot water emerging out of it. However, for a geologist, hot springs are geothermal (hydrothermal) springs or points on the surface of earth and these are actually the surface tip of a subterranean system that may extend for kilometers and discharge water which has a temperature above that of the normal local groundwater (Gold 1992). Hot springs are found in regions where volcanic activity is very young or has become inactive. In both the cases, the hot magma does not erupt onto the surface of the earth. In such instances magma from the magma reservoirs (magma chambers), have risen to shallow depths (5-10 km) beneath the surface (Banwell

1963). The water from recharge zones percolates through cracks or fractures and feeds the ground water. This water gets heated up by the hot magma chambers or deep magma flows and finds its way to surface of earth through different fissures and cracks, or an underground plumbing system (Saemundsson 2009). The pressure and temperature inside helps the water to move upwards to the surface of earth. At the earth's surface, the hot springs do not erupt, but may seem to churn and boil as gases from undergoing passes through them. Depending on the temperature produced in magma chamber below, hot springs can be tranquil, effervescent, or boiling in nature. It has been characterized that the there is a distinction of no less than 8.3°C hotter temperature of the water than the normal temperature of the adjacent air and can reach as high as 100°C (Banwell 1963; Walker 1993; Saemundsson 2009).

Hot spring water when passes through the fissures comes in contact with rocks adjoining and thus there are high chances of mineral dissolution from rocks which thus reaches the surface. It has been known that if there are volcanic rocks present in the adjoining path, then the water carries silica to the surface (Silica is the most abundant element found in magma) (Saemundsson 2009). When the water cools, the silica forms geyserite at the surface, a white or greyish deposit that creates rims or terraces around the spring known as sinter. (Campbell *et al.* 2015). The water and rocks in hot springs might be multi-shaded. Additionally, the diverse types of microorganisms prosper in the hot spring condition. These species form brilliant rings of red, blue, brown, green, orange, and yellow colors.

3.2.2. Geysers:

Geyser is the name of an erupting flow of heated water from the ground and is actually called as "gusher" in Iceland. The word geyser is actually an "eponym" and thus every geyser on the planet gets its name from gusher, structurally and functionally similar to it. A geyser is a type of hot spring that periodically erupts, shooting jetties of water and steam up to hundreds of feet above the ground into the air (Eppelbaum, Kutasov and Pilchin 2014). The geysers are rare as there must be few essential geological conditions for the existence of geysers such as it must be having an abundant water supply, an intense heat source, and a special plumbing system (Campbell *et al.* 2015). There are over one thousand geysers known to exist on earth. It has been found that, Yellowstone National Park, alone contains more than four hundred geysers. Also the geysers are distributed worldwide such as in Iceland; Valley of Geysers, Russia; Taupo Volcanic zone, New Zealand; El Tatio, Chile etc (Rinehart 1980).

3.2.3. Fumaroles:

A fumarole is mainly a small hole or vent in the Earth's surface through which volcanic gases discharge from underground. The fumarole releases most of the gas in the form of water vapour or steam and that is why they are also called as steam vents (Hunt 2001). They can be present at the base of volcanoes or in geothermal fields, both on land and on the floor of the ocean. They are more smoking than hot springs and fountains on the grounds that any groundwater that enters a fumarole is immediately transformed into steam. The temperature of the gas discharged from a fumarole may reach as high as 400°C.The fumaroles have very little water in their plumbing system (Menyailov *et al.* 1986). At the surface only steam and foul smelling gases (H_2S, SO_2) are permitted.

Sometimes, yellow deposits are formed around the vent, as the sulphur from sulphur dioxide cools and crystallize around the vent (Eppelbaum, Kutasov and Pilchin 2014).

3.3. Microbial diversity of hot springs.

One of the momentous physiognomies of hot springs is the diversity of microbes vested in them. A major term conferring microorganisms surviving in harsh or extreme ecosystems are known as extremophiles. These environments hold various combinations of extreme conditions such as enormously high or low pH or enormously high or low temperature, high pressure, high salinity or other mishmashes thereof (Aanniz *et al.* 2015; Elleuche *et al.* 2015). These extreme or exhilarating environments and the microorganisms living in them has open the doors for search of life exterior to earth and also the leeway to transfer life from one planet to another (Rothschild and Mancinelli 2001). The discovery of extremophiles has also poured vivacity into the biotech and other industries (Madigan and Marrs 1997). Around 1974, R.D. Mac Elroy coined the term "extremophiles" (Greek, philos means lovers) i.e., lovers of extreme environments (Macelroy 1974). The resulting environments, based on the elevated or low conditions, such as high temperature, low temperature, low pH, high pH and salinity are then known as thermophilic, psychrophilic, acidophilic, alkaliphiles and halophilic respectively (Rothschild and Mancinelli 2001). As the hot springs are high temperature ecosystems, thus our focus will be mainly on thermophiles.

Thermophile also carried a diction from Greek (*thermos* means heat and *philos* means lovers) and are thus the lovers of heat or high temperature. Thermophiles are generally designated as the organisms which can grow above 45°C (Madigan and Oren 1999). These possess an optimum range of temperature from 55-80°C, however, the microorganisms

growing above 80°C are referred as hyperthermophiles (Bertoldo and Antranikian 2002). The temperatures of thermophiles fluctuate and as per Aragno (1992) who characterized thermophilic prokaryotes into a few gatherings relying upon their ideal temperature (Aragno 1981, 1992).

- Facultative thermophile: any thermophile whose temperature run expands generally in the mesophilic extend (i.e. below 45°C).
- Thermotolerant: any organism with a temperature optimum ≤ 45°C but can grow at a temperature > 45°C.
- Moderate thermophile: any organism with a temperature optimum between 45°C and 60°C.
- Strict thermophile: any organism with a temperature optimum between 60°C and 90°C.
- Extreme thermophile/hyperthermophile: any organism with temperature optimum ≥ 90°C.

However, currently thermophiles are classified into moderate thermophiles (organisms possessing optimal growth temperatures between 50-64°C, with maximum growth at temperature below 70°C), extreme thermophiles (organisms possessing optimal growth temperatures between 65-85°C, with maximum growth at temperature above 70°C) and hyperthermophiles (organisms possessing optimal growth temperatures above 85°C, with maximum growth at temperature above 90°C) (Wiegel 2001). Although some eukaryotic organisms such as protozoa, metazoan, yeasts, fungi, and multicellular plants have partially colonized extreme habitats characterized by high temperature up to 60°C and/or of elevated hydrostatic pressure, however the organisms capable of thriving at the extreme limits of

temperature, pH, salt concentration and hydrostatic pressure are generally prokaryotes (Tansey and Brock 1972). Thermophiles may be Gram-positive or Gram negative, aerobic or anaerobic, spore or non-spore forming and motile or non-motile prokaryotes (Aragno 1992).

The main report of extremophiles goes back to 1880 when a US botanist Farlow confined halophilic species from salted fish, took after later by Eleazari Volcani in 1936, who detached microbial strains from high salt-amassed water in the Dead Sea (Oren and Ventosa 2015). Later in 1969, the US microbiologist Thomas Brock discovered *Thermus aquaticus*, a thermophilic bacterium living at 80°C from a hot spring in the Yellowstone National Park, and one year later he found *Sulfolobus acidocaldarius*, a hyperthermophilic archaea growing at 85°C and low pH (Brock and Freeze 1969; Brock *et al.* 1972). The study of Brock and his team at the Yellowstone National Park gave a new dimension to Microbiology and opened a new corridor of thought leading to bolt from the blue, that life at and above 100°C is possible. They are very hardy in nature as tolerating extreme conditions requires different physiological conditions as well as different gene blueprint. The protein folding and their enzymology is the main distinguishing factor which separates them from the rest (Brock 1997).

Thermophiles have a place with in two phylogenetically altogether different spaces of life, Bacteria and Archaea (Stetter 1999). It is generally confined that thermophilic Bacteria is dominating community at temperatures between 50°C and 90°C in most hydrothermal environments. Among bacteria, there are just couple of species that can be called hyperthermophiles, for example, *Thermotoga* and *Aquifex*, which have an ideal temperature in the scope of 90 to 95°C (Huber and Stetter 2000). In environments with

temperature above 90°C Archaea are dominating (Reysenbach and Shock 2002) e.g. *Pyrolobus fumarii,* that has a temperature optimum of 106°C and can live at temperature up to113°C. However, the first hyperthermophilic archaea found in enormously hot and acidic hot spring was *Sulfolobus acidocaldarius* (Brock *et al.* 1972). Up to this point, no hyperthermophilic microorganisms in the area Eukarya have been accounted for.

In view of their ideal temperature some thermophilic microorganisms having a place with direct thermophiles include *Bacillus caldolyticus*, *Geobacillus stearothermophilus*, *Thermoactinomyces vulgaris*, *Clostridium thermohydrosulfuricum*, *Thermoanaerobacter ethanolicus*, *Thermoplasma acidophilum* etc. The group termed extreme thermophiles includes both bacterial and archaeal thermophiles. Among aerobic bacteria several species, including *Bacillus caldolyticus*, *B. caldotenax, Bacillus Schlegelii, Hydrogenobacter thermophilus, Thermothrix thiopara, Thermus thermophilus*, *Thermus filiformis, Thermomicrobium roseum and Calderobacterium hydrogenophilum also some anaerobic thermophilic bacteria Dyctioglomus thermophilum, Thermosipho africanus, Thermotoga maritima and Thermotoga neapolitana*, *Fervidobacterium pennavorans*, *Acetomicrobium faecalis* and some archaea *Thermodesulfobacterium commune*, *Sulfolobus acidocaldarius*, *Methanococcus vulcanicus*, *Sulfurococcus mirabilis.* The hyperthermophiles including *Methanoccus jannaschii*, *Acidianus infernos*, *Archaeoglobus profundus*, *Methanopyrus kandleri*, *Pyrobaculum islandicum*, *Pyrococcus furiosus*, *Pyrodictiumoccultum*, *Pyrolobus fumarii*, *Thermococcus littoralis, Ignicoccus islandicum etc.* (Kristjansson, Hreggvidsson and Alfredsson 1986; Andrade, Jr and Antranikian 1999; Stetter 1999; Wiegel 2001; Reysenbach and Shock 2002; Ghosh *et al.* 2003).

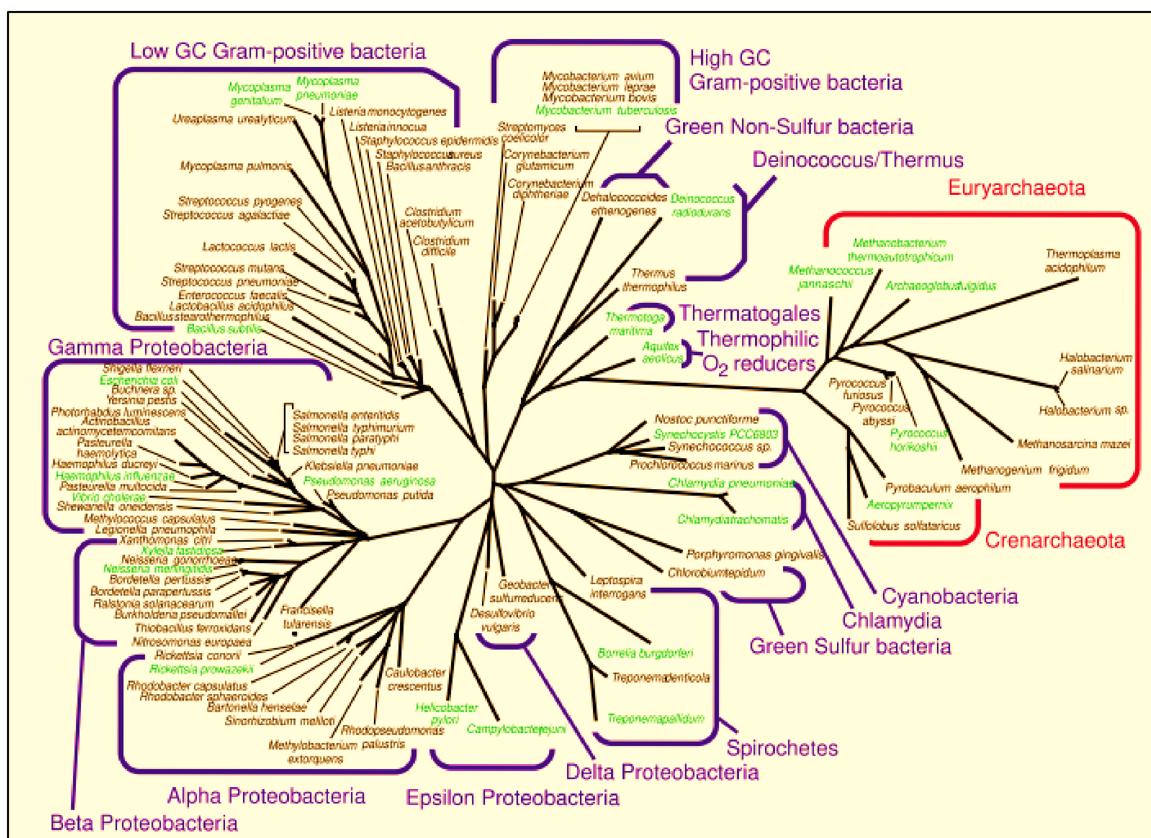

Fig.1. The universal phylogenetic tree and the position of some representative thermophiles (Nelson *et al.* 2000).

3.3.1. *Geobacillus* a moderate to extreme thermophile.

It has been found in India and all over the world that the hot springs with moderate temperatures ranging from 40-70°C possess bacterial diversity mainly comprising of Proteobacteria, Firmicutes, Bacteroidetes etc. However, the percentage of various phyla varied among various hot springs which might be due their geographical isolation. It has been observed that, only few specific genera can be found through culture dependent techniques in the hot springs, mostly belonging to phylum Firmicutes (Sahoo 2017). Generally, hot springs of India have temperature between 50°C and 70°C and have Firmicutes in predominance. Surprisingly, the predominance of Firmicutes (*Geobacillus* sp.) was reported in Bakreshwar hot spring, West Bengal, India and with increase in

temperature of this hot spring, abundance of Firmicutes (more specifically *Geobacillus* sp.), also increased. In this way, here we will be for the most part concentrating on the genus *Geobacillus.*

In 1991, Ash and co-workers, analyzed many species of *Bacillus* and found that they belong to five distinct groups based on 16S rRNA sequences. However, a few of the sporulating aerobes proved to be sequence outliers, which themselves might represent the new or previously unrecognized genera (Ash *et al.* 1991). In the meantime, a group of Moscow scientists working on the microbial ecology of high temperature oil fields in Kazakhstan. These scientists were led by N. A. Nazina at the Russian Academy of Sciences. These people found a huge source of thermophilic bacilli in the water samples drawn from a depth of over a kilometer at a temperature of 55-74°C (Nazina *et al.* 2007). Consequent investigations disengaged comparative microscopic organisms from oil fields in western Siberia and China (Nazina et al., 2000). In any case, Nazina et al. attempted an intensive, polyphasic examination of their "group 5" isolates. Their outcomes bolster the origination of a phylogenetically unmistakable, physiologically and morphologically predictable taxon, for which they have presented the truly depicted sort name of *Geobacillus* (Nazina *et al.* 2001). With high levels of 16S rRNA sequence similarity of 98.5 to 99.2%, the *Geobacillus* species include a cogent group of thermophilic bacilli *(Bacillus sterothermophilus, B. thermoleovorans, B. thermocatenulatus, B. kaustophillus, B. thermodenitrificans and B. thermoglucosidasius).*

Geobacillus bacteria are endospore-forming, Gram positive, aerobic or facultative anaerobic rods (Zeigler 2016). These bacteria usually grow at a temperature range between 35°C to 80°C, depending on the strain. The dwelling places of *Geobacillus* species can be

a number of hostile environments including high temperature oilfields (Kuisiene, Raugalas and Chitavichius 2004), marine vents (Maugeri *et al.* 2002), a corroded pipeline in an extremely deep well (Popova *et al.* 2002), African (Hawumba, Theron and Brözel 2002) and Russian (Nazina *et al.* 2004) hot springs, and the Mariana Trench (Takami *et al.* 2004), yet they can also be found in hay composts (Sung *et al.* 2002) and garden soils (Wiegand *et al.* 2013). The thermostable attributes make *Geobacillus* microorganisms appealing to the biotechnology business as wellsprings of thermostable catalysts (de Champdoré *et al.* 2007), as stages for biofuel creation (Cripps *et al.* 2009) and as sprouting constituents of bioremediation methodologies (Markossian *et al.* 2000).

3.4. Habitat of thermophiles.

The two major ecological factors determining the presence or absence of and the type of thermophilic bacteria in a given ecosystem are temperature and pH. Thus these bacteria have many habitats both geothermal and non-geothermal in nature (Aragno 1992). Thermophilic microorganisms can be easily isolated from natural environments such as hot springs which are ubiquitous. However other natural sites from which thermophilic and hyperthermophilic microorganisms have been isolated includes terrestrial volcanic sites (comprising solfataric fields), submarine hydrothermal systems (such as fumaroles, sediments, volcanoes and vents), subterranean sites such as oil reservoirs and solar heated surface soils. There is also some manmade environments such as compost piles, black smokers or chimneys, slag heaps, water heaters and industrial processes (Erauso *et al.* 1993; Stetter 1996; Oshima 2008).

3.5. Adaptations of thermophilic bacteria to temperature changes.

Thermophiles survive under extreme temperatures following many adaptations than their mesophilic counter parts. There bacteria possess various physical (membrane lipid modifications), genomic and proteomic adaptations in order to survive higher temperatures. Many of the adaptations are discussed below:

a) *Membrane permeability and fluidity*: these factors have a significant effect on diffusion of nutrients, function and mobility of membrane proteins and appropriate separation during cell division. The permeability and fluidity are the two typical membrane characteristics that are unfavourably affected by temperature changes. Higher temperature increases the fluidity and permeability of the membrane. Thus membrane integrity and fluidity must be fortified. However, thermophilic bacteria maintain this integrity by actively modifying their lipid composition (Vossenberg, Driessen and Konings 1998). There is a considerable distinction of structural lipids between the bacteria and archaea which represents a term called as "lipid divide". The bacteria and eukarya possess 'phosphatidic acids' whereas archaea hold 'archaetidic acids' in their membrane lipids (Koga 2012). The phosphatidic acid comprise of two fatty acid hydrocarbon chains esterified to glycerol-3-phosphate whereas archaetidic acid on the other hand possess two methyl-branched isoprenoids (phytanyls once saturated) linked by ether-bonds to glycerol-1-phosphate (Vossenberg, Driessen and Konings 1998; Siliakus, Oost and Kengen 2017). Anyway it is trusted that these distinctions are not straightforwardly identified with thermophiles. However, the thermophilic bacteria essentially regulate fluidity by increasing the amount of: (i) branched chain iso-fatty acids

(Sinensky 1974; Patel 1991) (ii) long chain fatty acids (Ray, White and Brock 1971) (iii) saturated fatty acids (M. Oshima and A. Miyagawa, 1973) (iv) polar carotenoid content (Yokoyama A, Shizuri Y, Hoshino T 1996). On the other hand, in case of thermophilic archaea, tetra-ether lipids are the principal lipids and are highly abundant. These lipids with hydrocarbon chains form monolayers and are thus highly stable. It was also shown in *Thermococci,* that the increase in temperature provokes the increase in tetra-ether/diether ratio (Cario et al., 2015; Matsuno et al., 2009).

The dependability of tetra-ether layers can be advanced by the expansion of pentacycli that can cause an up-move of the progress temperature (pentacyclization is a change incorporates the breaker of cyclopentane rings along the biphytanyl ties up to 4 rings for each chain. These rings are acknowledged to offset attach packaging and to decrease layer permeability (Alessandra Gliozzi et al, 1983; Siliakus et al., 2017) . Also macrocyclic archaeols may be present in heat adapted membranes (Kaneshiro and Clark 1995). These archaeols have isoprenoid chains cross connected at their last parts and hence making an upgraded layer obstruction water and film strength (Jain *et al.* 2014).

b) *Chemical stability and increase in proportion of lipids:* it is commonly assumed that the archaeal ether lipids are temperature resistant. However, this thermostability is due to the chemical stability of their membrane lipids. Also with the increase in temperature there is increase in proportions of certain lipids which are thus designated as 'thermophilic lipids'. It has been shown that a thermophilic strain growing on temperature between 45-70°C possess iso-C15 content (30-50%)

always higher than that of anteiso-C15 (lower than 10%) (Rilfors, Wieslander and Stahl 1978). It has also been shown that in *Methanocaldococcus jannaschii* a thermophilic archaeon, the diether lipids (archaeol based lipids) decrease from 80% to 40% when there is an increase an increase in temperature from 45-65°C. whereas the caldarchaeol based and cyclic archaeol based lipids increase from 10% to 40% (Sprott and James 1991).

c) G+C content: The G+C content of rRNA and tRNA of mesophiles is lower than that of thermophiles (Hector 2006). As the G+C pair of nucleic acids is thermostable than A+T pair due to the presence of additional hydrogen bond, thus the higher content of G+C in thermophiles will impart more thermostability in their nucleic acids (Paz *et al.* 2004).

d) Proteomics: Various studies have been performed to investigate the adaptations at proteomics level. The basic and most studies were conducted to check the changes in amino acids among thermophiles and their mesophilic analogues. To summarize it was shown that the foremost effect of thermophilly was the substantial reduction in the frequency of thermolabile amino acids in proteins like histidine, threonine and glutamine and simultaneous increase in both positively charged residues arginine and lysine and negatively charged glutamic acid (Singer and Hickey 2003). Thus the increase in oppositely charged residues help in the formation of additional ionic bonds, which may help to stabilize multimeric proteins at higher temperatures (Tekaia, Yeramian and Dujon 2002). The thermophiles also contain proteins with isoelectric points in the basic range (Kawashima *et al.* 2000). Further it has also been shown that the thermophilic proteins possess fewer non-charged polar amino

acids and more charged amino acids. These charged amino acids results in the formation of higher number of intermolecular salt bridges (Thompson and Eisenberg 1999). Also some structural genomic studies suggested the increase in intra-helical salt bridges and hydrogen bond formation among thermophiles (Kumar and Nussinov 2001; Suhre 2003). Finally, it has also been found that there are considerable differences in average length of proteins between thermophiles and mesophiles i.e. the proteins of thermophiles tend to be shorter than their mesophilic counter parts (Zhang 2000).

e) *Hydrophobicity and Ion pair interactions:* in case of thermostable proteins, it has been suggested by various studies that hydrophobic interactions stabilize them present in thermophilic bacteria (Gromiha *et al.* 2012). It has also been shown that the salt bridges (interaction between oppositely charged residues) increases the stability of thermostable proteins (Kumar, Tsai and Nussinov 2000). There is usually the network of ion pairs occur on both the surface and interface between subunits of these proteins (Missimer *et al.* 2007). Thus the combination of ion pair and hydrophobic interactions have positive effect on the stability of proteins which was also supported by molecular dynamic studies (Manjunath and Sekar 2013). Flexibility of protein was also reported to be essential for thermostability. As thermophilic proteins are more rigid due to strong molecular interactions thus contribute to thermostability (Mamonova 2010). In case of nucleic acids such as DNA, it has been shown that the monovalent or divalent ions provide stability to them. The studies show that the negative charge residing on phosphate groups are being neutralized by these mono or divalent salts in nucleic acids (Marguet and

Patrick 1998). Marguet and Forterre, 1994, have observed the robust reduction in the DNA thermodegradation in presence of physiological concentrations of monovalent (KCl/NaCl) and divalent ($MgCl_2$) salts (Marguet *et al.* 1994). It has also been studied that the physiological concentrations of two salts such as KCl and $MgCl_2$ induce chemical stability to double and single stranded DNA against heat induced cleavage by inhibiting depurination and hydrolysis (Marguet and Patrick 1998).

3.6. Applications of thermophiles.

Thermophilic microorganisms are a text style of remarkable curiosity as of late, in light of their thermostable proteins and catalysts. These essential metabolites do not get generally denatured at high temperature and thus are appropriate for many industrial applications (Zeikus 1979). It has been shown that most of the mesophilic enzymes gets denatured at around 40°C and are generally inactivated beyond 50°C-60°C whereas thermophilic enzymes can uphold at least half of their specific activity even at high temperatures of around 80°C (Vieillel, Burdette and Gregory 1996). Thermo catalysts or thermozymes got remarkable obligingness from the researchers everywhere throughout the world since these compounds are normally steady at high temperature, impervious to substance reagent and outrageous pH when contrasted with their mesophilic homologues.

3.6.1. Biotechnological Importance of thermophiles.

The biotechnology has clearly changed our lives in many captivating ways which is inexorable. Many of the reactions involved in biotechnological or industrial processes to develop out puts, need to take place on extremes of temperature, pH, pressure and salinity (Coker 2016). The mesophilic macromolecules can be utilized in these processes, but being

temperature susceptible, their macromolecules must be genetically or chemically modified in order to harvest the products. However, these modifications can be lengthy and cost effective (Siddiqui *et al.* 2009). In contrast, nature has fervently provided us substitutes in the form of extremozymes which are present in microorganisms that can bloom in extremes of temperature, pressure, salinity and pH (Kato *et al.* 1998; Horikoshi 1999). These naturally thermostable macromolecules are being already used in various industrial processes. Nonetheless, the chase has additionally filled in the previous quite a while by industry's acknowledgment that the "survival units" controlled by extremophiles can conceivably serve in a variety of uses (Madigan and Marrs, 1997). The various applications which have made paradigm shifts in the field of biotechnology are the discovery of polymerase from a thermophilic bacterium *Thermus aquaticus* (Ishino and Ishino 2014). Other applications include biofuel production using various thermophilic enzymes (Barnard *et al.* 2010), thermophilic microorganism used in bio-mining (Johnson 2014) and carotenoids used in food and cosmetic industries (Oren 2010).

3.6.2. DNA Polymerases.

DNA polymerase synthesizes complementary DNA strands by reading template DNA in living cells. The discovery of natural stability of DNA polymerase at higher temperature led to the introduction of robust PCR method (Saik *et al.* 1988). PCR is generally used to amplify the nucleic acid sequences, which in turn has found several applications directly or indirectly in biotechnological, genetical, medical, pharmaceutical and many other fields. Besides, PCR, DNA polymerase enzyme is exploited in DNA cloning, DNA sequencing, whole genome amplification (WGA), single nucleotide polymorphism (SNP) detection, molecular diagnostics and synthetic biology (Gardner and Kelman 2014). There are many

steadfast DNA polymerases used in above techniques such as Taq, Pfu and Vent which were isolated from thermophiles *Thermus aquaticus*, *Pyrococcus furiosus*, and *Thermococcus litoralis* respectively (Eckert and Kunkel 1990; Lundberg *et al.* 1991; Mattila *et al.* 1991). Taq polymerase was industrialized as a commercial product in the early age of PCR and $2 billion royalty was earned by PCR rights holders during its patent (Jr, Wiechers and Cook-deegan 2006). The distinguishing characteristics of each DNA polymerase may encourage the impending advancement of exclusive reagents and thus the exploration of novel DNA polymerase has been one of the foremost emphases in the research field (Ishino and Ishino 2014).

3.6.3. Biofuel production.

In order to support and replenish non-renewable resources such as fossil fuels, there is a strenuous effort to produce analogous fuels using biomass such as corn, sugar cane and wheat etc, which can be thus known as the biofuels (Coker 2016). The classification of the biofuels can be carried out on the basis of consumption of source and on the basis of product formed. Thus, on the basis of source utilization, biofuels are known as first generation biofuels which can be derived from easily hydrolyzing sugars like starches or oils or second generation biofuels which are generated from not easily hydrolyzed such as lignocellulosic material. However, on the basis of end product, biofuels can be classified as bioethanol, biodiesel, biobutanol, hydrogen and methane (Luque *et al.* 2008). It is known that various stages in biofuel production encompass extremes of many physical conditions such temperature and pH. Thus, thermophiles are the ideal entrants to replace their mesophilic analogues (Coker 2016). The other benefits using thermophilic microorganisms is that the thermophiles can readily ferment pentose and hexose sugars from biomass or

even complex carbohydrates and thus can be easily exploited in the production of second generation biofuels (Sommer, Georgieva and Ahring 2004). Moreover, thermophilic fermentations are less disposed to other microbial contaminations. Also, the product inhibition is reduced as the volatile products can be easily removed (Barnard *et al.* 2010).

Various thermophilic and hyperthermophilic microorganisms have been exploited in biofuel production. Although the earlier traditional methods of biofuel production such as biobutanol and bioethanol incorporates the use of chemical process complemented with mesophilic microbes such as *Saccharomyces cerevisiae* and *Clostridium* species (Lee *et al.* 2008). However, the exploitation of thermophilic microorganisms such as *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii* for large scale manufacture have been settled recently (De Vrije *et al.* 2002). Other thermophiles such as thermophilic *clostridia* (fermentative anaerobes) are able to degrade lignin-containing substances. They possess a multienzyme complex called as cellulosome in their cell membranes. This cellulosome complex has a potential to ensure the enzymatic degradation of cellulosic substances (Demain, Newcomb and Wu 2005). *Clostridium thermocellum*, therefore, is a good entrant for ethanol fermentation from cellulosic biomass. *Geobacillus* are the other promising thermophilic candidates for ethanol production. Certain species of *Geobacillus* are able to degrade complex carbohydrates such as xylan due to the production of xylanase enzymes by them (Wu, Liu and Zhang 2006). *Geobacillus stearothermophilus* is able to produce ethanol at higher temperatures and with a good yield comparable to those of *S. cerevisiae* (Bibi *et al.* 2014). Other species of *Geobacillus* can tolerate ethanol as high as 10% such as *Geobacillus thermoglucosidasius* (Fong *et al.* 2006). Thus, for such reasons, there is a great deal of interest of these thermophilic microorganisms in biofuel production.

3.6.4. Bioremediation and biomining.

Substantial metal contamination speaks to an essential issue because of its dangerous impact and aggregation all through the natural pecking order which prompts genuine environmental and medical issues (Iyer, Mody and Jha 2005). Removal and recovery of heavy metals are very important with respect to environmental and economic considerations (Nurba *et al.* 2002). Due to not being cost effective and non-eco-friendly nature of some conventional physiochemical methods such as electrochemical treatment, ion-exchange, precipitation, reverse osmosis etc., are not the methods of interest, however, biosorption using microbial biomass as the adsorbent has emerged as a potential alternative technique for heavy metal removal during the past few decades (Öztürk 2007; Özdemir *et al.* 2009). The main advantages of biological substrates are (a) the diversity of biological active binding sites, (b) small and uniform size and (c) less subject to interference from alkali and alkali-earth metals than ion-exchange resins (Madrid and Cámara 1997). Several reports on eubacteria and fungi are available which deals with the metal tolerance (Cánovas *et al.* 2003). However, thermophilic bacteria which are able to grow at higher temperatures and variable extreme conditions such as pH, salinity and higher concentrations of heavy metals were also established for bioremediation of heavy metals at higher temperatures (Rajendran, Muthukrishnan and Gunasekaran 2003; Sar *et al.* 2013). It has also been known that there is active (bioaccumulation) and passive (adsorption) uptake of heavy metals by microorganisms (Hussein *et al.* 2004). A thermophilic bacterium *Anoxybacillus flavithermus* possess metal binding capacity in order Mn » Ni < Zn < Cd < Pb » Cu, and it was shown metal binding was enhanced by forming complexes with carboxyl, phosphoryl, and hydroxyl moieties (Burnett *et al.* 2007). Also various thermophilic genera such as

Bacillus, Anoxybacillus, Brevibacillus, and *Geobacillus* were investigated for sensitivity and adsorption of Cd (Hetzer, Daughney and Morgan 2006). It was also shown that the parameters such as pH, contact time, biomass concentration, initial metal concentrations, and temperature were found to be the main conditions for equilibrium adsorption of Cd, Cu, Zn, Ni and Mn on various species of *Geobacillus* such as *G. toebii* ssp. *decanicus* and *G. thermoleovorans* ssp. *stromboliensis* (Özdemir *et al.* 2009). The other species of genus *Geobacillus* was also studied separately by Chatterjee et al. (2010) for biosorption of heavy metals from industrial wastewater (Chatterjee, Bhattacharjee and Chandra 2010).

Biomining generally called bioleaching, is the amputation of insoluble metal sulfides or oxides by using microorganisms (Donati, Castro and Urbieta 2016) . It has been estimated that the extraction rates using biomining are around 90% compared with 60% for traditional heap leaching (Vera, Schippers and Sand 2013) . Biomining has been potentially exploited in the mining of various metals such as gold, copper, silver, nickel, zinc and uranium (Donati, Castro and Urbieta 2016). The use of thermophiles has various advantages for biomining as compared to their mesophilic counter parts. The exploitation of thermophiles reduces the possibilities of acid mine drainage (AMD) which is sometimes caused by mesophiles is the acidic water, created by the oxidation of sulfides from the mine, starts streaming or filtering out of the mine and is cost effective (Sheoran, Sheoran and Choudhary 2010). Many thermophilic strains, such as *Sulfolobus* and *Metallosphaera* have also been employed in biomining (Vera, Schippers and Sand 2013). Thus, the above studies suggest that the thermophilic bacteria and archaea are suitable candidates which can be potentially exploited in bioremediation and biomining.

3.6.5. Thermozymes/thermophilic enzymes.

Enzymes got from thermophilic microorganisms have incomparable physiognomies, for example, temperature, pH and chemical stability. These proteins or enzymes are inherently more stable under extreme environments than those present in their mesophilic analogues (Ladenstein and Antranikian 1998). Thermal sensitivity has been the foremost problem to the widespread use of enzymes as far as industry is concerned. The foremost benefits of accomplishing processes at higher temperatures are reduced risk of microbial contamination, lower viscosity, improved transfer rates, and improved solubility of substrates (Bruins, Janssen and Boom 2001; Vieille and Zeikus 2001). From the biotechnological standpoint the thermophilic microorganisms are the most attractive due to their ability to produce enzymes proficient to catalyze industrial relevant process at higher temperature than corresponding enzymes from mesophiles. Thus, the ability of thermophilic enzymes to suitably work at high temperature implies many advantages for their applications in industry (Lasa and Berenguer 1993).Thus, these thermostable enzymes held an explicit allure for researchers all over the world. Thermostable extracellular-polymer-degrading enzymes (such as amylases, pullulanases, Cellulases, chitinases, xylanases, pectinases, isomerases, esterases, dehydrogenases and DNA-modifying enzymes are being potentially used in food, chemical and pharmaceutical industries and environmental biotechnology (Vieille and Zeikus 2001).Mesophilic hosts like *Escherichia coli*, *Bacillus subtilis and* yeasts have been successfully cloned by the thermo enzyme encoding genes for retrieving archaeal genes (Jørgensen, Vorgias and Antranikian 1997).

3.6.6. Starch hydrolyzing enzymes.

Starch is the most important carbohydrate polymer made of two fractions amylose and amylopectin (Koivula 1996). The amylose is a linear polysaccharide composed of D-glucose residues linked with α (1-4) linkages. Amylopectin is highly branched polysaccharide which is also composed of D-glucose residues linked with α (1-4) linkages and the branches are linked by α (1-6) linkages (Robyt 2008). The degree of polymerization and the comparative content of amylose and amylopectin depends on the source of starch (Koivula 1996). The depolymerization or degradation of starch is carried out by enzymes known as amylases. Amylases are synthesized by animals, plants and microorganisms and classified as alpha (α), beta (β) and gamma (γ) amylases. Alpha (α) amylases are endo-acting enzymes leading to the hydrolysis of α (1-4) linkages randomly and are unable to break α (1-6) linkages, thus α-amylases can lead to the formation of linear, branched oligosaccharides and limit dextrins. Beta (β) amylases are exo-acting and leading to the hydrolysis of only α (1-4) linkages. These act on polysaccharide chains from their non-reducing end thus results in the formation of major oligosaccharide maltose. Gamma (γ) amylases are also exo-acting and attacks the substrate from non-reducing ends and leads to the hydrolysis of both α (1-4) and α (1-6) linkages thus results in the formation of monosaccharides as a major product (Horváthová, Janeček and Šturdík 2001; Abd-Elhalem *et al.* 2015). The amylases can be exploited in a wide number of industrial applications such as fermentation, food, textile, detergent, pharmaceutical and paper industries (de Souza and e Magalhães 2010). Since the hydrolysis of starch related to industrial progressions require high temperature and pH. Thus, thermophilic amylases would be the first choice for industrial purposes. Various studies have shown the utilization of

thermophilic amylases in various processes (Coker 2016). A number of thermophilic microbes has shown significant amylolytic activity such as *Sulfolobales acidocaldarius*, *Sulfolobales solfataricus*, *Thermophilum, Desulfurococcus, Thermococcus* and *Thermotoga* (Bragger *et al.* 1989). It was shown that *Thermotoga maritima* a thermophilic bacterium possesses all the three amylolytic properties i.e. α, β, and γ amylase activities (Schumanni *et al.* 1991). Also, *Pyrococcus furiosus* and *Pyrococcus woesei,* hyperthermophilic bacteria were reported to possess highly thermostable amylolytic activities (Koch *et al.* 1991; Chung *et al.* 1995).

3.6.7. Proteases.

Proteases are the class of enzyme that converts the protein into amino acid and peptides. They are classified according to the nature of their catalytic activity (Ellaiah, Srinivasulu and Adinarayana 2002). Today the quantity of proteolytic enzymes produced throughout the world on a commercial scale is large as compare to the other biotechnologically modified enzymes. In the food, leather, pharmaceutical and textile industry, these are the major used enzymes (Li *et al.* 2012). Serine alkaline protease is used as addition to detergents for laundering. Proteinases having high keratinolytic and elastolytic activities are used for soaking in the leather industry (Rani, Rana and Datt 2012). The proteases that can catalyze responses under outrageous condition i.e, high temperature and extraordinary pH are profitable for modern application (Ladenstein and Antranikian, 1998). It has been found that most proteases from extremophiles belongs to the serine type and are stable at high temperature even in the presence of high concentration of detergents and denaturing agents (Ellaiah, Srinivasulu and Adinarayana 2002). Many studies have been done on thermophilic bacteria and archaea in order to get the promising proteolytic enzymes. A

hyperthermophilic archaeon *Thermococcus kodakarensis* KOD1 was studied and a highly heat stable protease enzyme Tk-subtilisin has been isolated. It has also been shown that after recombination, this enzyme shows optimal activity at 100°C and was readily stable under high concentrations of various denaturants (Koga *et al.* 2014). Another archaeal isolates thermopsin like protease SsMTP-1, stable at high temperature and pH has been isolated from *Sulfolobus solfataricus,* a thermophilic archaeon (Gogliettino *et al.* 2014). The thermophilic bacteria have also been exploited such as a thermophilic bacterium *Coprothermobacter proteolyticus* (Toplak *et al.* 2013). From this bacterium a serine protease proteolysin was isolated and is the excellent candidate in detergent industry due to its extreme stability at high temperature and elevated pH ranges.

3.6.8. Lipases.

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis and the amalgamation of esters shaped from glycerol and long-chain unsaturated fats. Lipases are viewed as the most flexible proteins of the enterprises which achieve a scope of bioconversion response (Sharma et al., 2011), which incorporates hydrolysis, inter esterification, esterification, acidolysis (Andualema and Gessesse, 2012). The esters produced by lipase catalysis play a vital role in the food industry as flavor and aroma constituents (Neena N, Sudhirprakash B and Jyeshtharaj B 1992). Other products formed from lipase activity such as long chain methyl and ethyl esters of carboxylic acid moieties may function as fuel for diesel engines (Jeong and Park 2008). On the other hand, ester of long chain carboxylic acid and alcohol moieties have application as lubricants and additives in cosmetic formulations (Andualema and Gessesse 2012). The lipases have been also exploited in various other applications such as in paper industry, for the hydrolysis of

milk fat in the dairy industry, removal of non-cellulosic impurities from raw cotton before processing into dyes and finished product, drug formulations in the pharmaceuticals industry and in the removal of subcutaneous fat in the lather industry (Andualema and Gessesse 2012). Lipases occur throughout the earth's flora and fauna. Extensively and abundantly, they are produced by various microbial communities like bacteria, fungi and yeast (Sharma, Sharma and Shukla 2011). There are many reports on the *Bacillus sp.*, as the major contributor and producer of lipolytic enzymes. In order to tolerate the extreme conditions of temperature or pH, the hunt for thermophilic lipolytic enzymes was carried out. The isolation of two thermostable and alkaline lipolytic enzymes were purified from two thermostable archaea *Sulfolobus acidophilus and Pyrobaculum sp,* (Shao, Xu and Yan 2014; Zhang *et al.* 2014). Other thermophilic species were also being exploited for lipolytic enzymes such as *Thermotoga maritima* and *Thermus thermophilus* (Wei *et al.* 2013). It has also been shown that the thermophilic *bacillus* produces lipase enzymes with greater activities and stabilities than their mesophilic analogues. Thus, these stabilities of thermophilic bacteria and their enzymes signify exceptional entrants for industrial applications.

3.6.9. Other enzymes.

There are various other enzymes studied from thermophilic bacteria and archaea such as cellulases, esterases, pullulanases, chitinases, xylanases, pectinases, isomerases, dehydrogenases and DNA-modifying enzymes. Cellulose being the most abundant polymer on earth, can be hydrolyzed by complex enzymes known as cellulases. Cellulase enzymes include endoglucanase, exoglucanase and β-glucosidase (Acharya and Chaudhary 2012). The cellulases are the important catalysts in various industrial applications such

food, detergent, textile, pulp and paper. These cellulases can also be exploited in ethanol production (Kuhad, Gupta and Singh 2011). Various thermophilic bacteria have been studied such as an anaerobic thermophile *Clostridium thermocellum* has shown cellulolytic activities (Mori 1992). Thermophilic *bacillus* isolated from hot springs (India) also have shown cellulolytic activity (Acharya and Chaudhary 2011). Thermophilic archaea have also been exploited and they have shown a considerably high thermostable activity such as cellulases isolated from *Pyrococcus furiosus* (Kengen et al., 1993) and *Pyrococcus horikoshii* (Ando *et al.* 2002a). *Sulfolobus solfataricus* MT4, *Sulfolobus acidocaldarius* and *Sulfolobus shibatae* (Grogan 1991). Other highly thermostable cellulases stable at temperatures between 95-115°C has been isolated from *Thermotoga maritima* MSB8 and *Thermotoga* sp. FjSS3-B1 (Ruttersmith and Daniel 1991; Bronnenmeier *et al.* 1995). Similarly, other enzyme such as esterase has been studied in some thermophilic microorganisms. Esterases are the class of enzymes that catalyze the hydrolysis (and formation) of ester bonds e.g. acetyl xylan esterases catalyze the removal of acetyl ester groups from C2 or C3 positions of D-xylopyranosyl residues (Bornscheuer 2002). Thermostable acetyl xylan esterases have been cleansed and portrayed from just couple of microscopic organisms including the anaerobic microbes *Thermoanaerobacterium* sp., *Clostridium thermocellum* etc (Correia et al., 2008). The phenolic acids released through the action of esterases are potential precursors to a variety of value-added products and may be exploited in bio-refineries and can also be exploited in food and cancer gene therapy (Sood, Sharma and Sharma 2016). Besides these above discussed applications of thermophilic microorganism and their thermostable enzymes, there are many other fields where they can be significantly exploited in the fields of agriculture, dairy, medical, cancer

treatments etc. Thus, the study of thermophilic microorganisms and their common sources such as hot springs are essential to study extensively. These thermophilic applications are one of the reasons which provoke us to study the pristine hot springs of Sikkim.

Table.1. Microorganisms possessing various enzymatic activities.

Enzymes	**Organism**	**Optimal temperature** (°C)	**References**
α-Amylase	*Bacillus amyloliquefaciens*	70	(Abd-Elhalem *et al.* 2015)
	Bacillus licheniformis	100	(Ivanova, Dobreva and Emanuilova 1993)
	Bacillus stearothermophilus	70-80	(Özcan and Özcan 2008)
	Pyrococcus furiosus	100	(Laderman *et al.* 1993)
	Bacillus circulans	60	(Nigam and Singh 1995)
Pullulanase	*Pyrococcus furiosus*	98	(Brown and Kelly 1993)
	Thermus caldophilus GK24	75	(Kim, Nashiru and Ko 1996)
Xylanases	*Bacillus amyloliquefaciens*	80	(Breccia *et al.* 1998)
	Pyrococcus furiosus	100	(Bauer, Driskill and Kelly 1999)
	Thermotoga neapolitana	102	(Zverlov *et al.* 1996)
Cellulases	*Bacillus subtilis*	70	(Mawadza *et al.* 2000)
	Pyrococcus furiosus	102-105	(Kengen *et al.* 1993)

	Rhodothermus marinus	95	(Halldórsdóttir *et al.* 1998)
Proteases	*Bacillus brevis*	60	(Banerjee *et al.* 1999)
	Bacillus stearothermophilus	85	(Raja *et al.* 1994)
	Staphylothermus marinus	100	(Mayr *et al.* 1996)
	Geobacillus Sp.	70	(Hawumba, Theron and Brözel 2002)
Lipases	*Bacillus acidocaldarius*	70	(Manco *et al.* 2000)
	Bacillus stearothermophilus	68	(Gupta *et al.* 1999)
	Geobacillus sp.	70	(Abdel-Fattah 2002)
	Pyrococcus horikoshii	97	(Ando *et al.* 2002b)

3.7. Antibiotic Resistance.

Although, thermophiles have been studied widely with respect to their applications in industries etc., much less is known about the antibiotic susceptibility patterns and their mechanisms of antibiotic resistance. The resistance to antibiotics is a major concern and threat to the present world which is causing inoperable infections, mortality and mounting healthcare costs (Miller *et al.* 2016). The research has shown that mortality rate per annum due to various drug resistant bacterial infections in various countries such as 23,000 in the US (United 2013), 25,000 in EU (Report, 2015), and 58,000 in India (Gelband *et al.* 2015). The essential discerning pressure (regardless of the existence of antibacterial agents) which drives the cumulative rates of resistance is eventually found in the abuse and misuse of

antibacterial agents weather used in an inappropriate prescribing, extensive agricultural use, availability of few antibiotics or regulatory barriers (Roca *et al.* 2015; Ventola 2015). Unfulfillment of the prescribed antibiotic course by patients may also lead to the development of antibiotic resistance. Over time there may be the accumulation of multiple resistance traits among bacteria which makes them resistant to several classes of antibiotics (Levy 1993). Thus, a huge research is going on and the antibiotic resistance causes, transfer and prevention has been extensively studied worldwide.

3.7.1. Antibiotics, their mode of action and resistance mechanisms.

The generic term "antibiotic" is used to denote any class of chemotherapeutic agents that inhibits or kills microbes (pathogens) by specific interactions with bacterial targets (Dafale *et al.* 2016). Antibiotics are usually the drug combinations made of chemical compounds that are synthesized by microorganisms and by chemical synthesis (Dafale *et al.* 2012). These substances are used in very small concentrations and are known to completely eradicate or moderately inhibit microorganisms. The antibiotics which are able to kill bacteria are called as bactericidal while as those that just inhibit the bacterial growth are called as bacteriostatic (Etebu and Arikekpar 2016). The antibiotics play a crucial role in defending against infectious diseases caused by microorganisms. However, the increasing rate of antibiotic resistance in microorganisms is critical and threating to human health (Dafale *et al.* 2015). The discovery of antibiotics dates back to a great scientist and bacteriologist Alexander Fleming, who accidently discover the antibiotic from a soil inhabiting fungus *penicillium notatum*. This antibiotic was then known as penicillin was first trialed on humans in 1940 (Aminov 2010). In 1940, the penicillin was prescribed to treat serious infections and was successful in combating the bacterial infections among

World War II soldiers (Ventola 2015). After the extensive use of penicillin antibiotic, resistant strains proficient of disarming the drug became prevalent. From that point this prompted the exploration of new anti-microbials and because of this disturbing opposition in microscopic organisms, the examination of novel anti-toxins and strategies to control anti-infection obstruction is still in advance (Davies and Davies 2010). There are various classes of antibiotics and their mode of action, some are discussed as below:

3.7.1.1. β-lactams.

These are the class of antibiotics which contain 3-carbon and 1-nitrogen ring in their structure (Etebu and Arikekpar 2016). The most vital members of the beta-lactam class include penicillins, cephalosporins, carbapenems and monobactams (Bycroft and Shute 1985). These antibiotics are bactericidal in nature as they kill the bacteria by inhibiting the production of cell wall (Spratt 1980). During the synthesis of peptidoglycan layer in cell wall, bacteria possess certain enzymes known as penicillin binding proteins (PBP) which help in the cross linking of peptide units. However, beta-lactams such as penicillin alter the function of these penicillin binding protein by binding them, thus hinder the formation of peptidoglycan resulting in lysis and cell death (Heesemann 1993). On the other hand, bacteria have acquired resistance to these beta-lactams and it is known that resistance to penicillin occurs in three different ways such as: (i) inhibition of drug from reaching its target: restricted permeability caused by bacterial cell wall to antibiotic. (ii) Target alteration: by altering the structure of penicillin binding proteins. (iii) Antibiotic inactivation: production of bacterial enzymes called as β-lactamases, causes the inactivation of antibiotic (Soares *et al.* 2012). There are various members of penicillin class

such as penicillin G, penicillin V, methicillin, ampicillin, oxacillin, amoxicillin, nafcillin, piperacillin, carbenicillin, mezlocillin and ticarcillin.

3.7.1.2. Aminoglycosides.

Aminoglycoside (AG) antibiotics are an important group of antibiotics which are operative against both Gram-positive and Gram-negative bacteria (Garneau-tsodikova and Labby 2016). The structure of aminoglycosides consists of 2-deoxystreptamine ring linked to two or more amino-modified sugars with the help of glycosidic bonds (Becker and Cooper 2012). It has been known that the aminoglycosides act by binding to the bacterial ribosome (30S subunit) and thus impairs the bacterial protein synthesis (Mingeot-Leclercq, Glupczynski and Tulkens 1999). In 1943. The first aminoglycoside discovered was streptomycin which used effectively used against *Mycobacterium tuberculosis* (Mahajan and Balachandran 2012). The diseases treated by using streptomycin were mainly tuberculosis, tularemia, and bubonic plague. However, streptomycin was found to be highly toxic and this led to the search and discovery of new aminoglycosides such as gentamycin, meomycin, amikacin and tobramycin (Etebu and Arikekpar 2016). In spite of their broad spectrum antibacterial activities, the bacterial resistance has invaded this class of antibiotics also. The antibiotic resistance mechanisms in bacteria against aminoglycosides are diverse and can be broadly occurs in four different ways such as (i) Ribosomal mutation: mutations in aminoglycoside target site (A-site) of 30S ribosomal subunit. (ii) Ribosomal modification by methyltransferases: A-site may be enzymatically modified by methylation of their 16S rRNA using methyltransferases. (iii) AG modifying enzymes: aminoglycosides may be chemically modified by aminoglycoside-modifying

enzymes (AMEs). (iv) Efflux pumps: the active transport of aminoglycosides out of the cell through efflux pumps (Becker and Cooper 2012; Etebu and Arikekpar 2016)

3.7.1.3. Macrolides.

Macrolides are wide spectrum antibiotics and possess diverse activities such as antibiotic, antifungal, prokinetics and immunosuppressants (Kanoh and Rubin 2010). These antibiotics consist of 14-, 15-, 16- membered macrocyclic lactose rings. These rings are linked with infrequent deoxy sugars such as L- cladinose and D- desosamine (Zuckerman, Qamar and Bono 2011). The first macrolide was isolated in 1952, and was known as erythromycin. Erythromycin was isolated from a fungus *Saccharopolyspora erythraea* (Shoemaker and Yow 1954). Macrolides such as erythromycin is often used in patients which are allergic to penicillin. These are mostly effective in Gram-positive bacteria and spirochetes (Seymour and Hogg 2008). The macrolides are known to act upon bacterial ribosomes and efficiently inhibit their protein synthesis by blocking the elongation (addition of amino acids) of polypeptide chains (Soares *et al.* 2012; Etebu and Arikekpar 2016). On the other hand, there are various resistance mechanisms developed by bacteria against macrolides such as: (i) Drug efflux: the active transport of macrolides out of the cell through efflux pumps. The active efflux is caused by ATP-binding-cassette (ABC) transporter superfamily and of the major facilitator superfamily (MFS). (ii) Target alteration: by ribosomal methylation, binding of erythromycin to its target site (23S rRNA) is prevented. (iii) Antibiotic inactivation: modifying the antibiotic molecules by various enzymes such as esterases and phosphotransferases (Leclercq 2002; Soares *et al.* 2012).

3.7.1.4. Quinolones.

Quinolones represent an important and most commonly prescribed class of antibiotics in the world. These antibiotics are used to treat infections caused by both Gram-positive and Gram-negative bacteria (Andriole 2005). The basic structure of quinolones consists of a heterocyclic two rings. However, recently used quinolones consists of an additional ring. Several other modifications have been carried out to enhance their spectrum of activity and potency (Etebu and Arikekpar 2016). Various bulky substituent are permissible on the positions 1 and 7 or 8, these substitutions led to the classification of quinolones into three categories such as piperazinyl-, pyrrolidinyl- and piperidinyl type side-chains (Hu *et al.* 2003). Also, the addition of fluorine at the position C6, these are often called as "fluoroquinolones" (Emmerson 2003; Andriole 2005). The first quinoline discovered in 1960's was nalidixic acid and the norfloxacin represents the first wide spectrum quinolone. The other members include ciprofloxacin, ofloxacin, temafloxacin, sparfloxacin, enoxacin etc (Aldred, Kerns and Osheroff 2014). The quinolones act by converting their targets such as bacterial type II topoisomerase, gyrases, and topoisomerase IV. These enzymes thus create fragments in the chromosome and thus inhibit the DNA synthesis (Fàbrega *et al.* 2009; Aldred, Kerns and Osheroff 2014). On the other hand, a number of quinoline resistant bacteria have evolved due to extensive use of these drugs. The resistance mechanisms found in bacteria against these antibiotics are given such as (i) Target mediated: specific mutations at target sites in gyrase and topoisomerase IV leads to quinoline resistance. Mutations occur in A and B subunits of both the enzymes usually at serine amino acid. (ii). Plasmid mediated: the types of quinoline resistance genes have been found in plasmid carrying resistant bacteria. The genes Qnr, aac and QepA1/A2 are

involves in quinoline resistance. (iii) Chromosome mediated: mutations in regulatory proteins involved in expression of chromosome encoded efflux, reduces the drug accumulation by increasing efflux or decreasing uptake of drug (Emmerson 2003; Fàbrega *et al.* 2009; Aldred, Kerns and Osheroff 2014).

There are other classes of antibiotics also such as tetracyclines, lincosamides, glycopeptides, chloramphenicol etc. The mode of action and resistance patterns of these antibiotics are given in table.

Table.2. Antibiotic classes with examples, their target sites and mode of resistance. Modified from (Zaman *et al.* 2017).

Antibiotic class	**Example(s)**	**Target sites/Mode of Inhibition**	**Mode(s) of resistance**
β-Lactams	Penicillins, Cephalosporins, Penems, Monobactams	Inhibiting Cell wall synthesis	Hydrolysis, efflux, altered target
Aminoglycosides	Gentamicin, Streptomycin, Spectinomycin	Inhibiting Protein synthesis (anti 30S ribosomal subunit)	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
Glycopeptides	Vancomycin, Teicoplanin	Inhibiting Cell wall synthesis	Reprogramming peptidoglycan biosynthesis
Tetracyclines	Minocycline, Tigecycline	Inhibiting Protein synthesis (anti 30S ribosomal subunit)	Monooxygenation, efflux, altered target
Macrolides	Erythromycin, azithromycin	Inhibiting Protein synthesis (anti 50S ribosomal subunit)	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Lincosamides	Clindamycin	Inhibiting Protein synthesis (anti 50S ribosomal subunit)	Nucleotidylation, efflux, altered target

Streptogramins	Synercid	Inhibiting Protein synthesis (anti 50S ribosomal subunit)	Carbon-Oxygen lyase, acetylation, efflux, altered target
Oxazolidinones	Linezolid	Inhibiting Protein synthesis (anti 50S ribosomal subunit)	Efflux, altered target
Phenicols	Chloramphenicol	Inhibiting Protein synthesis (anti 50S ribosomal subunit)	Acetylation, efflux, altered target
Quinolones	Ciprofloxacin	Inhibiting DNA synthesis	Acetylation, efflux, altered target
Sulfonamides	Sulfamethoxazole	Inhibiting Folic acid synthesis	Efflux, altered target
Rifamycins	Rifampin	Inhibiting RNA synthesis	ADP-ribosylation, efflux, altered target
Lipopeptides	Daptomycin, polymyxin	Inhibiting Cell wall synthesis	Altered target

3.8. Methodologies to study bacterial diversity.

The historical backdrop of the presence of microbial life is just around 300 years prior with Leeuwenhoek's innovation of the magnifying lens. However the pace of research in microbial diversity has increased (Pace 1997) due to the presence of industrially and biotechnologically important bacteria. The reason for poor understanding of the microbial world lies in the fact that microbes are tiny, individually invisible to the eye and such organisms are difficult, perhaps often impossible to cultivate (Pace 1997; Hugenholtz *et al.* 1998). Also, the natural taxonomic description of microbes was failed to designate on the basis of morphological and nutritional criteria. The customary established culture-subordinate approach was the essential and sole method to decide the geomicrobiology of the various ecosystems such as hot springs before the development of neo molecular techniques.

Rapid molecular methods such as PCR (Polymerase Chain Reaction) based technologies advanced our knowledge regarding the novel microbial community diversity (Baker and Cowan 2004). The all-inclusiveness of 16s rRNA qualities makes them a perfect focus for phylogenetic and taxonomic grouping. The consequent PCR of 16s rDNA and sequence analysis has facilitated a great understanding of microbial diversity but still many important taxa will continue to be missed if adequate universal primers will not be taken into account (Theron and Cloete 2000). Failure to culture most by far of microorganisms with the way of culture-dependent technique has scrutinized the system for comprehensive profiling of various condition (Amann *et al.* 1995). To setback this constraint, distinctive culture-free strategies including DGGE, PLFA, and Metagenomic ponders have uncovered a resulting increment in microbial molecular ecology studies. A well-known approach to give an idea about the total biomass and the microbial diversity present in harsh environments is based on the presence of various fatty acids present in bacteria, which is commonly known as Phospholipid Fatty Acid Analysis. This interesting non-culturable technique, i.e., Phospholipid Fatty Acid Analysis or PLFA is available and used since two decades to characterize microbial communities (Willers, Jansen van Rensburg and Claassens 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 D S and J. N. Ladd 1981). The main disadvantage of PLFA is that the PLFA profiles don't give any data on species organization; rather, they reveal the unique mark of network structure and have been broadly used to examine network structure in fluctuated scope of frameworks

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, Jordan and Maier 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, Rodríguez-Belmonte and González-Siso 2016). This method is precise for determining 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, Wentzel and Valla 2013). With the advent of next generation sequencing (NGS) technologies, more samples can be analyzed at lower sequencing cost and time, improving the production of 16S rRNA gene-based biodiversity studies. Furthermore, the utilization of NGS permits to recoup more data about the scientific categorization of the example. The progression of this high-throughput DNA sequencing has enabled the development and improvement of the genomic analysis of a population of microorganisms (Handelsman 2005). As the metagenomic approach is based on the total environmental DNA extraction which involves the DNA of both live and dead cells thus there is a kind of ambiguity in the results. Also the other important drawback of this approach is that high quality whole community DNA is needed, which makes the extraction a critical step in the process of generating metagenomic data (DeCastro, Rodríguez-Belmonte and González-Siso 2016).

4. MATERIALS

4.1. Materials used for measuring and mapping of the geographical features of the sample sites.

Global Positioning System - GPSMAP78S, Garmin©2010, Olathe, Kansas, United States of America (USA).

Google Earth – Software; Google ©, USA.

GSI (Geological Survey of India) Website: www.portal.gsi.gov.in

4.2. Materials used for sample collection.

Thermal Stainless-Steel Container (Thermo Flask), Capacity 0.75 liter, Mega Slim®.

4.3. Materials for the Isolation and Cultivation of the microorganisms.

4.3.1. Microbiological Media.

Thermus Agar	(Composition stated at Appendix I)
Nutrient Agar	(Composition stated at Appendix I)
Luria Bertani agar	(Composition stated at Appendix I)
Modified Luria Bertani agar	(Composition stated at Appendix I)
YTP-2 Medium	(Composition stated at Appendix I)
TR Medium	(Composition stated at Appendix I)
BP Medium	(Composition stated at Appendix I)
GYT Medium	(Composition stated at Appendix I)

Actinomycete Isolation Agar	(Composition stated at Appendix I)
R-2A Agar	(Composition stated at Appendix I)
Carbohydrate Fermentation Broth	(Composition stated at Appendix I)
Plate Count Agar	HiMedia, Mumbai, India.
Agar Agar Type-1	HiMedia, Mumbai, India.
Bacteriological Peptone	HiMedia, Mumbai, India.
Trypticase	HiMedia, Mumbai, India.
Yeast Extract	HiMedia, Mumbai, India.
Beef Extract	HiMedia, Mumbai, India.
Starch	HiMedia, Mumbai, India.
Trypticase Soya Agar	HiMedia, Mumbai, India.
Mueller Hinton Agar	HiMedia, Mumbai, India.
Gelatin	HiMedia, Mumbai, India.

4.4. Chemicals.

Ethyl Alcohol	Changshu Yangyuan Chemical, China.
Magnesium Chloride	Merck, Mumbai, India.
Mercuric Chloride	Merck, Mumbai, India.

Sodium Chloride	Merck, Mumbai, India.
Potassium Chloride	Merck, Mumbai, India.
Phenol Red	Merck, Mumbai, India.
Resublimed Iodine	Merck, Mumbai, India.
Urea	Merck, Mumbai, India.
Hydrogen Peroxide	Merck, Mumbai, India.
Dipotassium hydrogen phosphate (K_2HPO_4)	HiMedia, Mumbai, India.
Potassium dihydrogen phosphate (KH_2PO_4)	HiMedia, Mumbai, India.
Disodium hydrogen phosphate (Na_2HPO_4)	Merck, Mumbai, India.
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck, Mumbai, India.
Zinc Chloride ($ZnCl_2$)	HiMedia, Mumbai, India.
Manganese Chloride ($MgCl_2$)	HiMedia, Mumbai, India.
Phenol	HiMedia, Mumbai, India.
p-aminodimethylanaline oxalate	HiMedia, Mumbai, India.
Sulfanilic acid	HiMedia, Mumbai, India.
Acetic acid	HiMedia, Mumbai, India.
α-naphthylamine	HiMedia, Mumbai, India.
Formaldehyde	HiMedia, Mumbai, India.

4.5. Materials for the Staining of the microorganisms.

Gram stain kit (HiMedia K001-1KT) was used to stain bacterial isolates. Endospore Staining was carried out by using Schaeffer and Fulton's spore stain- kit (HiMedia K006-1KT). However manual methods were also used and the materials used manually are given below:

Crystal violet	HiMedia, Mumbai, India.
Copper Sulfate	HiMedia, Mumbai, India.
Gram's Iodine Solution	HiMedia, Mumbai, India.
Safranin	HiMedia, Mumbai, India.

4.6. Materials for the Biochemical Analysis of the microorganisms.

4.6.1. Carbohydrate Fermentation Test.

D (-) Arabinose	HiMedia, Mumbai, India.
D (+) Dextrose	HiMedia, Mumbai, India.
D (-) Fructose	HiMedia, Mumbai, India.
D (+) Galactose	HiMedia, Mumbai, India.
D (+) Lactose	HiMedia, Mumbai, India.
D (+) Mannose	HiMedia, Mumbai, India.
D (+) Maltose	HiMedia, Mumbai, India.

D (+) Rhamnose	HiMedia, Mumbai, India.
D (+) Sucrose	HiMedia, Mumbai, India.
D (+) Trehalose	HiMedia, Mumbai, India.
D (-) Sorbitol	HiMedia, Mumbai, India.
D (-) Mannitol	HiMedia, Mumbai, India.
D (+) Cellobiose	HiMedia, Mumbai, India.
D (+) Dulcitol	HiMedia, Mumbai, India.
D (+) Ribose	HiMedia, Mumbai, India.
D (+) Xylose	HiMedia, Mumbai, India.
D (+) Melizitose	HiMedia, Mumbai, India.
D (+) Melibiose	HiMedia, Mumbai, India.

4.6.2. Enzymatic Characterization

Starch Agar	(Composition stated at Appendix I)
Urea Agar	(Composition stated at Appendix I)
Gelatin Agar	(Composition stated at Appendix I)
Protease Agar	(Composition stated at Appendix I)
Oxidase	Discs Himedia
Nitrate	Discs Himedia

4.7. Materials for the DNA Isolation, PCR amplification and Sequencing

DNA was extracted using Qiagen QIAamp DNA Mini Kit (50)

For PCR amplification reactions GoTaq Green Master Mix 2X, (M712B) (Promega) was used.

PCR product was purified using a QIAquick PCR purifcation kit (Qiagen, 28106)

For sequencing BigDye^{TM}Terminator v3.1 cycle sequencing kit (Applied Biosystems) was used.

Agarose gel	(Sigma Aldrich, A9414)
Ethidium bromide	(Sigma Aldrich, MB071-1G)
50X TAE buffer	(Composition stated at Appendix I)
TE buffer	(Composition stated at Appendix I)
Tris Base	HiMedia, Mumbai, India
Glacial acetic acid	HiMedia, Mumbai, India
EDTA	HiMedia, Mumbai, India
Sodium acetate	HiMedia, Mumbai, India
Sodium dodecyl sulfate (SDS)	HiMedia, Mumbai, India
Chloroform	HiMedia, Mumbai, India.
Isoamyl alcohol	Merck, Mumbai, India.

4.8. Antibiotics used for Antibiotic Assay.

Amoxicillin (10mcg)	HiMedia, Mumbai, India.
Ampicillin (10mcg)	HiMedia, Mumbai, India.
Erythromycin (15mcg)	HiMedia, Mumbai, India.
Penicillin G (10U)	HiMedia, Mumbai, India.
Clindamycin (2mcg)	HiMedia, Mumbai, India.
Methicillin (10mcg)	HiMedia, Mumbai, India.
Chloramphenicol (30mcg)	HiMedia, Mumbai, India.
Streptomycin (10mcg)	HiMedia, Mumbai, India.
Gentamycin (10mcg)	HiMedia, Mumbai, India
Norfloxacin (10mcg)	HiMedia, Mumbai, India
Ciprofloxacin (10mcg)	HiMedia, Mumbai, India
Vancomycin (500mg) (Potency-950µg mg^{-1})	HiMedia, Mumbai, India.
Penicillin (1MU) (Potency-1440-1680 units mg^{-1})	HiMedia, Mumbai, India.
Erythromycin (1g) (Potency-920 units mg^{-1})	HiMedia, Mumbai, India.
Oxacillin (5mg) (Potency 816-950 µg mg^{-1})	HiMedia, Mumbai, India.
Chloramphenicol (Potency-900 µg mg^{-1})	HiMedia, Mumbai, India.
Gentamycin (1g) (Potency-590 µg mg^{-1})	HiMedia, Mumbai, India.

4.9. Materials used for Heavy metal tolerance tests:

$MgCl_2$	HiMedia, Mumbai, India
HgCl2	HiMedia, Mumbai, India
MnSO4	HiMedia, Mumbai, India
ZnSO4	Merck, Mumbai, India.
$CuSO_4$	Merck, Mumbai, India.
$COCl_2$	Merck, Mumbai, India.

4.10. Laboratory Equipment's and Instruments.

Environmental Chamber	Indian Instruments, Kolkata, India. Model-ETC-10D,285 LITER
Spectrophotometer (uv-vis range)	Perkin Elmer, USA, Model-LAMBDA25+PTP (1+1)
Thermo Cycler	BIORAD thermal cycler (MJ Mini), Model-PTC-1148
Automated DNA Sequencer	Model-ABS/Genetic 3500 Analyzer.
Cooling Centrifuge	Thermo Fisher Scientific LR56495/D-37520.
BIOLOG	Toshniwal Technologies, India (OmniLog Combo Plus ID System)

Electrophoretic Chamber	Genei, India
Laminar Air Flow System	Thermo Scientific-Model-1386
Weighing Balance	Mettler Toledo, Switzerland Model-Mettler-202-S/03
Autoclave	Instrumentation India, Kolkata, India.
Incubator cum Shaker	REMI, Kolkata, India. REMI/RIS/24BL.
Cooling Incubator	REMI/CIS-6S, Kolkata, India.
Digital Incubator	Digilab, India,
Freezer -80°C	Thermo Scientific, 8606
Freezer -20°C	Voltas, India.
Freezer 4°C	Samsung, India.
Compound Microscope	Olympus, Model - 808209.
Phase Contrast Microscope	Olympus, Model – CKX41
Water distillation unit	Riviera/Double-vertical panel mounted, Mumbai India.
Hot Air Oven	N.A. Instruments & Equipment's, Kolkata, India/NAHAO/031/09
Microwave	Samsung, India.

4.11. Glass wares

Petri plates	Borosil, India.
Test Tubes	Borosil, India.
Spreader	Borosil, India.
Pipettes	Borosil, India.
Conical Flask	Borosil, India.
Reagent Bottle	Borosil, India.
Glass slides	HiMedia, Mumbai, India.
Cover Slip	Blue Star, India.
Dropper	Borosil, India.

4.12. Other Materials

Inoculum Loop	HiMedia, Mumbai, India.
Spirit Lamp	Borosil, India.
Absorbent Cotton	Bengal Surgical, West Bengal, India.
Non-Absorbent Cotton	Bengal Surgical, West Bengal, India.
Test Tube Rack	Tarson, India.
Cryo Vials	Tarson, India.
Micropipette	Gilson, France.

Micro tips	Tarson, India.
PCR tubes	Axygen, USA, PCR-02-C.
Cryo Vial Stand	Tarson, India.
Paraffin Tape	Bengal Surgical, West Bengal, India.
Blotting Paper	Bengal Surgical, West Bengal, India.
Autoclavable bags	HiMedia, Mumbai, India.

5. METHODS

5.1. Mapping of the sampling site.

By the help of GPS78SMAP device (Global Positioning System), the geographical coordinates were determined. To describe any geographical location, one has to measure the latitude and longitude of the area. Thus, on locating the coordinates, the exact area gets mapped. Next, the elevation of the land from the sea level is calculated to position the area of the land. By the help of compass, the exact direction of the site studied can be easily quantified. After measuring all the co-ordinates, elevation, latitude, longitude, the elevation of the site, the ascent of the site and the atmospheric temperature of the site, hence were mapped in Google Earth and Arc GSI Software (Nagendra *et al.* 2013).

5.2. Methods used for sample collection.

A quantity of 3L water samples were collected twice from each hot spring aseptically in 1L sterile thermal flasks in triplicates from each sampling sites (Sherpa, Das and Thakur 2013). The Hot Springs of south Sikkim were visited and samples were collected on 11th - 12th March, 2015. However, sampling from Yumthang hot spring located in North district, Sikkim was done from 23rd -25th, March 2015. Again samples for the second time from south Sikkim including Reshi, Borong and Polok hot springs were collected on 14th June 2017 and also the water samples from Yumthang hot spring located in north Sikkim was collected on 18th -19th June 2017. 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 4ºC in order to maintain the microbes' viability and reduce their metabolic activity (Najar *et al.* 2018a).

5.3. Physicochemical Analysis of the water samples.

5.3.1. Methods used for the analysis and determination of the physical characteristics of the water.

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) (Lang *et al.* 2016). The above mentioned parameters are checked for at least three water samples.

5.3.2. Methods used for the analysis and determination of the chemical characteristics of the water.

A total of 25 elements were analyzed in all the hot springs with the help of (Inductively Coupled Mass Spectroscopy) ICPMS. Besides the elemental exploration, various other aspects such as total hardness of water, total alkalinity, phenolic compounds, biochemical oxygen demand (BOD) and chemical oxygen demand (COD) were considered and analyzed. The measurement of temperature, pH, color, turbidity, electrical conductivity, and total dissolved solids (TDS) was also done by using ICPMS (Inductively Coupled Plasma Mass Spectroscopy Model: PerkinElmer Model Nex-ION 300X ICPMS System, USA) as per the reported protocol (Ewa and Bosnak 2012).

5.3.3. Statistical analysis

Piper analysis was done to check the chemical characteristics of hot spring according to the relative concentration of its constituents (Piper M, 1944). A Piper plot is a method for imagining the science of a stone, soil, or water test. In view of different components these charts are valuable in bringing out concoction connections among groundwater tests in more positive terms (Teng *et al.* 2016). The piper diagram was ploted using AqQA v1.x. (Rockware) software. The matrix diagram was formed to compare the elemental concentrations in four hot springs using PAST software.

5.4. Culture-Dependent Analysis

5.4.1. Isolation of Bacterial Strains

For culture dependent microbial diversity studies, the samples were enriched immediately after the collection. The enrichment was done in conical flasks using Nutrient broth and were then incubated at 60± 2 ºC for 72 hours. 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 an **Appendix.1**. The isolation was done by the standard spread and streak plate methods (Arya, Joshi and Gupta 2015; Mohammad *et al.* 2017). The culture plates were incubated at 60 ± 2 ºC for 24 - 72 hours. As we were interested in isolation of true thermophilic bacteria and the literature review suggests the optimum temperature of 60ºC for true thermophiles. Thus we also incubated the isolates at 60± 2 ºC. 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 ºC till further use (Brumm, Land and Mead 2016).

5.4.2. Characterization of the bacterial samples from the Hot Spring water.

5.4.2.1. Culture characteristics and morphology.

a. General Morphology of the microorganisms as observed under the compound microscope.

Shape: Spheres / Short rods / Long rods / Filaments / Commas / Spirals

Arrangement: Single / Pair/ Chains / Clusters

Gram Stain: Positive or Negative

b. Cultural Characteristics of the colonies on Agar plate.

Colony color: Golden; Yellow; White; Glistening; Other pigmentation.

Form: Circular; Irregular; Rhizoid

Margins: Entire; Lobate; Undulate; Serrate; Filamentous

Elevation: Flat; Raised; Convex; Umbonate

Density: Opaque; Translucent; Transparent

c. Staining

Gram-staining was carried out using a Gram stains- kit (Himedia) K001-1KT, Mumbai India, for each of the isolates as per the guide lines given by the manufacturer. Crystal Violet (primary stain) was flooded to the heat fixed, air dried smear and was kept for 30s.

Then it was washed with distilled water and Gram's Iodine solution was flooded to the stained slide and kept for 60 s after which the iodine was washed off with 95% Ethanol followed by distilled water. Later on, counter stain safranin (0.5%) was added to the smear and kept for 30 s and was washed off by distilled water and the slide was air dried. The slides were then observed under oil emersion lens 100X light microscope and the observations were noted down.

d. Endospore Staining

Endospore Staining was carried out for each of the isolates using Schaeffer and Fulton's spore stain- kit. Bacterial smears were made in clean grease free slides. It was air dried and then heat fixed. Slides were then placed over hot water bath for 10 minutes (with the bacterial film on upper side). When large droplets condense on the lower side of the slide, the slide was flooded with Schaeffer & Fluton spore stain A and then steamed for 3-6 minutes. Then rinsed under running tap water and then counter stained with Schaeffer & Fluton spore stain B for 30 seconds. The slides were then washed with water. After drying were observed under oil emersion lens 100X light microscope.

5.5. Growth Profile at various physical parameters

5.5.1. Tolerance to various degrees of Temperature.

A 200µl of freshly prepared cultures were inoculated in the 10ml of Thermus Broth and were incubated at 30°C, 40°C, 50°C, 60°C and 80°C at a pH of 7±2 and NaCl concentration of 0.2% in an Environmental Chamber. Thermus broth was taken as standard for this experiment. However, many isolates were able to grown in only specific mediums (as indicated in appendix 1) and they were grown respectively in their mediums. They were

kept for 48 hours incubation. After incubation the Optical Density (O.D.) of the broth cultures were measured at 600 nm in spectrophotometer (Perkin Elmer, USA). Hence the optimum temperature of the isolated bacteria were seen. A media tube without culture inoculation was kept as blank and was also taken as a negative control (Elnasser *et al.* 2006).

5.5.2. Tolerance to various pH.

The isolates were similarly inoculated as per their different specific broths. The broths were prepared having various pH range, i.e., from 4-10. The pH was adjusted by adding 0.1 N HCl for making the condition acidic and 0.1 N NaOH to make the condition alkaline. The cultures were inoculated in given ranges of pH at a constant temperature of 60°C in an incubator (as most of the isolates shown maximum growth at 60°C). They were kept for 48 hours of incubation. After incubation the Optical Density (O.D.) of the broth cultures were measured at 600 nm. The readings were noted down against the control which was prepared by taking the uninoculated media broth. Thus, the pH tolerance capability and the optimum pH of the bacteria was interpreted (Elnasser et al, 2006).

5.5.3. Tolerance to various concentrations of NaCl.

The isolates were inoculated in different specific broths respectively and were incubated at various concentrations of NaCl. The cultures were inoculated at various NaCl concentrations of 0.5-5% at a constant temperature of 60°C in an incubator (as most of the isolates shown maximum growth at 60°C). They were kept for 48 hours of incubation. After incubation the Optical Density (O.D.) of the broth cultures were measured at 600 nm in a spectrophotometer (Perkin Elmer, USA). The tolerance capability and optimum NaCl

concentrations of NaCl were interpreted against the control which was prepared by taking the uninoculated media broth (Elnasser *et al.* 2006).

5.6. Biochemical characterization of the bacterial samples.

5.6.1. Carbohydrate Fermentation Test.

Fermentation involves the anaerobic dissimilation and production of an organic acids such as lactic, formic or acetic acid by utilizing substrates usually sugars and that may be accompanied by gases such as hydrogen or carbon dioxide. Fermentative degradation of various carbohydrates by microbes under anaerobic conditions is carried out in a fermentation tube. A fermentation tube is a culture tube that contains a Durham tube (a little tube set in an altered position in the way of culture tube) for the recognition of gas creation as a final result of metabolism. The fermentation broth contains the ingredients of nutrient broth, a specific carbohydrate and a pH indicator (Phenol red), which is red at neutral pH (7.0) and turns yellow at or below a pH of 6.8 due to the production of an organic acid. The 200µl of all the individual bacterial cultures were inoculated separately in 10ml of carbohydrate fermentation broth and was kept at 60°C for 48 hours. After incubation, the broths were observed for any growth and color change from red to yellow for positive reaction and gas production (Adetunji, Ajani and Umanah 2012) .The color change from red to yellow and the production of gas indicated the positive result that means bacteria is able to ferment the considered sugar.

5.6.2. Catalase Activity.

During aerobic respiration in the presence of oxygen, microbes produce hydrogen peroxide (H_2O_2) which is lethal to the cell. The enzyme catalase breaks down hydrogen peroxide to

water and oxygen. Release of free oxygen gas in the form of bubbles is a positive catalase test. The freshly grown over night cultural isolates were used. The clean slides were taken and few drops of 3% H_2O_2 was flooded on the slide and after then a drop of culture was also added. After adding the culture the catalase positive bacteria produced bubbles which were seen through naked eye. The observations were then noted down (Adetunji, Ajani and Umanah 2012).

5.6.3. Protease Activity.

The ability of microbes to degrade protein refers to their proteolytic activity. The bacterial isolates were streaked on protease agar and were incubated at 60°C for 48 hours and the observations were noted down. Formation of colonies and halo zones around the colonies indicated that the microbes possess protease activity (Shuai *et al.* 2012).

5.6.4. Amylase Activity.

Starch is a complex sugar (polysaccharide) made out of two constituents – amylose, a straight chain polymer of 200-300 glucose units and amylopectin, a bigger spread polymer with phosphate gatherings. Starch consists of eight or more monosaccharide units and disaccharide units. These monosaccharides and disaccharides, enter into the cytoplasm of the bacterial cell through the semi permeable membrane and thereby used by the endoenzymes. Amylase is an exoenzyme that hydrolysis or cleaves starch into its composite units of monosaccharide and disaccharide sugars. The ability to degrade starch is used as a criterion for the determination of amylase production by the microbes. Iodine solution is used as an indicator. Starch within the sight of iodine solution delivers a strong blue shade of the medium and the development of a yellow radiance zone around the

settlement demonstrates the amylolytic movement of the organisms. All the individual isolates were streak plated on Starch Agar Media and was kept at 60°C for 48 hours. After incubation, the 2% Iodine solution was flooded to the plates and were observed for halo zone formation (Kumar *et al.* 2014).

5.6.5. Gelatin Hydrolysis Test.

Gelatin is a protein produced by the hydrolysis of collagen, a major component of connective tissue and tendons in humans and other animals. It dissolves in warm water (45-50°C) and exists as a liquid above 25°C and solidifies when cooled below 25°C. Hydrolysis of gelatin is brought about by microbes capable of producing a proteolytic exoenzyme known as gelatinase, which acts to hydrolyze this protein into amino acids. Hydrolysis of gelatin is observed by flooding the Gelatin Agar medium with saturated ammonium sulfate to precipitate unhydrolyzed gelatin, making the clear zones easier to see. Results are often observed within 5 to 10 minutes after flooding with saturated ammonium sulfate and observing the plates for clear halo zones along the line of growth. All the individual isolates were streak plated in Gelatin Agar Media and were kept at 60°C for 48 hours. After incubation, the saturated ammonium sulfate was flooded to the plates and was observed for any zone formation (Clarke 1953; Mohammad *et al.* 2017)

5.6.6. Urease Activity.

Urea is a noteworthy natural waste result of protein absorption in many vertebrates and is discharged in the urine. A few microorganisms can create the enzyme urease. The urease is a hydrolytic chemical which act on the carbon and nitrogen bonds in amide mixes to free a basic item, for example, ammonia. It is a helpful demonstrative test for recognizing microscopic organisms, particularly to recognize individuals from the variety *Proteus* from

the Gram-negative pathogens. *Proteus vulgaris* is a vital and quick maker of urease. Urease test is performed on Urea Agar medium containing the pH pointer Phenol red (pH 6.8).Due to the utilization of urea, ammonia an alkaline product will be produced, which will increase the pH of the medium and creates an alkaline environment. And in this alkaline environment the indicator Phenol red changes from yellow color to a red or deep pink color. Failure of the development of a characteristic deep pink color infers that microbe lacks urease activity. All the individual isolates were streak plated in Urea Agar Media and was kept at 60°C for 48 hours. After incubation, the plates were observed for any color change (Aparna, Sujoy and Kamal 2015).

5.6.7. Oxidase Test

The oxidase test is utilized to recognize microscopic organisms that deliver cytochrome c oxidase, a protein of the bacterial electron transport chain. The oxidase test helps in separation among individuals from the genera Neisseria and Pseudomonas, which are oxidase-positive and Enterobacteriaceae, which are oxidase negative. Whenever introduce, the cytochrome c oxidase oxidizes the reagent (tetra methyl-p-phenylenediamine) to purple shading (indophenols) finished result. At the point when the catalyst is absent, the reagent stays colorless. The oxidase tests were performed utilizing readymade oxidase discs (Bacteriological separation discs for oxidase testing) (HiMedia) according to maker rules. Oxidase response was done by contacting and spreading an all-around detached province on the oxidase disc. The reaction is observed within 5-10 seconds at 25-30°C. If the color of the disc changes to purple that indicates the positive result. A change later than 10 seconds or no change at all is considered negative reaction.

5.6.8. Nitrate Reductase Test

Nitrate reduction is a process in which nitrate (NO_3^-) is being reduced to nitrite (NO_2^-). The nitrate reduction test is based on the detection of nitrite. The nitrate has the ability to form a complex with sulfanilic acid (Reagent A). The complex formed (nitrite-sulfanilic acid) which then reacts with α-naphthylamine (Reagent B) to give a red precipitate. This reaction is catalyzed by zinc powder.

Fresh cultures were grown in Trypticase nitrate broth for 24 hours at 60 ºC. After incubation 5 drops of Reagent A and 5 drops of Reagent B to all nitrate broth cultures were added. The development of red color represents the positive result. Further a minute quantity of zinc was added to cultures in which no red color developed. At this step if the color changes to red after adding zinc this verifies that nitrates were not reduced to nitrites by the organism thus giving the negative result, and if addition of zinc produces no color change this verifies that the nitrates in the medium were reduced beyond nitrites to ammonia or nitrogen gas, thus giving a positive result.

5.6.9. Methyl Red Test

Methyl red test is the test performed to demonstrate whether the microorganisms is capable to perform mixed acid fermentation when provided with glucose. The substantial amount of acids created causes a noteworthy reduction in the pH of the way of culture medium. The culture medium turns red after adding the methyl red indicator (p-dimethylaminoaeobenzene-O-carboxylic acid) due to the formation of acids that is decrease in pH and thus giving the positive result. No change in color from yellow to red

in the culture medium, which may occur due to less acid production (higher pH), indicates negative result (Mcdevitt 2016).

Glucose phosphate broth inoculated with different bacterial isolates were prepared. The test tubes were incubated for 24 hours at 60°C. After incubation, 5 drops of methyl red indicator was added to 5 ml of each cultured broth. Acid production was indicated by formation of red coloration (Irdawati *et al.* 2018).

5.7. Identification of bacterial isolates by Culture dependent Techniques

5.7.1. DNA extraction.

For culture-dependent techniques, the DNA was extracted using Qiagen QIAamp DNA Mini Kit (Qiagen, USA). The extracted DNA was stored at -80ºC for further analysis. The step wise procedure of extraction of genomic DNA was followed as per manufacturer guidelines.

1.1 ml of bacterial culture was added into a 1.5 ml microcentrifuge tube, and centrifuged for 5 min at 7500 rpm.

2. Then ATL buffer (supplied in the QIAamp DNA Mini Kit) was added to a total volume of 180µl to a total volume of 180 µl. For Gram-positive bacteria, Suspend bacterial pellet in 180 µl of the appropriate enzyme solution (20 mg ml^{-1} lysozyme or 200 µg ml^{-1} lysostaphin; 20 mM Tris·HCl, pH 8.0; 2 mM EDTA; 1.2% Triton).

3. After that incubate for at least 30 min at 37°C was done and 20 µl Proteinase K and 200 µl Buffer AL was added and mixed by vortex.

4. Again Incubate at 56°C for 30 min and then for a further 15 min at 95°C was done. Note: Extended incubation at 95°C can lead to some DNA degradation then Centrifuge for a few seconds.

5. After that 200µl ethanol (96–100%) to the sample was added, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

6. Then carefully the mixture from step 5 (including the precipitate) was transferred to the QIAamp spin column (in a 2 ml collection tube) without wetting the rim and centrifuged at (8000 rpm) for 1 min and then spin column was placed in a clean 2 ml collection tube (provided), and the tube containing the filtrate was discarded.

7. After then 500 µl Buffer AW1 was added without wetting the rim and centrifuged at (8000 rpm) for 1 min. Then column was placed in a clean 2 ml collection tube (provided), and the collection tube containing the filtrate was discarded.

8. After that carefully 500µl Buffer AW2 was added without wetting the rim and centrifuged at full speed (14,000 rpm) for 3 min.

9. Then column was placed in a new 2 ml collection tube (not provided) and the collection tube containing the filtrate was discarded. Centrifuge at full speed for 1 min.

10. At last the column was placed in a clean 1.5 ml micro-centrifuge tube (not provided), and discard the collection tube containing the filtrate. And carefully 30µl Buffer AE or distilled water was added. Incubation at room temperature for 1 min was done and then centrifuge at 6000 x g (8000 rpm) for 1 min and step 10 was repeated again.

5.7.2. 16S rRNA Sequencing.

The 16S rRNA gene amplifications were done by using two universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') (Devereux and Wilkinson 2004) and 1406R(GACGGGCGGTGTGTRCA) (Baker and Cowan 2004). The polymerase chain reaction was performed in 25µL volume using 12.5µL GoTaq Green Master Mix 2X (Promega), 10-20pM of forward and reverse primers and 2µL of the template. The PCR cycle was designed as, 5 min at 94°C; 35 cycles of 1min at 95°C, 1min at 55°C, and 2 min at 72°C; and a final extension step of 10 min at 72°C. The PCR products were analyzed by electrophoresis at 100V for 1hr in a 0.8% Agarose gel (Sigma Aldrich, USA).

5.7.3. DNA purification.

The PCR product was purified using a QIAquick PCR purification kit (Qiagen, USA) for cycle sequencing as per the manufacturer guidelines. All centrifugation steps were carried out at 13,000 rpm in a table top micro centrifuge at room temperature. Before starting 96-100% ethanol was added to buffer PE before use (given in the kit):

a. 5 volumes of buffer PB to 1 volume of the PCR reaction and then were mixed (If the color of the mixture became orange or violet, then 10µl 3M sodium acetate was added at pH of 5, and mixed so that the color changes to yellow.
b. QIAquick column was placed in a 2 ml collection tube provided and then sample was applied to the column and centrifuged for 30-60s.
c. After that washing was done by adding 750 µl buffer PE to the QIAquick column and then centrifuged for 30-60s. The flow through was discarded and the column

was placed in the same tube. Then the column was again centrifuged for 1 minute to remove residual wash buffer.

d. Before eluting QIAquick column was placed in clean 1.5ml micro centrifuge tube. DNA was then eluted by adding 30 µl buffer EB or Milli Q water and then centrifuged the column for 1 minute.

5.7.4. DNA sequencing.

The purified 16S rDNA was then sequenced using a BigDye^{TM}Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) as manufacturer's instructions using Automated DNA Sequencer (ABS/Genetic 3500 Analyzer). After 16S rRNA PCR amplifications, the sequencing of good DNA products were performed. Prior to sequencing the next round of PCR amplifications were performed using only one primer at a time with the reaction mixture. That means two reaction mixture PCR tubes one containing 27F primer and other tube containing 1492R primer. The total reaction mixture was of 10µl containing 1µl Master Mix RR100, 1.5µl Terminate Buffer (provided in the kit), 2µl primer, 1µl PCR purified DNA and 4.5µl Milli Q. The reaction conditions were as

The second purification of the PCR product was also done by manufacturer guidelines as:

a. 10 µl of MilliQ water was added to the PCR tube containing the product and then mixed well and transferred to 1.5ml eppendorf tube.

b. After that 2 µl of 125mM EDTA, 2 µl 3M Sodium acetate, and 50 µl 100% ethanol was added and incubated for 15 minutes at room temperature. After incubation the eppendorf tubes were kept at 4 ºC for 30 minutes.

c. Then centrifugation was done for 15 minutes at 14000 rpm and the flow through was decanted.

d. After that 70% ethanol was added and centrifuged for 10 minutes at 14,000 rpm.

e. After then the supernatant was removed and dried and 14 µl HiDi Formamide (provided in the kit) was added.

f. The tubes were then heated at 55 ºC for five minutes then immediately transferred to ice for 5 minutes.

g. After that 12-14 µl of that reaction mixture was added to sequencer 96 well plate and the sequencing was performed.

5.7.5. Statistical analysis.

The sequence obtained was assembled with Codon Code Aligner (ver. 5.2). Assembled sequences were compared with nr/nt database of NCBI using BLAST sequence homology search for taxonomic identification. A phylogenetic tree was constructed to identify the evolutionary lineage of the isolates. The tree was constructed using neighbor-joining method (Saitou and Nei 1987) with jukes-cantor evolutionary distance measurement (Erickson, 2010) in MEGA 7.0 software.

5.8. Fatty acid methyl-ester Analysis.

Fatty acid Methyl Ester (FAME) analysis was done as per the standard protocol (Buyer and Sasser 2012) and were analyzed using Sherlock-MIDI identification system. The calibrated standards were used by the microbial identification system (MIDI) for annotation of generated phospholipid peaks. The cultured bacteria is the prerequisite for Sherlock System. The FAME involves many steps such as the extraction of fatty acids by saponification in dilute sodium hydroxide/methanol solution followed by methylation with

dilute hydrochloric acid/methanol solution to give the respective methyl esters (FAMEs). The FAMEs are then separated from the water phase by the utilization of an organic solvent and the subsequent concentrate is analyzed by GC. FAMEs are more appropriate to GC analysis because they are more volatile than their respective fatty acids. The Sherlock programming at that point utilizes a particular strategy recognition algorithm and consequently coordinates the obscure FAME profile to the put away sections for Identification. The results in Sherlock system is represented by a numerical value called as Similarity Index (SI). The SI index is actually a comparison of fatty acid esters of an unknown microorganism with the mean fatty acid composition of other closely related strains used to create a library entry listed as its match (Kunitsky, Osterhout and Sasser; Sharmili and Ramasamy 2016)

5.9. Whole genome sequencing and analysis.

The whole genome sequencing was carried out in two isolates AYN2 and LYN3 isolated from Yumthang hot spring. The whole genome sequencing was performed by using Illumina Hiseq 4000 sequencing technology with a paired end sequencing module. For high quality data and genome assembly, the data was filtered by employing Next Generation Sequencing Quality Control (NGSQC) Toolkit and SQIT (Patel and Jain 2012). The primary genome assembly was carried out by Velvet (V 1.2.10) (Zerbino and Birney 2008). The genome was assembled into scaffolds using SSPACE v3.0 scaffolder (Boetzer *et al.* 2011). Bowtie2 (v 2.2.2) (Langmead and Salzberg 2012) was used for *de novo* genome validation and quality control. The genomic elements of assembled genome was done by using ARAGORN v1.2.36 (Laslett and Canback 2004) and RNAmmer 1.2 Server (Lagesen *et al.* 2007). Non-core genomic elements were also screened and using Plasmid

Finder(V 1.3) (Carattoli *et al.* 2014). The draft genome was annotated and its functional characterization was done. The annotation tool used was Rapid Annotations using Subsystems Technology RAST (V 2.0) (Aziz *et al.* 2008). The taxonomy identification method was performed using EzTaxon (Kim *et al.* 2012) and MEGA6.

5.10. Identification of Novel thermophilic bacteria

A novel bacterium was isolated and identified. Polyphasic characterization was done and according to guidelines given by "The International Committee on Systematic Bacteriology (ICSB)" (Logan *et al.* 2009), various phenotypic and genotypic experiments has been done. Those experiments are discussed below:

5.10.1. Morphological characterization

Morphological characteristics such as colony morphology such as colony color, form, margins elevation and density were checked. The general morphology including shape, arrangement and motility was also checked. Staining was done as given above.

5.10.2. Scanning Electron Microscope (SEM)

The Scanning Electron Microscope (SEM) analysis i.e., FESEM was performed to measure the size and to check the shape of the novel isolate as per (Hagen *et al.* 1968; Golding *et al.* 2016) using JCM-5700 Scanning Electron Microscope (JEOL USA, Peabody, MA, USA).

5.10.3. Growth Profile at various physical parameters

The growth at various physical parameters such as temperature (ranging from 40-70°C), pH (ranging from 6-10) and NaCl concentration (ranging from 0-<4) was checked as per the above given protocols.

5.10.4. Biochemical characterization (using manual experimentation)

Various biochemical parameters were checked such as carbohydrate fermentation, enzymatic tests such as amylase, protease, catalase, oxidase, nitrate reductase, methyl red test etc. as per the procedures given above.

5.10.5. Biochemical characterization using BIOLOG

All of the physical and biochemical tests were also performed by BIOLOG system as per the guidelines given by manufacturer (Miller 1991)

5.10.6. Fatty Acid Methyl Ester (FAME) analysis

The fatty acid analysis of the novel strain was performed at 50°C. Fatty acids were extracted and analyzed following the instructions of the Microbial Identification System (MIDI)(Buyer and Sasser 2012) The RTSBA6 method was used and the results were analyzed by Sherlock Version 6.2.

5.10.7. Identification by 16S rDNA and *rpoB*, *dnak, dnaJ* gene sequence analysis.

The 16S rDNA sequence was amplified by using two universal primers (27F and 1492 R) as described by Hugenholtz P, *et al*, 1998 (Hugenholtz *et al.* 1998). The 16S rDNA PCR product was purified by using QIAquick PCR purification kit (Qiagen). The purified 16S rDNA was further sequenced by using a BigDye^{TM} Terminator v3.1 cycle sequencing kit

(Applied Biosystems) in automated DNA Sequencer (ABS/Genetic 3500 Analyzer). Also, three more conserved genes, i.e., *rpoB, DnaK* and *dnaJ* genes DNA sequence was also checked using primers (*rpoB*1698 F, 5'-AACATCGGTTTGATCAAC-3'; corresponding to *E.coli position* 1643 and *rpoB*2041 R, 5'-CGTTGCATGTTGGTACCCAT-3'; corresponding to *E. coli* position 2041) (Dahllöf *et al.* 2000); (*dnaK* F, 5'-CTCCGTGGACCTTCTCTTGG -3' and *dnaK* R, 5'-ATGATCTGCTTGTGGGCCTC-3') (Cusick *et al.* 2015); *dnaJ* F, 5' CAGATCGAGGTSACCTTCGAC-3' and *dnaJ* R, 5' CGTCRYCATMGAGATCGGCAC-3') (Diouf *et al.* 2015)

5.11. Identification of bacterial diversity by Culture Independent Techniques

5.11.1. Phospholipid Fatty Acid Analysis (PLFA)

Phospholipids are crucial constituents of microbial membranes (Powl, East and Lee 2007) and they vary between different species among prokaryotes (Joergensen and Wichern 2008). The PLFA results are based on live microbiota present in the sample of any ecosystem because after cell death the phospholipids are unstable and decompose rapidly (Lanekoff and Karlsson 2010). PLFA analysis thus provides significant information on the microbial community structure, the abundance of viable microbial groups, and their physiological status (Nielsen and Petersen 2000). The method of PLFA is based on four key steps: 1. Extraction of lipids from samples with a single-phase chloroform mixture, 2. Fractionation using solid phase extraction columns to isolate phospholipids from other extracted lipids, 3. Production of fatty acid methyl esters (FAMEs) by methanolysis, and 4. FAME analysis by capillary gas chromatography (GC analysis). Two measures are utilized, including 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine (PC(19:0/19:0)) to

evaluate the general recuperation of the extraction technique, and methyl decanoate (MeC10:0) as an inward standard (ISTD) for the GC examination (Quideau et al., 2016). For PLFA analysis, the phospholipids were extracted according to the standard protocol (Quideau *et al.* 2016; Fan, Zhang and Morrill 2017) and were analyzed using Sherlock-MIDI identification system. The calibrated standards were used by the microbial identification system (MIDI) for annotation of generated phospholipid peaks. The Equivalent carbon length (ECL) values in comparison to the expected ECL value in the PLFA peak are mentioned in Appendix.2. The minimum limit of detection (LOD) for the MIDI-PLFA method per 2uL injection, is 1ng of fatty acid.

5.11.2. Next Generation Sequencing

5.11.2.1. Metagenomic DNA extraction

Environmental DNA was extracted using DNeasy PowerWater Kit (MO BIO Laboratories, Carlsbad, CA, USA) in accordance with the manufacturer's instruction. Quality of the DNA was checked on 0.8% agarose gel and DNA was quantified using Qubit Fluorometer (Thermofisher Scientific, USA), with a detection limit of 10 -100 ng μl^{-1}.

5.11.2.2. 16S Metagenomic Sequencing Library Preparation and Sequencing

Amplifications of the V3 and V4 regions of bacterial 16S rRNA gene were done using two primers (16SV3F=5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC WGCAG;16SV3R=5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTA CHVGGGTATCTAATCC3') (Klindworth *et al.* 2013). The amplicon libraries were prepared using Nextera XT Index Kit (Illumina inc.), accordance with 16S metagenomic sequencing library preparation protocol (Illumina, n.d.). The amplicon library was purified

with AMPure XP beads. The amplified library was checked by Bioanalyzer 2100 (Agilent Technologies) using High Sensitivity (HS) DNA chips and concentration was quantified by Qubit fluorometer. Based on the data obtained from the Qubit fluorometer and the bioanalyzer, 500ul of the 10pM library was loaded into MiSeq cartridge for cluster generation and sequencing. Paired-end sequencing method was used. After the sequencing, high-quality metagenome reads were trimmed to remove the barcode and adaptor sequences.

5.11.2.3. Metagenomic Data Analysis of Polok and Borong Hot Spring

The adapter trimmed sequences were subjected to pre-processing for De-replication, Singleton removal, OTU Clustering, Chimera filtering with SolexaQA. Sequences with Phred score lower than 20 and ambiguous bases having primer mismatch and low read length less than 100bp were removed. Annotation and normalization of operational taxonomic unit (OTU) was done using UPARSE OTU clustering and QIIME at 97% similarity (Edgar 2013). For normalization, inbuilt script as well as METAGEN assist was used. The resulting representative OTU was aligned and given taxonomic classing using Greengenes database (http://greengenes.lbl.gov/). The output of this workflow is a classification of reads at several taxonomic levels: kingdom, phylum, class, order, family, genus, and species. Sequences without homologous pair were classified as unknown.

5.11.2.4. Diversity and Functional Metagenomic Data Analysis of Reshi and Yumthang Hot Springs.

The sequence data was generated using Illumina HiSeq. Data quality was checked using FastQC and MultiQC (Ewels *et al.* 2016) software. The data was checked for base call quality distribution, % bases above Q20, Q30, %GC, and sequencing adapter

contamination. The microbial abundance was estimated using Metaphlan2 (Truong *et al.* 2015), a tool that profiles and classifies the sequencing data from shotgun metagenome samples, with species-level resolution using a set of 1 million clade-specific marker genes from more than 17,000 different microbes (~13,500 bacteria and archaea, ~3,500 viri, and ~110 eukaryotes). To generate the metagenome assembly, metaSPAdes (Nurk *et al.* 2017) and IDBA_UD (Peng *et al.* 2012) were used with multiple kmer values set from 41 to 120 with increments of 20. Later these assemblies were scaffolded to get the best contigs from all the assemblies. The best assembly was identified by considering the parameters N50, a number of contigs, length distribution of contigs, size of assembly and % reads mapped back onto the assembly. The assembly statistics were calculated using Quast (Gurevich *et al.* 2013). The assembled contigs were annotated using blastn (Altschul *et al.* 1990) against the NCBI nt custom metagenome database that included all nt entries for organisms that belong to the Taxonomy Archaea, Bacteria, Fungi and Viruses; with minimum sequence similarity of 60% and e-value lesser than 1e-03. The gene prediction and annotation of the assembled contigs were carried out using PROKKA (Seemann 2014). The predicted genes functional classification based on KEGG orthology was done using FMAP (Kim *et al.* 2016) and COG (Cluster of Orthologous Groups) classification by blastx against the COG (Tatusov 2000) database.

5.11.2.5. Metagenomic studies of antibiotic resistance genes (ARGs) and metal resistance genes (MRGs)

Putative Antibacterial Resistance genes from the Prokka predicted genes were identified with the ardbAnno V.1.0 (Liu and Pop 2009) script available from the ARDB (Antibiotic Resistance Genes Database) consortium using the non-redundant Resistance Genes as

reference and Putative Metal resistance genes were identified by using BacMetScan V.1.0 (Pal *et al.* 2014) script available from the BacMet AntiBacterial Biocide & Metal resistance gene database. The manually curated database of genes with experimentally confirmed resistance function was used in BacMet-Scan as a reference. Also, in case of both Antibiotic and Metal resistance, the predicted resistance gene classification was also done based on COG (Cluster of Orthologous Groups) classification by blastx against the COG (Tatusov 2000) database.

5.11.2.6. Data Availability and Accession Number

Raw metagenomics reads were submitted to Sequence Read Archive (SRA), NCBI under accession numbers SAMN08038921, SRA: SRS2697425 for Polok Hot Spring with sample name as POLV4 and SAMN08038632, SRA: SRS2697438 for Borong Hot Spring with sample name as BORV4. The BioSample records will be accessible with the following links for Polok and Borong Hot Springs respectively http://www.ncbi.nlm.nih.gov/biosample/8038921 and http://www.ncbi.nlm.nih.gov/biosample/8038632. The Bio- sample and Sequence Read Archive (SRA) accession numbers are given as SAMN08940114, SRP140681, and SRS3178356 for Reshi Hot Spring with sample name as RESMETAV4 and SAMN08940367, SRP140682, SRS3178357 for Yumthang Hot Spring with sample name as YUMMETAV4. The SRA records will be accessible with the following links for Reshi and Yumthang Hot Springs respectively https://www.ncbi.nlm.nih.gov/sra/SRP140681 and https://trace.ncbi.nlm.nih.gov/ Traces/sra/?run=SRR7015754.

5.11.2.7. Statistical analysis

The principal component analysis (PCA) was used to correlate between bacterial diversity and physicochemical parameters of the samples using PAST. The Analysis of variance was used to check the significance of various data with the help of Graph Pad Prism and XLSTAT. The Shannon diversity indices, chao1 were calculated with the help of EstimateS and PAST software (Chao *et al.* 2006). The heat map was used to analyze the comparative bacterial diversity among each of the two hot springs and between previously reported microbial diversity of different hot springs from Central India, North- east India, and Tibet with the help of R software using Bray Curtis Dissimilarity matrix (package: ggplot, function: heatmap.2).

5.12. Antibiotic Sensitivity of Isolates.

The isolates were tested for their response to various antibiotics by Disc Diffusion Method. Hence, a series of Antibiotic Sensitivity was carried out for the isolates. Kirby Bauer Antibiotic Sensitivity method was followed. 0.1 ml of isolate (as per McFarland 0.5 turbidity standard. 0.5 turbidity is equivalent to 1.5×10^{8} cfu ml^{-1}) was streaked by sterile cotton swabs in different respectively labelled Mueller Hinton Agar (MHA) plates. Then after 15 minutes of standing, one sterile antibiotic disc was placed at the center of the MHA plate. For a single isolate, its Antibiotic Sensitivity was measured against 10 various antibiotics. Thus, all the isolates were tested against the following antibiotics; ampicillin (10mcg), penicillin (10U), methicillin (10mcg), amoxicillin (10mcg), erythromycin (15mcg), chloramphenicol (30mcg), gentamycin (10mcg), clindamycin (2mcg), norfloxacin (10mcg), and ciprofloxacin (10mcg). All the Antibiotics were tested against various isolates isolated from four hot springs. The two known thermophilic bacteria

Geobacillus stearothermophilus (MTCC37) and *Geobacillus thermoleovorans* (MTCC4219) were taken as positive control whereas, negative Control was set up by placing sterile 0.4µm membrane filter paper discs immersed in sterile autoclaved water on the swabbed plate. The plates were incubated at 60°C for 48 hours. After incubation, the plates were observed for their zone of inhibition and their diameter was measured and recorded. By comparing the diameter size of the zone of inhibition, with the standard chart provided for the Assay, the isolates were interpreted as Resistant (R) or Intermediate (I) or Susceptible (S) to the respective antibiotics (Kristjansson 1994; Bjornsdottir *et al.* 2009).

5.12.1. Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentrations of various antibiotics used were carried out by standard methods (Mazzola *et al.* 2009). The MIC is defined as the lowest concentration of antibiotic at which there is no visible growth. The MIC of penicillin, vancomycin, erythromycin, chloramphenicol and methicillin was checked. The antibiotic stock solution was prepared as per the formula including potency (already given on antibiotic powder pack) given below:

$$\mathbf{W=1000/P\times V\times C}$$

Where W=weight of antibiotic to be dissolved in V, V=volume (ml) required, P= potency (already given on antibiotic pack) and C= final concentration of solution in multiples of 1000. The stock solutions were freshly prepared and kept at 4°C for later use. After that various dilutions from stock antibiotic solution were made such as 0.25, 0.5, 1, 2, 4, 8, 16 mgl^{-1} using formula:

$$C1 \times V1 = C2 \times V2$$

Where C1= concentration of antibiotic stock solution, V1=volume we have to calculate, C2= concentration required and V2= final volume of media to be poured in petri plate. For example, if we have the concentration of antibiotic stock solution as 1000 mg l^{-1}, and if we have to make the dilution of 8 mg l^{-1} for 30ml of final volume used to pour in petri plate. Then we have to use 240µl of volume (V1) from antibiotic sock solution.

The media was autoclaved separately and after autoclave the media were kept for some time in laminar air flow to cool down and after cooling the specific antibiotic (dilution) was added to the media and were shacked gently so that antibiotic solution will mix well with the media. The media was then poured into the petri plates and kept in laminar airflow to solidify. After solidification, 1-2 µl of inoculum was spread plated to the agar plates containing specific antibiotic dilutions. The inoculum was prepared as per McFarland 0.5 turbidity standard. 0.5 turbidity is equivalent to 1.5×10^{8} cfu ml^{-1}. McFarland reagent consists of 1% barium chloride and 1% sulfuric acid which together form a precipitate of barium sulfate ($BaSO_4$). For 0.5 turbidity, 0.05ml of 1%$BaCl_2$ + 9.95ml of 1% H_2SO_4 are mixed.

5.12.2. Detection of various antibiotic resistance causing genes.

The detection of resistance causing genes were carried out for various antibiotics such as ampicillin, penicillin, and methicillin. Various genes were targeted in each antibiotic. In case of penicillin the *PBP1A, PBP2A* were targeted. PBPs are the enzymes that catalyze polymerization and cross-linking of peptidoglycan precursors in the bacterial cell wall biosynthesis. Alterations of the PBPs reduce the affinities of β-lactam antibiotics, resulting

in resistance. Among six PBPS it has been shown in *Streptococcus pneumoniae*, PBPs 1A, 2B and 2X are the major proteins involved in resistance. The primes used for the detection and amplification of these *PBP1A* and *PBP2A* genes were pbp1F-CCAGCAACAGGTGAGAGTC, pbp1R-GTAAACACAAGCCAAGACAC (Sanbongi *et al.* 2004).

In case of ampicillin, *ampC* genes were targeted. *ampC* genes encode enzyme β-lactamases, which confer resistance to aminopenicillins such as ampicillin, cephalosporins, oxyimino-cephalosporins (ceftriaxone, cefotaxime and ceftazidime), cephamycins (cefoxitin and cefotetan) and monobactam (aztreonam). AmpC enzymes may be encoded by either chromosomal or plasmid-mediated genes. The primes used for the detection and amplification of *ampC* genes were *ampC* F-TGAGTTAGGTTCGGTCAGCA, *ampC* R-AGTATTTTGTTGCGGGATCG (Fernando *et al.* 2016).

Similarly, in case of methicillin *mecA1* and *mecA2* were targeted. Resistance to methicillin is determined by the gene mecA, which encodes a penicillin-binding protein (PBP2a) that has low affinity for beta-lactamic compounds. The primes used for the detection and amplification of *mecA1* and *mecA2* were mecA1- AAA ATC GAT GGT AAA GGT TGG C, mecA2- AGT TCT GCA GTA CCG GAT TTG C (Cuteri, Mezzasoma and Valente 2003).

5.12.3. Isolation of Plasmids.

Isolation of plasmids were done by PURE YIELD PLASMID KIT (promega) system as per the guidelines of manufacturer. *E coli* competent cells possessing plasmid were taken as positive control.

5.13. Heavy metal tolerance to isolates.

5.13.1. Screening and assessment of metal toxicity.

Based on distinct locations the two hot springs, Reshi and Yumthang were selected. Among 84 isolates of Reshi and Yumthang hot springs, a total of 27 isolates were screened and assessed for metal toxicity. The heavy metal solutions were prepared from their chloride and sulfate salts as $CuSO_4$ [Copper (II) Sulfate], $MnSO_4$ [Manganese (II) Sulfate], $ZnCl_2$ [Zinc Chloride], $HgCl_2$ [Mercury (II) Chloride] and $CoCl_2$ [Cobalt (II) Chloride]. Stock solutions were prepared in distilled water, slightly acidified with HNO_3 and were autoclaved. These solutions, in various concentrations according to the metal tested, were kept at 4°C for no longer than one month.

5.13.2. Tube dilution method

Tube dilution method was performed to check the minimal inhibitory concentrations (Hassen *et al.* 1998). Culture medium and metallic solution were autoclaved. 0.5mL of appropriate metals concentrations were dissolved in TR broth with a final volume of 10mL. Three tubes were prepared for each metallic concentration, and then inoculated with 200µL of an18hr old culture (O.D. at 600nm = >1) of the studied bacterial strain. A positive control consisted of TR broth (as it is heavy metal-deficient medium) inoculated with the isolate and a negative control consisted of a metal-supplemented medium without adding the isolate. To constitute the metal supplemented medium, one mL of respective metallic solution was added to 9mL of TR broth. The glassware used for the experiments was leached in 2N HNO_3 and then was rinsed three times with deionized double distilled water (MilliQ) so as to rule out any possibilities of heavy metal contamination. Tubes were read

in *uv/vis* spectrophotometer (Perkin Elmer LAMBDA 40) at 600nm after incubation at 60°C for 24hrs (Kim *et al.* 2007).

5.13.3. Statistical Analysis

Analysis of variance (ANOVA) was performed by using statistical analysis software - Graph Pad Prism. Effective Concentration (EC) values (statistically derived estimate of a concentration of a substance resulting in 50% reduction of the growth within a specified time) were estimated. The data was obtained from three replicate samples. Principal Component analysis (PCA) was done using XLSTAT 2014.03 software.

6. RESULTS

6.1. Description of sampling sites of four hot springs:

The hot springs of Sikkim are located in the Himalayan geothermal belt (HGB). The three hot springs i.e., Reshi, Polok, and Borong are located in south district of Sikkim, whereas the fourth one is located in north district of Sikkim. The Reshi, Polok and Borong hot springs are present on the bank of river Rangit in south Sikkim whereas the Yumthang Hot spring is located in Yumthang valley alongside on river Lachung. These hot springs are believed to be as a sacred places with medicinal properties due to rich sulfur content, which has opened a door to tourism and people from different regions visit these places (Das *et al.* 2012). The geographical location of all the four-hot spring were determined with the help of GPSMAP 78S (Garmin, USA) as per the guidelines are given by the manufacturer. The coordinates of the Polok hot spring is 27°21'00.29" **N** longitude and 88°19'21.99" **E** latitude. The Borong is located at 27°21'93.57" **N** longitude and 88°19'67.01" **E** latitude. The elevation range of the locations are 884m and 998m for Polok and Borong hot springs respectively. These two hot springs are nearer to each other. The coordinates of the sites of Reshi *Tatopani* and its water source lies between 27°14.957'N longitude and 88°18.176'E latitude. The Yumthang *Tatopani* is located at 27°47.575'N longitude and 88°42.516'E latitude. The elevation range of the locations are 515m and 3404m for Reshi and Yumthang hot springs respectively above the sea level. The maps of these hot springs are given in Fig.2a,b.

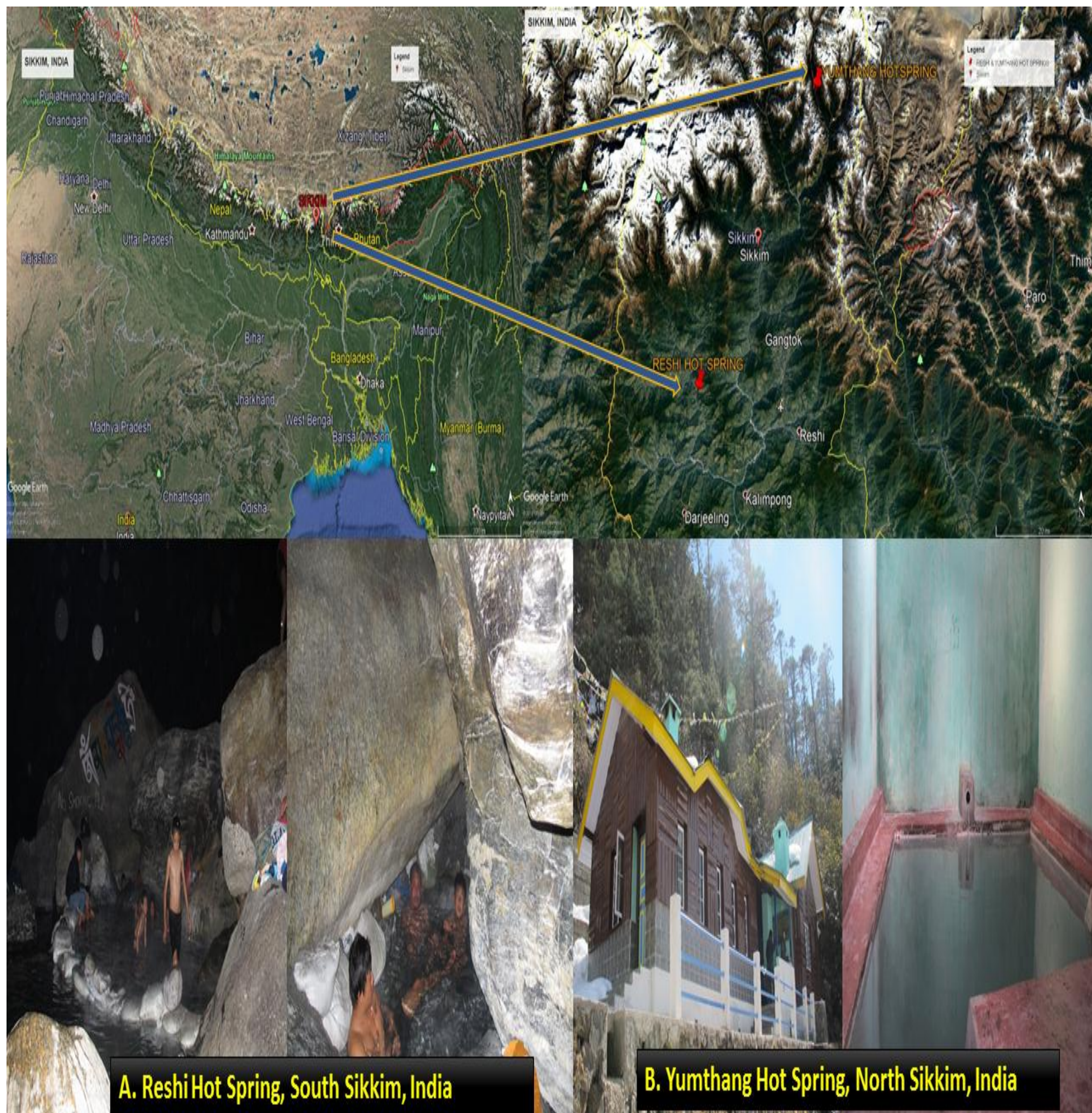

Fig.2a. Map showing the location of Reshi and Yumthang Hot Spring.

Fig.2b. Map showing the location of Polok and Boron Hot Spring.

6.2. Sampling and analysis of Physical parameters

Prior to sampling, the physical parameters were checked such as temperature, pH, dissolved oxygen, total dissolved solids, conductivity etc. at the sampling site of all the hot springs with the help of Multi Water Quality Checker U-50 Series Instrument (Horiba Japan). The water samples were then collected aseptically in a 1-liter sterile thermal flasks in triplicates from all the four hot springs. The samples were grouped into three groups based on the experiments to be carried out such as one group was kept for culture dependent bacterial isolation, second group for chemical analysis through ICPMS (Inductive Coupled Plasma Mass Spectroscopy) and the third group for PLFA (Phospholipid Fatty Acid Analysis) and metagenomic analysis. The samples were then immediately transferred to the laboratory and kept at 4°C. The samples kept for cultural dependent bacterial isolations and for PFLA and metagenomic analysis were immediately processed.

The temperatures of the four hot springs were found considerably different. The Polok hot spring was hotter than the rest of hot springs with a temperature of 75-77°C, Borong was recorded with second highest temperature bearing, with 50-52°C. It was observed that the temperature of the Reshi hot spring varies from 47-48°C where as that of Yumthang hot spring temperature was found to be around 41°C. The pH of the three hot springs such as Polok, Reshi and Yumthang was somehow similar from normal to little alkaline. The pH of Polok hot spring was in between 7.5 – 8.5 and that of Reshi and Yumthang was 6.5-7 and 7.5 respectively. However, the pH of Borong hot spring is slightly acidic in nature with the pH of 5.1 – 5.6. The conductivity was similar in case of Borong and Reshi hot spring and was measured to be 0.20 ms cm^{-1} and 0.23 ms cm^{-1} respectively whereas the conductivity of Polok and Yumthang hot spring was 0.75 ms cm^{-1} and 0.93 ms cm^{-1} respectively. Conductivity is used to measure the concentration of dissolved solids which have been ionized in a polar solution such as water whereas total dissolved solids (TDS) is a measure of the combined content of all inorganic and organic substances contained in a liquid in molecular, ionized or micro granular suspended form.The DO (Dissolved Oxygen) was similar in case of Reshi and Yumthang hot spring with 7.07 mg l^{-1} and 7.5 mg l^{-1} respectively. In case of Polok and Borong the DO (Dissolved Oxygen) was also relatively similar with 4.5-5.5 mg l^{-1} and 5.5-6.2 mg l^{-1} respectively. The total dissolved solids (TDS) was found to be similar in case of Polok and Yumthang hot springs with 0.4 g l^{-1} and 0.59 g l^{-1} respectively. However, the Borong and Reshi hot springs possess similar TDS such as 0.13 g l^{-1} and 0.15 g l^{-1} respectively. The physical parameters are given in **Table.3.**

Table.3. Physical Parameters of Polok and Borong Hot Springs

Table.3. Physical Parameters of Polok and Borong Hot Springs						
Hot Spring	**Temperature (in°C)**	**pH**	**Conductivity mS cm^{-1}**	**D.O. (mg L^{-1})**	**D.O. (%)**	**TDS(g L^{-1})**
Polok	76.3	7.52	0.756	5.56	92	0.483
Borong	52.3	5.32	0.205	6.56	98.3	0.133
Reshi	47.4	6.57	0.935	7.07	104.09	0.598
Yumthang	41	7.5	0.234	7.5	116.5	0.15

6.2.1. Chemical characterization by Inductive coupled mass spectroscopy (ICPMS)

A total of 25 elements were analyzed in all the hot springs with the help of (Inductively Coupled Mass Spectroscopy) ICPMS. Besides the elemental exploration, various other parameters such as total hardness of water, total alkalinity, phenolic compounds, biochemical oxygen demand (BOD) and chemical oxygen demand (COD) were considered and analyzed. The elemental analysis with ICP-MS showed more or less similar pattern of elemental composition in all the springs (**Table.4, Fig. 3**). However, the Reshi hot spring was found to be rich in chloride (111 mg l^{-1}), calcium (69 mg l^{-1}), magnesium (23 mg l^{-1}) and sulfate (24 mg l^{-1}). Also, the parameter like total alkalinity (136 mg l^{-1}), total hardness (233 mg l^{-1}) and total dissolved solids (750 mg l^{-1}) were higher in case of Reshi hot spring as shown in Table 4. After Reshi, Polok hot spring was rich in elements such as chloride (59 mg l^{-1}), nitrate (19 mg l^{-1}), sulfate (24 mg l^{-1}), COD (20 mg l^{-1}) and BOD (8.4 mg l^{-1}). The COD and BOD were higher in Polok hot spring followed by Borong. In case of Yumthang hot spring sulfur (11 mg l^{-1}) was present in higher concentrations. However, Borong and Yumthang also possess these elements but in lesser concentrations.

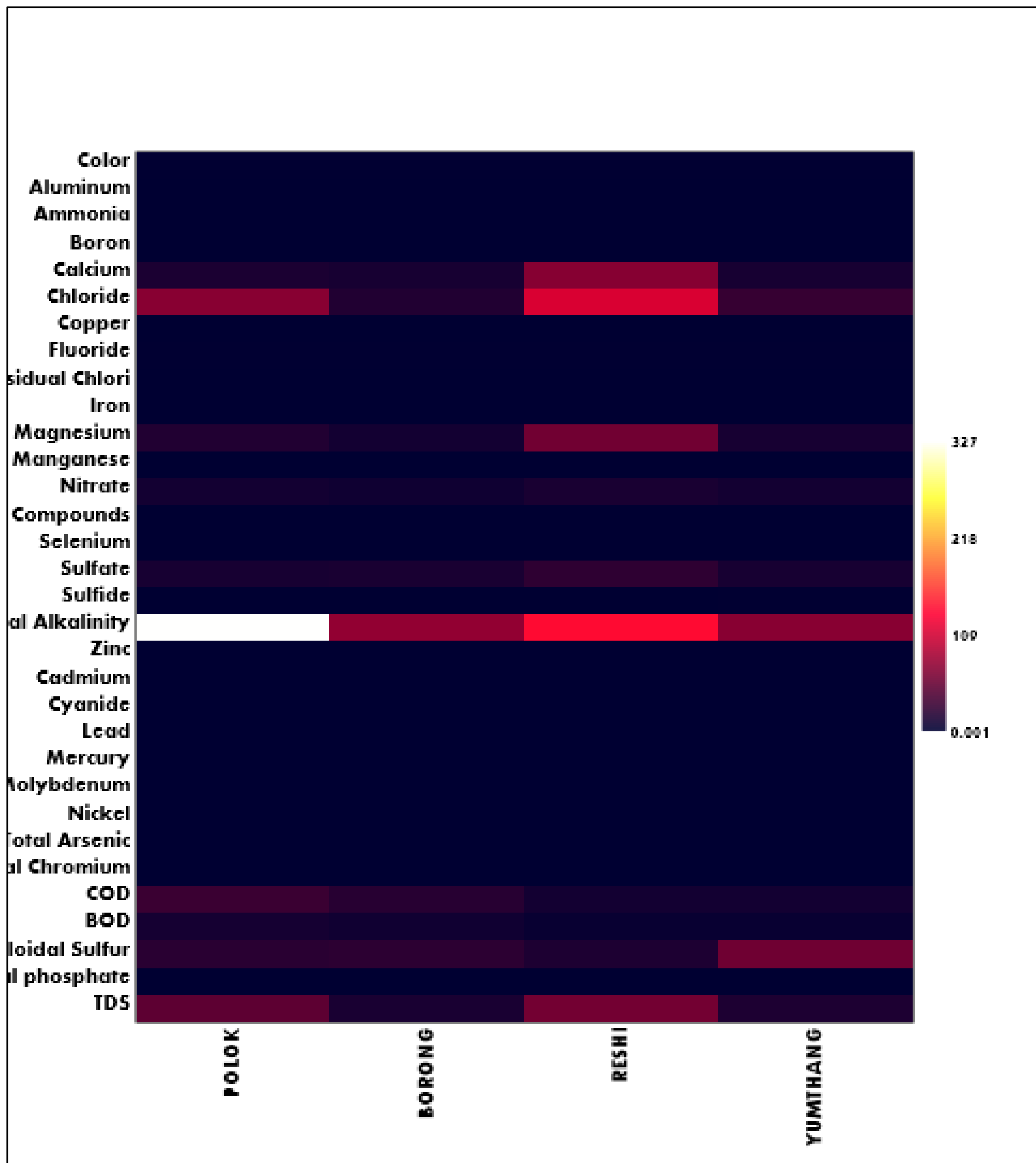

Fig.3. Comparative analysis of elemental concentrations in all the four hot springs.

Table 4. Elemental Analysis of Polok and Borong Hot Springs

SNO.	Test Parameters	Unit	POLOK	BORONG	RESHI	YUMTHANG
1	**Color**	Hazen	<1	<1	<1	<1
2	**Aluminum**	mgL^{-1}	<0.03	<0.03	<0.03	<0.03
3	**Ammonia**	mgL^{-1}	<0.5	<0.5	<0.5	<0.5
4	**Boron**	mgL^{-1}	<0.5	<0.5	<0.5	<0.5
5	**Calcium**	mgL^{-1}	14	12	69	12
6	**Chloride**	mgL^{-1}	70	17	111	28
7	**Copper**	mgL^{-1}	<0.05	<0.05	<0.05	<0.05
8	**Fluoride**	mgL^{-1}	<1	<1	<1	<1
9	**Free Residual Chlorine**	mgL^{-1}	0.22	0.2	0.24	0.2
10	**Iron**	mgL^{-1}	0.08	0.06	0.11	0.06
11	**Magnesium**	mgL^{-1}	7	4	23	5
12	**Manganese**	mgL^{-1}	<0.1	<0.1	<0.1	<0.1
13	**Nitrate**	mgL^{-1}	10	8	13	10
14	**Phenolic Compounds**	mgL^{-1}	0.07	0.18	0.21	0.22
15	**Selenium**	mgL^{-1}	<0.01	<0.01	<0.01	<0.01
16	**Sulfate**	mgL^{-1}	12	13	24	12
17	**Sulfide**	mgL^{-1}	0.4	0.4	0.4	0.8
18	**Total Alkalinity**	mgL^{-1}	327	75	136	70
19	**Zinc**	mgL^{-1}	<0.05	<0.05	<0.05	<0.05
20	**Cadmium**	mgL^{-1}	<0.003	<0.003	<0.003	<0.003
21	**Cyanide**	mgL^{-1}	<0.05	<0.05	<0.05	<0.05
22	**Lead**	mgL^{-1}	<0.01	<0.01	<0.01	<0.01
23	**Mercury**	mgL^{-1}	<0.001	<0.001	<0.001	<0.001
24	**Molybdenum**	mgL^{-1}	<0.07	<0.07	<0.07	<0.07
25	**Nickel**	mgL^{-1}	<0.02	<0.02	<0.02	<0.02
26	**Total Arsenic**	mgL^{-1}	<0.01	<0.01	<0.01	<0.01
27	**Total Chromium**	mgL^{-1}	<0.05	<0.05	<0.05	<0.05
28	**COD**	mgL^{-1}	30	20	10	10
29	**BOD**	mgL^{-1}	11	8.4	4.4	4.5
30	**Colloidal Sulfur**	mgL^{-1}	8.7	10.2	6.1	11
31	**Total phosphate**	mgL^{-1}	<0.05	<0.05	<0.05	<0.05

6.2.2. Statistics and comparative analysis

Ionic concentration of elements in the hot springs were plotted as piper diagram **(Fig.4,5.)** for classification on the basis of chemical composition (Piper 1944). Piper diagram is a combination of triangle plots representing anionic and cationic element on a common baseline. The apexes of the cation plot were magnesium, calcium, sodium, and potassium cations, while the apexes of the anion plot were chloride, sulfate, carbonate and hydrogen carbonate anions. The two ternary plots are then anticipated onto a diamond which can be used to describe different water types. Piper divided water into four basic types conferring to their location near the four corners of the diamond. Water that plots at the top of the diamond is high in Ca^{2+} + Mg^{2+} and Cl^{-} + $SO4^{2-}$, which results in an area of permanent hardness. The water that plots near the left corner is rich in Ca^{2+} + Mg^{2+} and HCO^{3-} and is in an area of temporary hardness. Water plotted at the lower corner of the diamond is mainly composed of alkali carbonates (Na^{+} + K^{+} and HCO^{3-} + $CO3^{2-}$). Water present near the right-hand side of the diamond may be reflected as saline (Na^{+} + K^{+} and Cl^{-} + $SO4^{2-}$).

The piper diagram suggested that the water of Reshi hot spring is Na-Cl type whereas that of the Yumthang hot Spring Na-HCO^{3-} type and thus it can be predicted that the Reshi hot spring water is marine or deep ancient ground water whereas Yumthang hot spring water is deep ground water influenced by ion exchange. On the other hand, the piper diagram suggested that the water of both the Polok and Borong hot springs are Ca-HCO^{3-} type and can be predicted as shallow fresh ground waters (**Fig.5**).

Comparative analysis shows that all the hot springs possess relatively similar elemental conformation. However, the Reshi hot spring is rich in elements followed by Polok hot

spring. The Yumthang and Borong hot springs also possess the various elements but in relatively less quantities **(Fig.3.)**

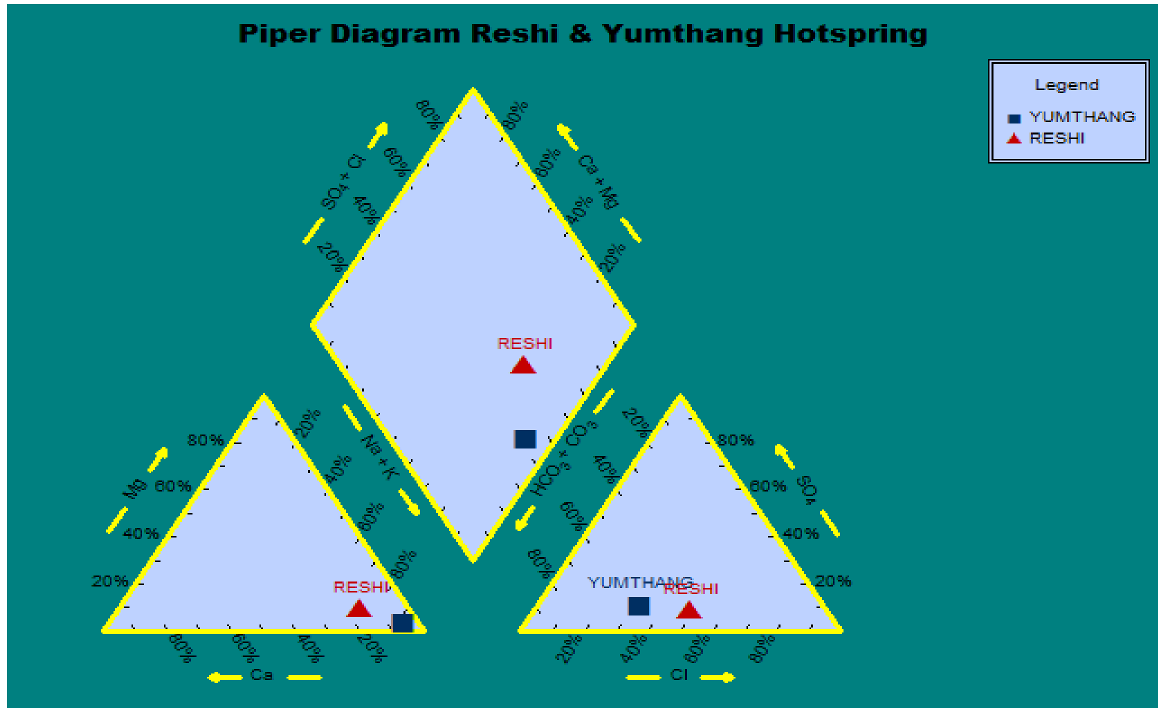

Fig. 4. Piper diagram showing Yumthang and Reshi hot springs.

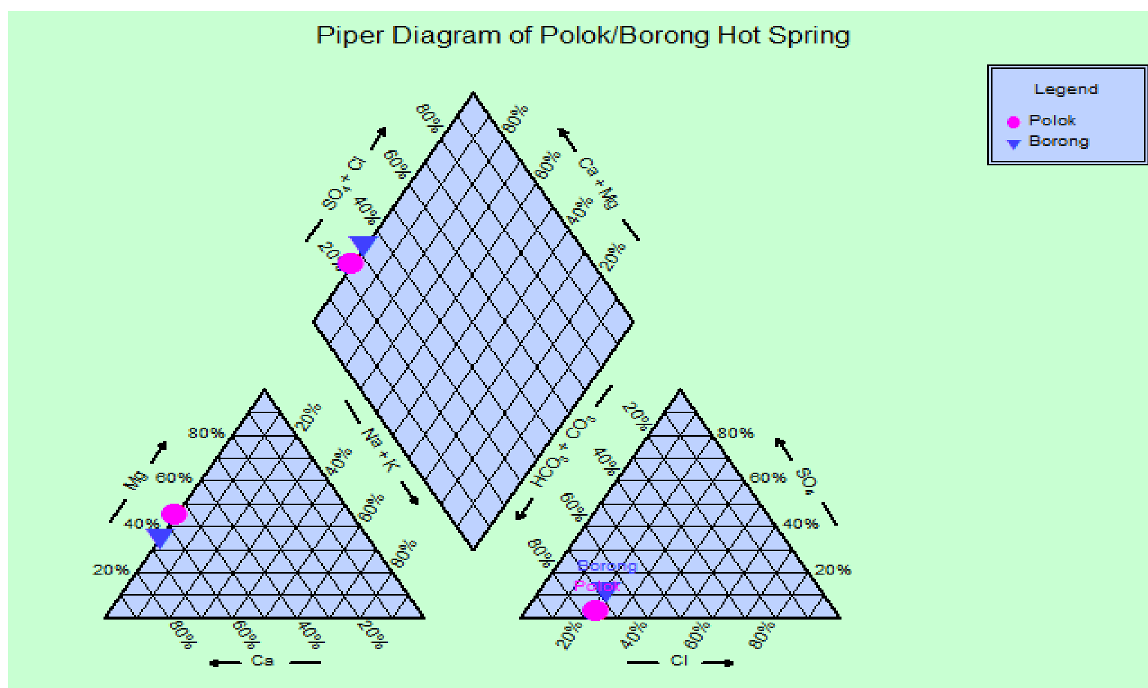

Fig. 5. Piper diagram showing Polok and Borong hot springs.

6.3. Culture-Dependent Analysis

6.3.1. Isolation of Bacterial Strains

A total of 218 thermophilic bacterial isolates were isolated from the four hot springs of Sikkim and among them 152 were selected for further analysis on the basis of morphological and biochemical characterization. Ten different media were used for isolation. In case of Yumthang 37 isolates were selected for further studies, whereas in case of Reshi hot spring 47 isolates were selected. However, in case of Polok and Borong, 17 and 23 isolates were selected respectively for further analysis.

6.3.2. General Morphology of the bacterial isolates

When observed under compound microscope, it was found that all the isolates were rod shaped, however the size of the rods vary among different isolates such as some were large

rods, some medium and some were small in size as shown in **Table.5.** Colony characteristics were checked on agar plates and it was found that the colony color of almost all the isolates was white creamy, off white or pale yellow. The colonies were having circular or round form. The margins were mostly entire with opaque density and with flat elevation as shown in **Table.5.**

6.3.3. Staining

Gram-staining and spore staining was performed as per protocol given in material and methods. Among all the isolates, almost all the isolates were Gram-positive rods. Some of the isolates were Gram variable also. Out of 152 isolates, it was found that 89 isolates were endospore formers except few such as TYN2, TYN6, TYN7, TP9, TB10, TB7, TB1, XTR1, XTR2, XTR13, XTR14, XTR15, XTR16, XTR23, XTR24, XTR24, TY1, TY2, TY3, TY4, LYN1, LYN2, CTRL1, CTRL3 etc. as shown in **Table.5.**

Table.5a. Morphological characterization of isolated bacteria from Yumthang hot spring.

Yumthang	Staining			Colony Morphology				
ISOLATES	Simple Staining	Gram Staining	Spore Staining	Color	Shape	Margins	Elevation	Density
TY1	Small rods	+	-	white	circular	entire	flat	opaque
TY2	Small rods	+	-	white creamy	oval	entire	flat	opaque
TY3	Small rods	+	-	white	circular	entire	flat	opaque
TY4	Small rods	+	-	off white	circular	entire	flat	opaque
TY5	Small rods	+	+	white	circular	entire	flat	opaque

TY6	Small rods	+	-	yellowish	circular	entire	flat	opaque
TY7	Large rods	+	-	white	circular	entire	flat	opaque
TY8	Small rods	+	+	white creamy	circular	entire	flat	opaque
TY9	Medium rods	+	-	yellowish	circular	entire	flat	opaque
TY10	Medium rods	+	-	off yellow	circular	entire	flat	opaque
TYNT4	Medium rods	+	-	white creamy	round undulate	entire	flat	opaque
TYNT6	Medium rods	+	+	off yellow	circular	entire	flat	opaque
TYNT 10	Small rods	+	+	white creamy	circular	entire	flat	opaque
LYNT1	Small rods	+	-	white creamy	circular	entire	flat	opaque
LYNT2	Small rods	+	-	white creamy	circular	entire	flat	opaque
LYNT3	Large rods	+	+	white	undulate	entire	flat	opaque
LYNT5	Small rods	+	+	white creamy	circular	entire	flat	opaque
LYNT9	Small rods	+	+	white creamy	circular	entire	flat	opaque
LYNT10	Medium rods	+	+	white creamy	circular	entire	flat	opaque
AYN2	Medium rods	+	+	white	circular	entire	flat	opaque
CTRL1	Small rods	+	-	off yellow	circular	entire	flat	opaque
CTRL2	Small rods	+	-	off yellow	circular	entire	flat	opaque
CTRL3	Small rods	+	-	white creamy	circular	entire	flat	opaque
CTRL4	Medium Rod	+	+	white creamy	circular	entire	flat	opaque
CTRL5	Medium rods	+	+	white creamy	circular	entire	flat	opaque
CTRL6	Medium rods	+	+	off yellow	circular	entire	flat	opaque
CTRL7	Large rods	+	+	white creamy	circular	entire	flat	opaque

Table.5b. Morphological characterization of isolated bacteria from Reshi hot spring.

Reshi	**Staining**			**Colony Morphology**				
ISOLATAS	**Simple Staining**	**Gram Stain**	**Spore stain**	**Color**	**Shape**	**Margins**	**Elevation**	**Density**
XTR1	medium rods	+	-	pale yellow	circular	entire	flat	opaque
XTR2	medium rods	+	-	white	circular	entire	flat	opaque
XTR3	medium rods	+		white	circular	entire	flat	opaque
XTR4	medium rods	+	+	white	circular	entire	flat	opaque
XTR5	medium rods	+	+	pale yellow	circular	entire	flat	opaque
XTR6	large rods	+	+	pale yellow	circular	entire	flat	opaque
XTR7	small rods	+	+	pale yellow	circular	entire	flat	opaque
XTR8	small rods	+	+	white creamy	circular	entire	flat	opaque
XTR9	small rods	+	+	white creamy	circular	entire	flat	opaque
XTR10	large rods	+	+	white	circular	entire	flat	opaque
XTR11	medium rods	+	+	white creamy	circular	entire	flat	opaque
XTR12	medium rods	+	+	pale yellow	circular	entire	flat	opaque
XTR13	large rods	+	-	white	circular	entire	flat	opaque
XTR14	medium rods	+	-	white creamy	circular	entire	flat	opaque
XTR15	large rods	+	-	white	circular	entire	flat	opaque
XTR16	medium rods	+	-	white creamy	circular	entire	flat	opaque
XTR17	large rods	+	-	pale yellow	circular	entire	flat	opaque
XTR18	medium rods	+	-	white	circular	entire	flat	opaque
XTR19	large rods	+	-	white creamy	circular	entire	flat	opaque
XTR20	medium rods	+	-	pale yellow	circular	entire	flat	opaque
XTR21	large rods	+	+	white creamy	circular	entire	flat	opaque
XTR22	medium rods	+	+	white	circular	entire	flat	opaque
XTR23	medium rods	+	-	white creamy	circular	entire	flat	opaque
XTR24	small rods	+	-	pale yellow	circular	entire	flat	opaque

XTR25	medium rods	+	-	pale yellow	circular	entire	flat	opaque
XTR26	medium rods	+	+	white creamy	circular	entire	flat	Opaque
XTR27	small rods	+	+	white	circular	undulate	flat	opaque
XTR28	small rods	+	-	white	circular	entire	flat	opaque
XTR31	medium rods	+	-	pale yellow	circular	entire	flat	opaque
XTR32	medium rods	+	-	white	circular	entire	flat	opaque
XTR34	medium rods	+	+	white	circular	entire	flat	opaque
XTR36	medium rods	+	+	pale yellow	circular	entire	flat	opaque
XTR37	small rods	+	+	pale yellow	circular	entire	flat	opaque
XTR38	medium rods	+	+	pale yellow	circular	entire	flat	opaque
XTR39	medium rods	+	+	white	circular	entire	flat	opaque
XTR40	small rods	+	-	white creamy	circular	entire	flat	opaque
XTR41	medium rods	+	-	pale yellow	circular	entire	flat	opaque
XTR51	medium rods	+	-	pale yellow	circular	entire	flat	opaque
XTR52	medium rods	+	+	pale yellow	circular	entire	flat	opaque

Table.5c. Morphological characterization of isolated bacteria from Polok/Borong hot spring.

Polok/ Borong	**Staining**			**Growth on agar plates**				
Isolates	**Simple Stain**	**Gram Stain**	**Spore Stain**	**Colony colour**	**Shape**	**Margin**	**Elevation**	**Density**
TP1	Medium rods	+	+	White	circular	Entire	Flat	Opaque
TP2	Medium rods	+	+	White Creamy	circular	Entire	Flat	Opaque
TP3	Medium rods	+	+	Off White	circular	Entire	Flat	Translucent
TP4	Very Small rods	+	-	Off White	circular	Entire	Flat	Opaque
TP5	Small rods	+	+	Off White	circular	Entire	Flat	Opaque
TP6	Large rods	+	-	White Creamy	Circular	Entire	Flat	Opaque

TP7	Small rods	+	-	Pale Yellow	Circular	Entire	Flat	Opaque
TP8	Small rods	+	-	Off White	Circular	Entire	Flat	Opaque
TP9	Medium rods	+	-	Off White	Circular	Entire	Flat	Opaque
TP10	Medium rods	+	+	White	Circular	Entire	Flat	Opaque
TP11	Small rods	+	+	White	Circular	Entire	Flat	Opaque
TP12	Small rods	+	-	White Creamy	Circular	Entire	Flat	Opaque
TP13	Medium rods	+	-	White	Circular	Entire	Flat	Opaque
BPP1	Small rods	+	+	Off White	Circular	Entire	Flat	Opaque
BPP2	Medium rods	+	+	Off White	Circular	Entire	Flat	Opaque
BPP3	Large rods	+	-	Off White	Circular	Entire	Flat	Opaque
BPP4	Medium rods	+	-	Off White	Circular	Entire	Flat	Opaque
BPP5	Medium rods	+	-	Off White	Circular	Entire	Flat	Opaque
10PHP1	Large rods	+	+	White Creamy	Circular	Entire	Flat	Opaque
10PHP2	Small rods	+	-	White Creamy	Circular	Entire	Flat	Opaque
10PHP3	Small rods	+	+	White Creamy	Circular	Entire	Flat	Opaque
10PHP4	Medium rods	+	+	Off White	Circular	Entire	Flat	Opaque
10PHP5	Medium rods	+	-	Off White	Circular	Entire	Flat	Opaque
TB1	Medium rods	+	-	Off White	Circular	Entire	Flat	Opaque
TB2	Small rods	+	-	White	Circular	Entire	Flat	Opaque
TB3	Medium rods	+	+	White	Circular	Entire	Flat	Opaque
TB4	Medium rods	+	+	White	Circular	Entire	Flat	Opaque
TB5	Small rods	+	-	White	Circular	Entire	Flat	Opaque
TB6	Medium rods	+	+	Off White	Circular	Entire	Flat	Opaque
TB7	Medium rods	+	-	Off White	Circular	Entire	Flat	Opaque
TB8	Medium rods	+	-	Off White	Circular	Entire	Flat	Opaque
TB9	Medium rods	+	+	White	Circular	Entire	Flat	Opaque
TB10	Large rods	+	+	Off White	Circular	Entire	Flat	Opaque

TB11	Medium rods	+	+	White	Circular	Entire	Flat	Opaque
TB12	Small rods	+	-	Off White	Circular	Entire	Flat	Opaque
BPB1	Medium rods	+	+	Off White	Circular	Entire	Flat	Opaque
BPB2	Small rods	+	+	Off White	Circular	Entire	Flat	Opaque
BPB3	Small rods	+	+	Off White	Circular	Entire	Flat	Opaque
BPB4	Small rods	+	+	Off White	Circular	Entire	Flat	Opaque
BPB5	Medium rods	+	+	Off White	Circular	Entire	Flat	Opaque
17B1	Long rods	+	+	White	Circular	Entire	Flat	Opaque
TRB1	Small rods	+	+	Off White	Circular	Entire	Flat	Opaque
TRB2	Small rods	+	-	White Creamy	Circular	Entire	Flat	Opaque
YTPB1	Medium rods	+	-	White Creamy	Circular	Entire	Flat	Opaque
YTPB2	Medium rods	+	-	Off White	Circular	Entire	Flat	Opaque

6.3.4. Growth Profile at various physical parameters

Bacterial growth curve for any bacteria indicate how fast it can reproduce at particular temperature, pH and NaCl concentration in a given medium. The growth profiles of all the isolates were checked at different temperature, pH and NaCl concentrations. The growth of isolates at various temperatures were carried out such as at 30°C, 40°C, 50°C, 60°C and 80°C. The cultures were incubated for 48 hours in a shaker incubator. After incubation the Optical Density (O.D.) of the broth cultures were measured at 600 nm. The results have shown that the isolates were able to grow at a wide range of temperatures from 40°C-65°C, However, the optimum temperature of most of the isolates was 60°C as shown in **Fig.6.** Almost all the isolates were showing optimal growth at 60°C such as TY1, TY2, TY10, .LYN2, LYN3, LYN9, TP1, TP2, TP5, TP10, TP12, TB1, TB2, TB4, TB6, BPB1, BPB2, BPP1, BPP2, XTR1, XTR2, XTR15, XTR17, XTR18, XTR32, XTR41, XTR51, YTPR1, TRR2, YTPB1, 17R1, 17R2, 10PHP1, 10PHB1 etc. pH conditions play an important role

in deciphering the physiology of the thermophilic bacteria. Depending on the bacterial optimum pH conditions, they are either classified as Acidophiles or Alkaliphiles. Acidophiles requires acidic conditions and Alkaliphiles requires basic conditions of pH. Thus the growth profile of isolates have been checked at various pH ranges of 3-10. At constant temperature of 60°C the results have shown that the isolates were able to grow at a wide range of pH from 3-9. However, the optimum pH range was between 7 and 8 as shown in **Fig.7.** Most of the isolates were having neutral pH, however few of the isolates were having optimum pH of 9-10 such as isolates 10PHP1, 10PHP2, 10PHB1, 10PHB2 and 10PHB3. Saline conditions are most important in deciphering the physiology of the thermophilic bacteria. The growth of isolates at various NaCl concentrations was carried out at a range of 0.5-5% at 60°C in a shaker incubator at constant temperature of 60°C and pH7. The results have showed that the most of the isolates were able to grow up to 4% NaCl concentrations **Fig.8.** No growth was seen above 5% NaCl concentration, however, the optimum growth was found to be at 2% NaCl concentration. Most of the isolates such as TY1, TY2, TY10, .LYN2, LYN3, LYN9, TP1, TP2, TP5, TP10, TP12, TB1, TB2, TB4, TB6, BPB1, BPB2, BPP1, BPP2, XTR1, XTR2, XTR15, XTR17, XTR18, XTR32, XTR51 etc. were having optimum NaCl concentrations of 1-2%. However few of the isolates such as 17R2, 17R3, 17R6, 17B1, 10PHP1, 10PHB2, 10PHB3 were showing higher growth at concentration of 5% NaCl.

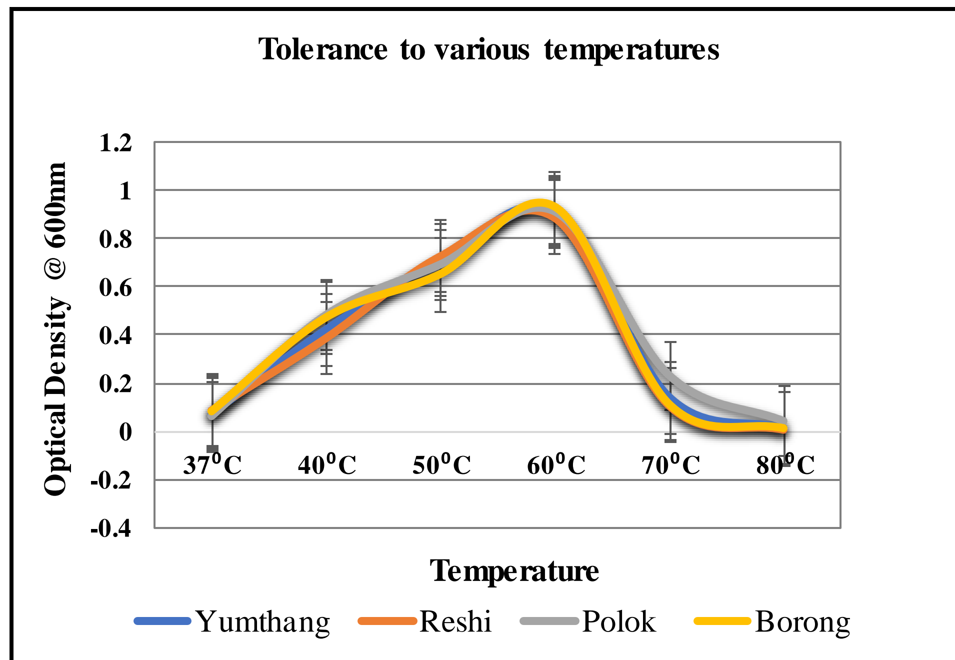

Fig.6. Tolerance to various temperature ranges.

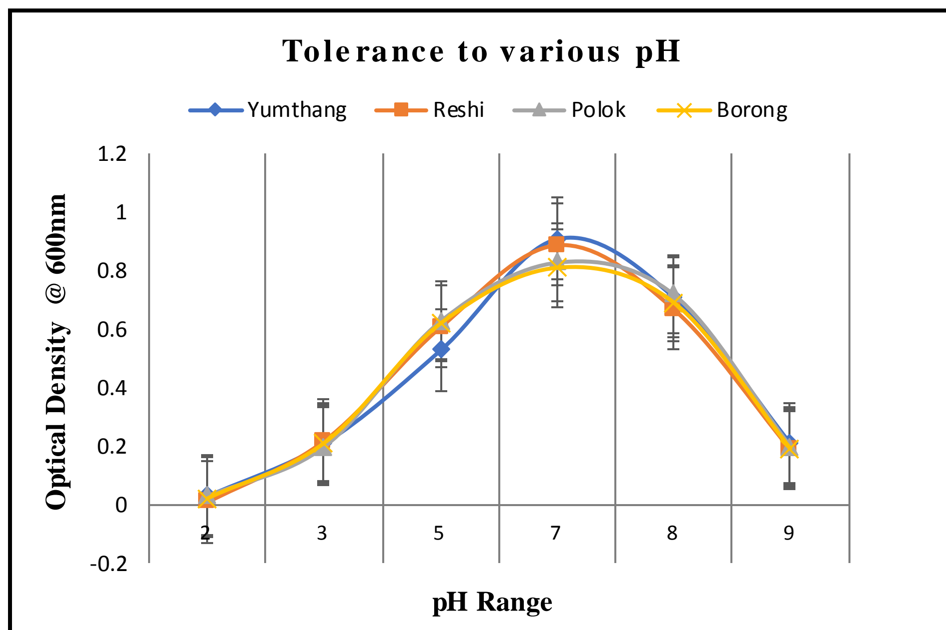

Fig.7. Tolerance to various pH ranges.

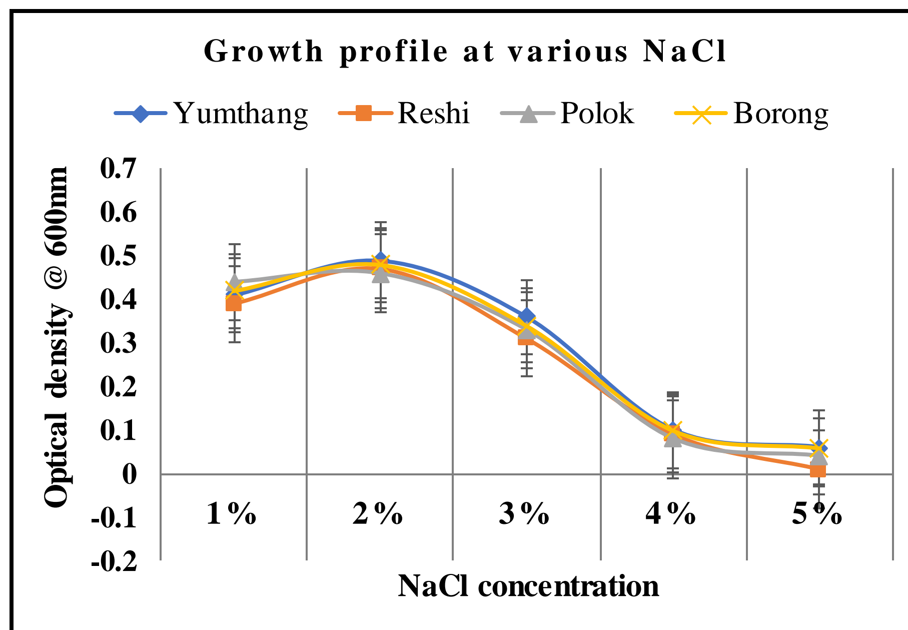

Fig.8. Tolerance to various NaCl concentrations.

6.4. Biochemical characterization of the bacterial samples.

6.4.1. Carbohydrate Fermentation Test.

According to Bergey's classification of bacteria, carbohydrate utilization is an important characteristic feature of bacteria through which differentiation and group classification can be done. Thus, carbohydrate fermentation test of 16 sugars were performed. The results have shown that isolates were active utilizers of simple sugars like dextrose, maltose, ribose, fructose, mannitol, while they were unable to utilize complex sugars like sucrose, arabinose, raffinose, cellobiose, dulcitol, melizitose, galactose and inositol. **Fig.9, Table.6.** Fig.9. suggest that around 90 isolates were able to utilize maltose, 80 isolates were able to utilize fructose and dextrose.

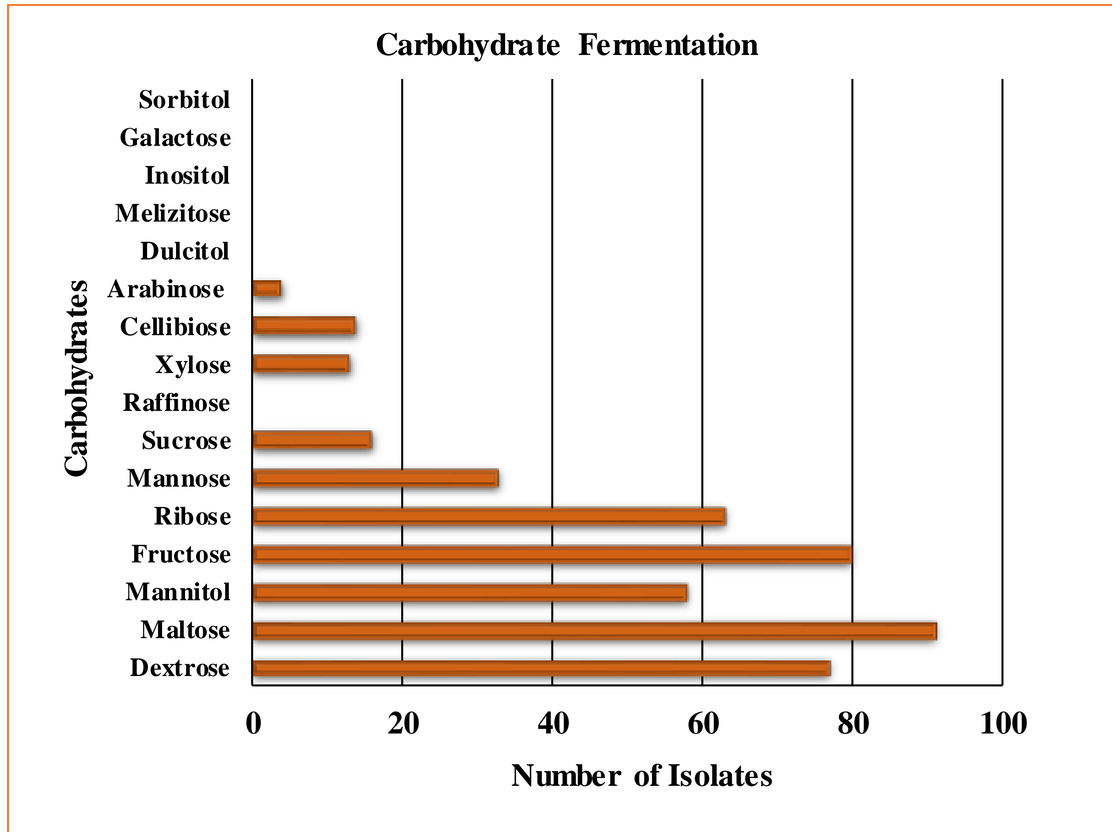

Fig.9. Carbohydrate fermentation of bacterial isolates.

Table.6. Carbohydrate fermentation test of bacteria isolated from four hot springs

ISOLATES	DEXTROSE	MALTOSE	MANNITOL	FRUCTOSE	MANNOSE	SUCROSE	RAFFINOSE	XYLOSE	RIBOSE	CELLIBIOSE	DULCITOL	MELIZITOSE
TY1	–	+	–	–	+	–	–	–	–	–	–	–
TY2	+	+	–	+	+	–	–	–	+	–	–	–
TY3	+	+	–	+	+	–	–	+	–	+	–	–
TY4	–	+	–	+	+	–	–	–	+	–	–	–
TY5	+	+	–	+	+	–	–	–	–	–	–	–
TY6	–	+	–	+	+	–	–	+	+	–	–	–
TY7	+	+	–	+	+	–	–	–	–	+	–	–
TY8	+	+	–	+	+	–	–	–	–	–	–	–
TY9	+	+	–	+	–	–	–	–	–	+	–	–
TY10	+	+	–	+	+	–	–	–	–	–	–	–
TYNT4	–	+	–	–	–	+	–	–	–	–	–	–
TYNT6	+	+	–	–	+	–	–	–	–	–	–	–
TYNT 10	–	–	–	–	–	+	–	–	–	–	–	–
LYNT1	–	+	–	–	+	–	–	–	–	–	–	–
LYNT2	–	+	–	–	–	–	–	–	–	–	–	–
LYNT3	–	–	–	–	–	+	–	–	–	–	–	–
LYNT5	–	–	–	–	–	–	–	–	–	–	–	–
LYNT9	–	–	–	–	–	+	–	–	–	+	–	–
LYNT10	–	+	–	–	+	–	–	–	–	–	–	–
AYN2	+	+	–	+	–	–	–	–	+	–	–	–
CTRL1	+	+	–	+	+	–	–	–	+	–	–	–
CTRL2	+	+	+	+	–	–	–	–	+	–	–	–
CTRL3	+	+	+	+	+	–	–	–	–	–	–	–
CTRL4	+	+	+	+	–	+	–	–	–	–	–	–
CTRL5	+	+	+	+	–	–	–	–	+	–	–	–
CTRL6	+	+	+	+	–	+	–	+	+	+	–	–
CTRL7	+	+	+	+	–	+	–	–	–	–	–	–
XTR1	+	+	–	+	+	–	–	–	+	–	–	–
XTR2	+	+	+	+	–	–	–	–	+	–	–	–
XTR3	+	+	+	+	–	–	–	–	+	–	–	–
XTR4	+	+	+	+	+	–	–	–	+	+	–	–
XTR5	+	+	+	+	–	–	–	–	+	–	–	–
XTR6	+	+	+	+	–	–	–	–	+	–	–	–
XTR7	+	+	+	+	–	–	–	–	+	+	–	–
XTR8	+	+	+	+	–	–	–	–	+	–	–	–
XTR9	+	+	+	+	–	–	–	–	+	–	–	–
XTR10	+	+	+	+	–	–	–	–	+	–	–	–

XTR11	+	+	+	+	–	–	–	–	+	–	–	–
XTR12	+	+	+	+	–	–	–	–	+	–	–	–
XTR13	+	+	–	+	–	–	–	–	+	–	–	–
XTR14	+	+	+	+	–	–	–	–	+	–	–	–
XTR15	+	+	–	+	–	–	–	–	+	+	–	–
XTR16	+	+	+	+	–	–	–	–	+	–	–	–
XTR17	+	+	+	+	–	–	–	–	+	–	–	–
XTR18	+	+	+	+	–	–	–	–	+	–	–	–
XTR19	+	+	+	+	–	+	–	–	+	+	–	–
XTR20	+	+	–	+	–	–	–	–	+	–	–	–
XTR21	+	+	+	+	–	–	–	–	+	–	–	–
XTR22	+	+	+	+	–	–	–	–	+	–	–	–
XTR23	+	+	–	+	+	–	–	–	+	–	–	–
XTR24	+	+	–	+	+	–	–	–	+	+	–	–
XTR25	+	+	+	+	–	–	–	–	+	–	–	–
XTR26	+	+	+	+	–	–	–	–	+	–	–	–
XTR27	+	+	+	+	–	–	–	–	+	–	–	–
XTR28	+	+	+	+	–	–	–	–	+	–	–	–
XTR29	+	+	+	+	–	–	–	–	+	–	–	–
XTR30	+	+	+	+	–	–	–	–	+	+	–	–
XTR31	+	+	+	+	–	–	–	–	+	–	–	–
XTR32	+	+	+	+	–	–	–	–	+	–	–	–
XTR33	+	+	+	+	–	–	–	–	+	–	–	–
XTR34	+	+	+	+	–	–	–	–	+	+	–	–
XTR35	+	+	–	+	–	–	–	–	+	–	–	–
XTR36	+	+	+	+	–	+	–	–	+	–	–	–
XTR37	+	+	–	+	+	–	–	–	+	–	–	–
XTR38	+	+	+	+	–	–	–	–	+	–	–	–
TP1	+	+	–	+	+	–	–	–	+	–	–	–
TP2	+	+	+	+	–	–	–	–	–	–	–	–
TP3	+	+	+	+	–	–	–	–	+	–	–	–
TP4	+	+	+	+	–	–	–	–	–	–	–	–
TP5	+	+	+	+	–	–	–	–	+	–	–	–
TP6	+	+	+	+	–	+	–	–	–	–	–	–
TP7	–	–	–	–	–	+	–	+	–	–	–	–
TP8	+	+	+	+	–	–	–	–	+	–	–	–
TP9	+	+	+	+	–	+	–	+	–	–	–	–
TP10	+	+	+	+	–	–	–	+	+	–	–	–
TP11	+	+	+	+	+	–	–	+	+	–	–	–
TP12	+	+	+	+	–	–	–	–	–	–	–	–
TP13	+	+	+	+	+	–	–	–	+	–	–	–
BPP1	–	+	–	–	+	+	–	–	–	–	–	–
BPP2	–	+	–	+	+	–	–	–	–	+	–	–

BPP3	+	+	+	+	+	–	–	–	–	–	–	–
BPP4	+	+	+	+	–	–	–	+	–	–	–	–
BPP5	+	+	+	+	–	–	–	–	–	–	–	–
TB1	+	+	+	+	+	–	–	–	+	–	–	–
TB2	+	+	+	+	+	–	–	–	+	–	–	–
TB3	+	+	+	+	–	–	–	–	+	–	–	–
TB4	–	–	–	+	–	–	–	+	–	–	–	–
TB5	+	+	+	+	+	–	–	+	+	–	–	–
TB6	+	+	+	+	–	–	–	+	+	–	–	–
TB7	+	+	+	+	+	+	–	–	+	–	–	–
TB8	+	+	–	+	+	–	–	–	+	–	–	–
TB9	+	+	+	+	+	–	–	–	+	–	–	–
TB10	+	+	+	+	+	–	–	+	+	–	–	–
TB11	+	+	+	+	+	–	–	+	–	–	–	–
TB12	+	+	+	+	–	–	–	+	+	–	–	–
BPB1	–	+	–	–	–	+	–	–	–	+	–	–
BPB2	–	+	–	–	–	–	–	–	–	–	–	–
BPB3	–	+	–	–	–	+	–	–	–	–	–	–
BPB4	–	+	–	–	+	–	–	–	–	–	–	–
BPB5	–	+	+	–	–	–	–	–	–	–	–	–

Isolates	Glucose	Maltose	Fructose	Mannose	Arabinose	Galactose	Trehalose	Ribose	Ribulose	Cellobiose	Rhaminose	Sucrose	Raffinose
SY1	+	+	+	+	–	–	+	+	+	–	–	–	–
SY2	+	+	+	+	–	–	+	+	+	–	–	–	–
SY3	+	+	+	+	–	–	+	+	+	–	–	–	–
SY4	+	+	+	+	–	–	+	+	+	–	–	–	–
SY5	+	+	+	+	–	–	+	+	+	–	–	–	–
SY6	+	+	+	–	–	–	+	+	+	–	–	–	–
SY7	+	+	+	+	–	–	+	+	+	–	–	–	–
SY8	+	+	+	+	–	–	+	+	+	–	–	–	–
SY9	+	+	+	+	–	–	+	+	+	–	–	–	–
SY10	+	+	+	+	–	–	+	+	+	–	–	–	–
SY11	+	+	+	+	–	–	+	+	+	–	–	–	–
SY12	+	+	+	+	–	–	+	+	+	–	–	–	–
SY13	+	+	+	+	–	–	+	+	+	–	–	–	–
SY14	+	+	+	+	–	–	+	+	+	–	–	–	–
SY15	+	+	+	+	–	–	+	+	+	–	–	–	–
SY16	+	+	+	+	–	–	+	+	+	–	–	–	–
SY17	+	+	+	+	–	–	+	+	+	–	–	–	–

6.4.2. Enzymatic analysis of bacterial isolates

The further key to identify a bacterium and distinguish it from the rest is the detection of various enzyme activities of the isolates. Depending on the enzyme activity, they can be classified into their respective groups as per the Bergey's Systemic Classification. Catalase activity of the isolates were carried out and it was found that most of the isolates were having catalase activity as they produced bubbles when treated with 3% Hydrogen peroxide **Fig.10, Table.7.** The results have shown that 113 isolates were catalase positive. The proteolytic activity of isolates were checked by growing these isolates on Thermus Agar supplemented with 10% Double toned milk. Positive result in protease test infers that the microorganisms are able to degrade protein present in milk with the enzyme protease, and can utilize it for its growth. It was found that very less number of isolates, i.e., only seven isolates (LYN2, XTR13, XTR15, XTR17, XTR21, XTR32 and XTR33) where having protease activity **Fig.10, Table.7.** The starch hydrolysis activity or amylase activity was checked by growing these isolates on Starch Agar plates at 60°C and pH7 for 48 hours and later flooded with iodine solution The results indicated that the large number of isolates where having amylase activity. Among all the isolates 47 isolates were showing positive amylase activity and most of them were belonging to Reshi and Yumthang hot springs as shown in **Fig.10, Table.7.** Gelatin hydrolysis test was performed and it was shown that none of the isolates were having gelatinase activity as shown in **Fig.10, Table.7.** The isolates were also checked for their oxidase activity and it was found that some of the isolates were positive for the test. Among all the isolates 41 isolates were having oxidase activity as shown in **Fig.10, Table.7.** Nitrate reductase activities were also checked and

around 51 isolates showed positive result for nitrate reductase activity as shown in **Fig.10, Table.7.**

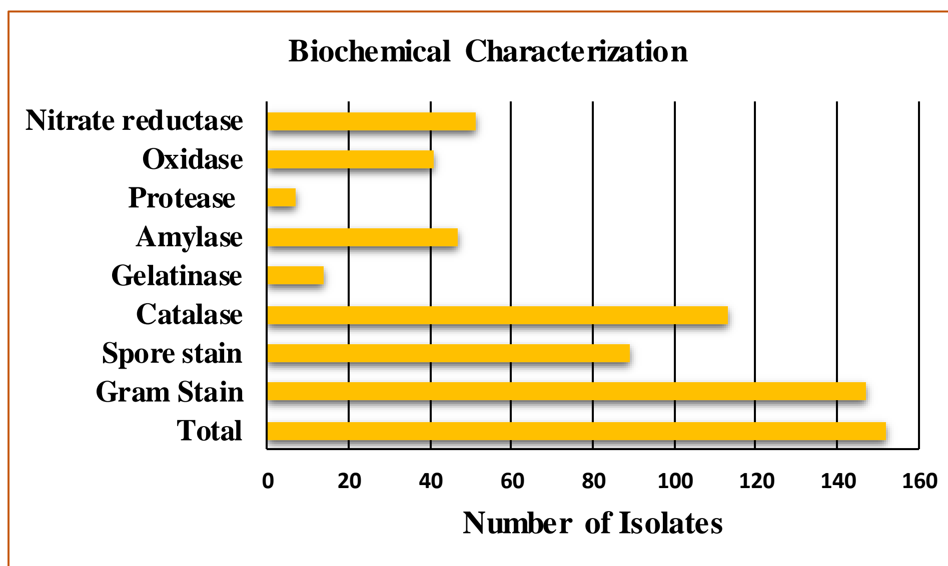

Fig.10. Biochemical Characterization of bacterial isolates.

Table.7. Enzymatic analysis of bacterial isolates.

Isolates	Catalase	Amylase	Protease	Gelatinase	Nitrate Reductase	Oxidase
TY1	+	-	-	-	+	+
TY2	-	+	-	-	-	+
TY3	+	+	-	-	-	-
TY4	+	+	-	-	+	-
TY5	+	-	-	-	+	-
TY6	+	-	-	-	-	-
TY7	-	-	-	-	+	-
TY8	+	-	-	-	-	-
TY9	+	+	-	-	-	-
TY10	+	-	-	-	+	-
TYNT4	+	+	-	-	+	-
TYNT6	+	-	-	-	-	-
TYNT 10	+	-	-	-	-	-

LYNT1	+	+	-	-	-	-
LYNT2	+	+	+	-	+	-
LYNT3	+	+	-	-	-	+
LYNT5	+	+	-	-	-	-
LYNT9	+	+	-	-	+	-
LYNT10	+	+	-	-	-	+
AYN2	+	-	-	-	-	-
CTRL1	+	-	-	-	+	-
CTRL2	+	-	-	-	+	-
CTRL3	+	-	-	-	-	+
CTRL4	-	-	-	-	+	-
CTRL5	+	-	-	-	-	+
CTRL6	-	-	-	-	+	-
CTRL7	+	-	-	-	-	-
XTR1	+	+	-	-	+	+
XTR2	+	+	-	-	+	-
XTR3	+	+	-	-	+	-
XTR4	+	+	-	-	+	+
XTR5	+	+	-	-	+	+
XTR6	+	-	-	-	+	+
XTR7	+	+	-	-	-	+
XTR8	+	+	-	-	-	-
XTR9	+	+	-	-	-	+
XTR10	-	+	-	-	+	-
XTR11	+	-	-	-	-	+
XTR12	+	+	-	-	+	-
XTR13	-	+	+	-	-	+
XTR14	+	+	-	-	+	-
XTR15	+	+	+	-	+	+
XTR16	+	+	-	-	-	-
XTR17	+	+	+	-	+	-
XTR18	+	-	-	-	-	+
XTR19	+	+	-	-	+	+

XTR20	+	+	-	-	-	-
XTR21	+	+	+	-	+	+
XTR22	+	-	-	-	+	+
XTR23	+	+	-	-	-	+
XTR24	+	+	-	-	+	+
XTR25	+	+	-	-	+	-
XTR26	+	+	-	-	-	+
XTR27	+	-	-	-	+	-
XTR28	+	-	-	-	+	+
XTR29	+	+	-	-	+	-
XTR30	+	+	-	-	-	+
XTR31	+	+	-	-	-	+
XTR32	-	+	+	-	+	+
XTR33	+	+	+	-	+	+
XTR34	-	+	-	-	-	-
XTR35	+	-	-	-	+	+
XTR36	+	+	-	-	+	+
XTR37	+	-	-	-	-	+
XTR38	-	-	-	-	+	-
TP1	+	-	-	-	+	-
TP2	+	-	-	-	-	-
TP3	+	-	-	-	-	-
TP4	-	-	-	-	-	-
TP5	+	-	-	-	+	-
TP6	+	+	-	-	-	-
TP7	+	+	-	-	-	-
TP8	-	-	-	-	-	+
TP9	+	+	-	-	-	+
TP10	+	+	-	-	-	-
TP11	+	-	-	-	+	+
TP12	+	+	-	-	-	-
TP13	+	-	-	-	+	-

BPP1	-	-	-	-	-	+
BPP2	+	-	-	-	+	-
BPP3	+	-	-	-	-	-
BPP4	-	-	-	-	-	+
BPP5	+	-	-	-	-	-
TB1	+	-	-	-	+	-
TB2	+	-	-	-	+	+
TB3	+	-	-	-	+	-
TB4	+	+	-	-	-	+
TB5	+	-	-	-	+	-
TB6	+	-	-	-	-	-
TB7	+	-	-	-	-	+
TB8	+	+	-	-	-	-
TB9	+	-	-	-	-	+
TB10	+	-	-	-	-	-
TB11	+	-	-	-	-	+
TB12	+	-	-	-	+	-
BPB1	-	-	-	-	+	-
BPB2	+	-	-	-	+	+
BPB3	+	-	-	-	+	-
BPB4	-	-	-	-	+	+
BPB5	+	-	-	-	+	-

6.5. Identification of bacterial isolates by culture dependent techniques

6.5.1. 16SrRNA Sequencing.

One of the most acceptable tool for identification of bacterial isolates is 16s rRNA sequencing. 16S rDNA sequencing was carried out by Automated DNA Sequencer (ABS/Genetic 3500 Analyzer) using a BigDye^{TM}Terminator v3.1 cycle sequencing kit. The obtained sequence were assembled with Codon Code Aligner (ver. 5.2) and compared with

nr/nt database of NCBI using BLAST sequence homology search for taxonomic identification. Molecular identification showed the unusual dominance of phylum *Firmicutes.* Major genus found in the study is *Geobacillus* with a few representatives of genus *Anoxybacillus* and *Bacillus.* Identified isolates of *Geobacillus* were as *G. stearothermophilus* XTR25, *G. Kaustophilus* YTPR1, *G. subterraneus* 17R4*, G. lituanicus* TP11, various strains of *G. toebii* such as *G. toebii* TYN4, LYN10, LYN3, TY3, TY1, TP3, TP5 etc, *Parageobacillus toebii* 10PHP2, *G. Kaustophillus* YTPB1*, G.* sp, BPP2*,* and *G sp,* TB7. The representative isolates of genus *Anoxybacillus* were *Anoxybacillus gonensis* TP9 and *Anoxybacillus Caldiproteolyticus* TRB1. The representative isolates of genus *Bacillus* were *Bacillus simithi* 17R6 and *Bacillus* sp, 17R5. Also, some uncultured bacteria were reported such as Uncultured 17R2 and Uncultured TRR2. The alignment and similarity search of 16S rRNA sequence with nr/nt database of NCBI have shown that many of the isolates have a distinct percentage of identity from <95%. These results suggested the novelty of these isolated bacteria. The various identified isolates along with their accession numbers are given in **Table.8.** A phylogenetic tree was constructed using the neighbor-joining method with jukes-cantor evolutionary distance measurement is shown in **Fig.11a,b,c.**

Table.8. Identified bacterial isolates with percentage identity and accession numbers.

Isolates	Identification based on 16SrRNA	%Identity	Accession No.
TP3	*Geobacillus toebii* TP3	94	MG603308
TP2	*Geobacillus* sp. TP2	91	MG603309
BPP2	*Geobacillus* sp. BPP2	91	MG603313
TP5	*Geobacillus toebii* TP5	93	MH535464
TP1	*Geobacillus toebii* TP1	91	MH535463
TB5	*Geobacillus* sp. TB5	89	MH535462
10PHB2	*Geobacillus* sp. 10PHB2	92	MH535460
10PHB3	*Geobacillus* sp. 10PHB3	90	MH535461
10PHP1	*Geobacillus toebii* TP5	88	MG603315
10PHP2	*Parageobacillus toebii* 10PHP2	95	MG731573
TP9	*Anoxybacillus gonensis* TP9	100	KX894322
TP11	*Geobacillus Lituanicus* TP11	99	MG603317
BPP1	*Geobacillus toebii* BPP1	95	MG731574
TB10	*Geobacillus* sp. TB10	91	MG603310
TB7	*Geobacillus* sp.TB7	91	MG603311
TB3	*Geobacillus toebii* TB3	90	MG603312
BPB1	*Geobacillus* sp.BPB1	90	MG603314
10PHB1	*Geobacillus toebii* 10PHB1	90	MG603316
YTPB1	*Geobacillus kaustophillus* YTPB1	99	MG603318
TRB1	*Anoxybacillus caldiproteolyticus* TRB1	98	MG603319
TB9	*Geobacillus* sp.TB9	93	MG731576
TB1	*Geobacillus* sp.TB1	95	MG731575
SY1	*Geobacillus toebii* strain R-35642	94	MF360785
SY3	*Geobacillus toebii* strain R-35643	96	MF278569
SY4	*Geobacillus toebii* strain R-35644	98	MF278570
SY5	*Geobacillus toebii* strain R-35645	97	MF278564
SY6	*Geobacillus toebii* strain R-35646	96	MF278563
SY8	*Geobacillus toebii* strain R-35647	97	MF278571

SY12	*Geobacillus toebii* strain R-35648	97	MF278565
SY14	*Geobacillus toebii* strain R-35649	95	MF278566
SY15	*Geobacillus toebii* strain R-35650	97	MF278572
SY17	*Geobacillus toebii* strain R-35651	96	MF278573
17R2	Uncultured compost bacteria 17R2	99%	MG709464
17R4	*Geobacillus subterraneus* 17R4	99%	MG709465
17R5	*Bacillus* sp. 17R5	100%	MG709466
17R6	*Bacillus simithi* 17R6	99%	MG709467
TRR2	Uncultured compost bacteria TRR2	99%	MG709468
XTR1	*Geobacillus* sp. XTR1	98%	MG709469
XTR3	*Geobacillus* sp. XTR3	94%	MG709470
XTR5	*Geobacillus* sp. XTR5	94%	MG709471
XTR6	*Geobacillus toebii* XTR6	95%	MG709472
XTR11	*Geobacillus* sp.XTR11	95%	MG709473
XTR12	*Geobacillus* sp.XTR12	92%	MG709474
XTR14	*Geobacillus* sp. XTR14	93%	MG709475
XTR17	*Geobacillus* sp. XTR17	98%	MG709476
XTR19	*Geobacillus* sp. XTR19	93%	MG709477
XTR20	*Geobacillus toebii* XTR20	94%	MG709478
XTR21	*Geobacillus* sp.XTR21	93%	MG709479
XTR24	*Geobacillus* sp.XTR24	99%	MG709480
XTR25	*Geobacillus stearothermophilus* XTR25	99%	MG709481
XTR26	*Geobacillus* sp.XTR26	93%	MG709482
XTR27	*Geobacillus* sp.XTR27	95%	MG709483
XTR28	*Geobacillus* sp.XTR28	96%	MG709484
XTR32	*Geobacillus toebii* XTR32	93%	MG709485
XTR39	*Geobacillus* sp.XTR39	93%	MG709486
XTR40	*Geobacillus* sp.XTR40	95%	MG709487
XTR52	*Geobacillus* sp.XTR52	95%	MG709488
XTR54	*Geobacillus* sp.XTR54	94%	MG709489

YTPR1	*Geobacillus kaustophilus* YTPR1	99%	MG709490
XTR22	*Geobacillus toebii* XTR22	99%	MG709491
TY1	*Geobacillus* sp.TY1	96%	MG725734
TY2	*Parageobacillus toebii* TY2	92%	MG725735
TY3	*Geobacillus toebii* TY3	92%	MG725736
TY4	*Geobacillus* sp.TY4	95%	MG725737
TY6	*Geobacillus* sp.TY6	96%	MG725738
TY7	*Geobacillus* sp.TY7	94%	MG725739
TY11	*Geobacillus* sp.TY11	92%	MG725740
TYN6	*Geobacillus* sp.TYN6	94%	MG725741
TYN4	*Parageobacillus toebii* TYN4	92%	MG725742
TY8	*Parageobacillus toebii* TY8	93%	MG725743
TY9	*Parageobacillus toebii* TY9	93%	MG725744
LYN3	*Geobacillus toebii* LYN3	93%	MG725745
LYN5	*Geobacillus* sp.LYN5	90%	MG725746
LYN10	*Geobacillus toebii* LYN10	91%	MG725747

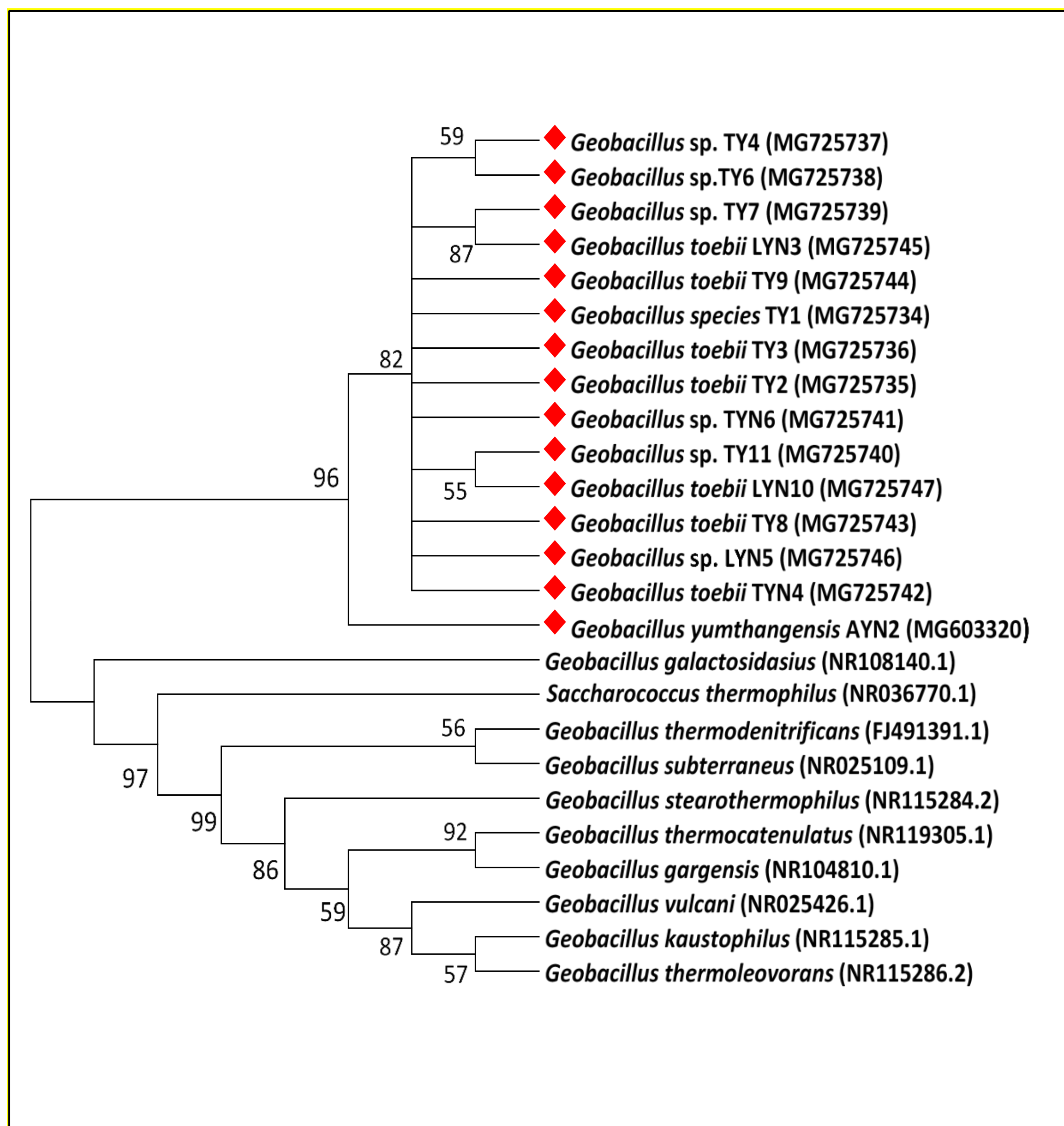

Fig.11a. Phylogenetic tree of some Yumthang Isolates. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis involved 25 nucleotide sequences. All positions containing gaps and missing data were eliminated. There. Evolutionary analyses were conducted in MEGA7.

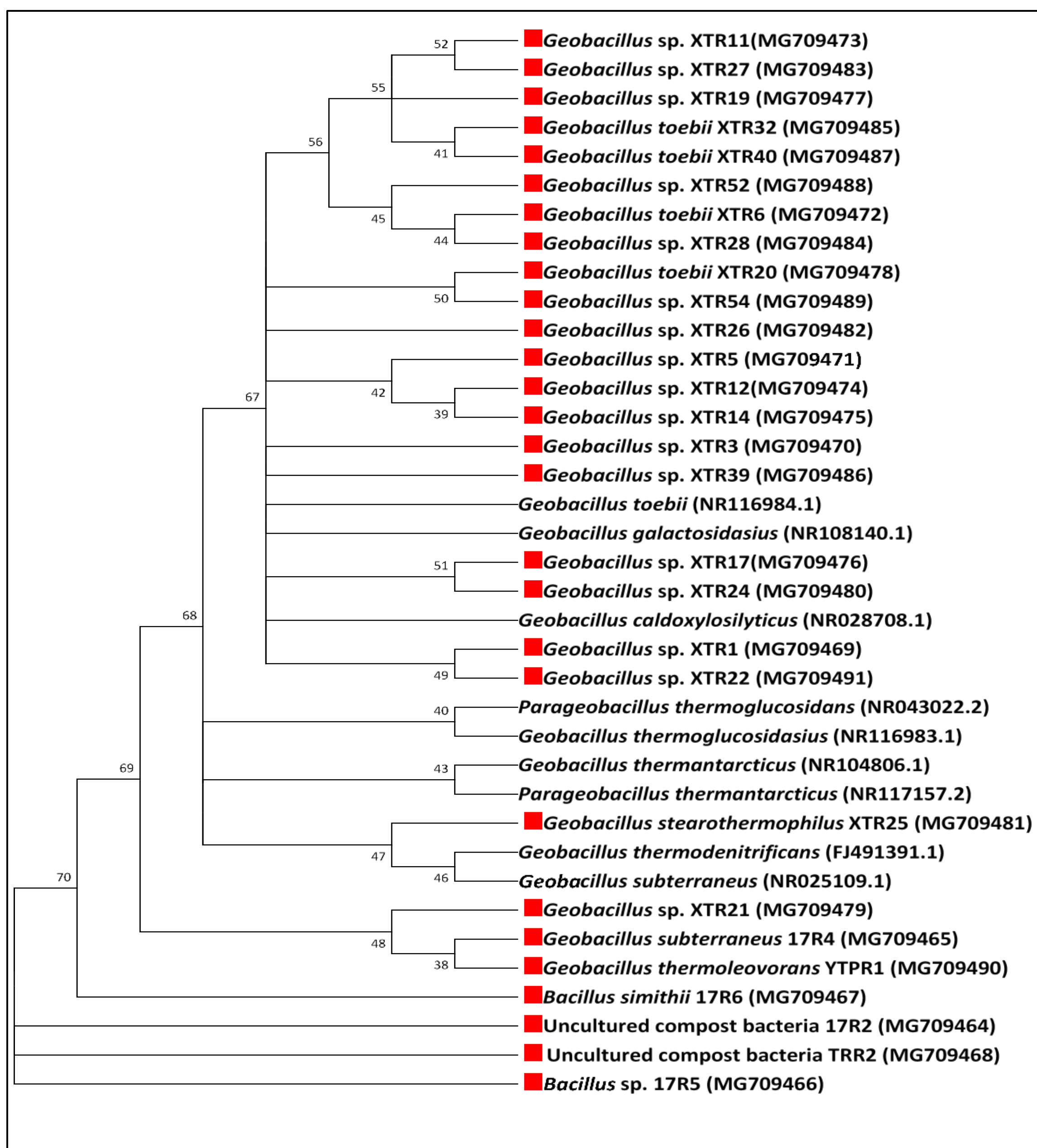

Fig.11b. Phylogenetic tree of Reshi isolates. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis involved 37 nucleotide sequences. All positions containing gaps and missing data were eliminated. There. Evolutionary analyses were conducted in MEGA7.

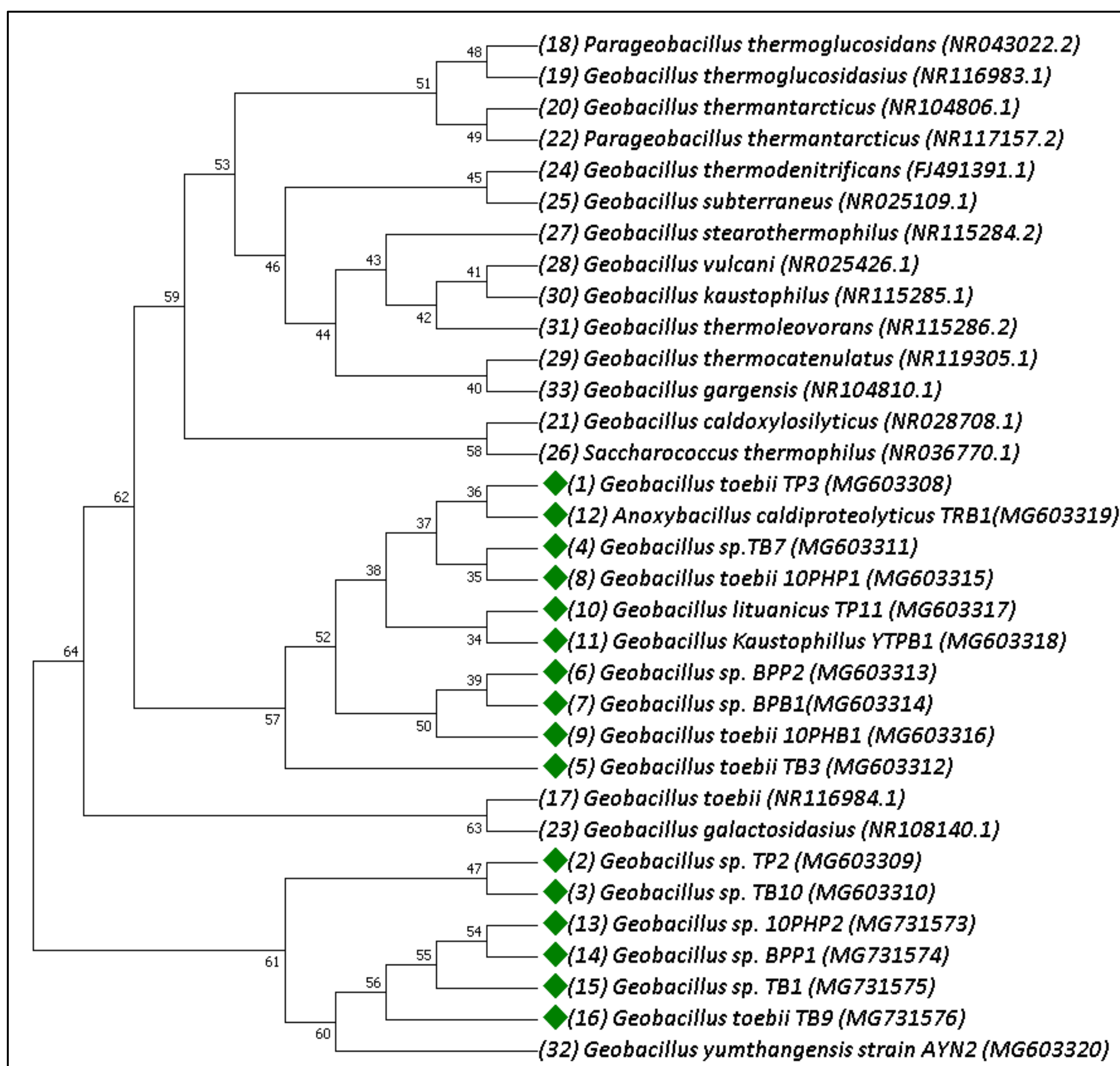

Fig.11c. Phylogenetic tree of Polok and Borong isolates. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.39 is shown. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis involved 33 nucleotide sequences. All positions containing gaps and missing data were eliminated. There. Evolutionary analyses were conducted in MEGA7.

6.5.2. Whole genome sequencing and analysis of AYN2 and LYN3 bacterial isolates

16s rRNA sequencing had provide molecular identification of our bacterial isolates, however to get more insights about the genetic make-up of these isolates, whole genome sequencing was carried out for two randomly selected isolates, i.e., AYN2 and LYN3. The whole genome sequencing was performed by using Illumina Hiseq 4000 sequencing technology with a paired end sequencing module **(Table 9).** The whole genome sequencing of AYN2 produced a total of 68,563,860 bp (68.56 Million) paired end reads with a maximum read length of 101 bp. For high quality data and genome assembly, the data was filtered by employing Next Generation Sequencing Quality Control (NGSQC) Toolkit and SQIT (Patel and Jain, 2012). The total QC passed high quality reads were 52,028,822 (52.03 Million). The overall quality of the data was good with more than 75.88% high quality reads. The primary genome assembly was carried out by Velvet (V 1.2.10) (Zerbino and Birney, 2008). The primary genome assembly statistics revealed the K-mer length of 71 with total number of 454 contigs. The average contig length was 7487.7 bp (~0.07 Mb) with N50 contig size of 24,353 (~0.02 Mb). Based on paired-end directional information, the genome was assembled into 264 scaffolds, with N length 27,853 bp (0.03 Mb) and average scaffold length 12,863.51 bp (~0.01 Mb) using SSPACE v3.0 scaffolder (Boetzer *et al.* 2011). The obtained draft genome was assembled resulting in a total genome size of ~3.3 Mb with (G + C) content of 42.28%. The final genome draft consists of 124 scaffolds with average scaffold length of 27,499.73 bp (~0.02 Mb) and N50 contig size of 2,988,775 bp (~ 3.0 Mb), constituting 3,409,966 bp (~ 3.4 Mb) of the genome with (G + C) content of 42.11%.

All the three types of rRNAs and possible tRNAs were identified, indicating a high degree of completeness in the genome assembly. Using ARAGORN tRNA identification method, the number of tRNA genes identified were 71. All the three RNAs (5S rRNA, 23S rRNA and 16S rRNA) were identified. Non-core genomic elements were also screened and using PlasmidFinder (V 1.3) (Carattoli *et al.* 2014), no plasmid sequences were found. The draft genome was annotated and its functional characterization was done. The number of genes predicted was 3712, with 2609 characterized proteins and 1090 hypothetical/putative proteins. The number of rRNA genes and tRNA genes were 5 and 71 respectively **(Table 9).** The circular representation of complete genome of AYN2 is given in **Fig. 12a.** The taxonomy identification method was performed using EzTaxon (Kim *et al.* 2012) and MEGA6, from which it was found that AYN2 is the putative species (as per sequence homology) and *Geobacillus toebii* NBRC 107807 is the closest homolog of the assembled genome (based on 16S rRNA match). *Geobacillus thermoglucosidasius* DSM 2542, complete genome was used as a reference genome (Ref Seq accession ID: NZ_CP012712.1). Using RAST, 962 proteins were annotated. **Fig. 13a** presents an overview of the count of each subsystem feature and the subsystem coverage. RAST functional annotation predicted (357) genes were linked to carbohydrate metabolism. The absence of any photosynthetic apparatus and gene clusters indicated that AYN2 may be adapted to a heterotrophic metabolism.

In case of LYN3 similar methodology was followed and it was found that Illumina Hiseq 4000 sequencing produced a total of 71,567,268 (71.57 Million) paired end reads with a maximum read length of 100 bp. The total QC passed high quality reads were 53,022,906 (53.02 Million). The overall quality of the data was good with more than 74.09% high

quality reads. The primary genome assembly statistics revealed the K-mer length of 73 with total number of 373 contigs. The average contig length was 8651.42 bp (~0.08 Mb) with N50 contig size of 25,681 (~0.03 Mb). Based on paired-end directional information, the genome was assembled into 249 scaffolds, with N length 28,453 bp (0.02 Mb) and average scaffold length 12,955.14 bp (~0.01 Mb) using SSPACE v3.0 scaffolder (Boetzer *et al.* 2011). The obtained draft genome was assembled resulting in a total genome size of ~3.2 Mb with (G + C) content of 42.33%. The final genome draft consists of 106 scaffolds with average scaffold length of 30,567.26 bp (~0.03 Mb) and N50 contig size of 2,927,468 bp (~ 3.0 Mb), constituting 3,240,130 bp (~ 3.2 Mb) of the genome with (G + C) content of 42.33%.

Using ARAGORN tRNA identification method, the number of tRNA genes identified were 80. All the three RNAs (5S rRNA, 23S rRNA and 16S rRNA) were identified. Non-core genomic elements were also screened and using PlasmidFinder (V 1.3) (Carattoli *et al.* 2014), no plasmid sequences were found. The draft genome was annotated and its functional characterization was done. The number of genes predicted was 3549, with 2405 characterized proteins and 1037 hypothetical/putative proteins. The number of rRNA genes and tRNA genes were 8 and 80 respectively **(Table 9).** The circular representation of complete genome of LYN3 is given in **Fig.12b.** The taxonomy identification method was performed using EzTaxon (Kim *et al.* 2012) and MEGA6, from which it was found that the bacterial strain LYN3 is the putative species (as per sequence homology) and *Geobacillus toebii* NBRC 107807 is the closest homolog of the assembled genome (based on 16S rRNA match). *Geobacillus thermoglucosidasius* DSM 2542, complete genome was used as a reference genome (Ref Seq accession ID: NZ_CP012712.1). Using RAST, 905

proteins were annotated. **Fig. 13b** presents an overview of the count of each subsystem feature and the subsystem coverage.

Table.9. Whole Genome characterization and accession numbers

Genome Characteristics	*Geobacillus yumthangensis* AYN2	*Geobacillus* sp. LYN3
NCBI Bioproject Id	PRJNA407404	PRJNA450255
NCBI Biosample Id	SAMN07653191	SAMN08933550
NCBI Genome Accession Number	NWUZ00000000	QCWL00000000
Sequencing Platform	ILLUMINA HISEQ 4000	ILLUMINA HISEQ 4000
Sequencing Module	PAIRED END	PAIRED END
Total Number Of Reads	6,856,386,000 (~ 6856 Mbp)	71,567,268 (~7156 Mbp)
Read Length	101 bp	100bp
Average Coverage	99.94%	99.93%
Estimated Genome Size	3,409,966 (~3.4 Mb)	3,240,130 bp (~ 3.2 Mb)
G+C Content	42.11%	42.33%
Protein Coding Genes	3631	3456
tRNA Coding Genes	71	80
rRNA Coding Genes	5	8
Plasmid Sequences	NONE	NONE

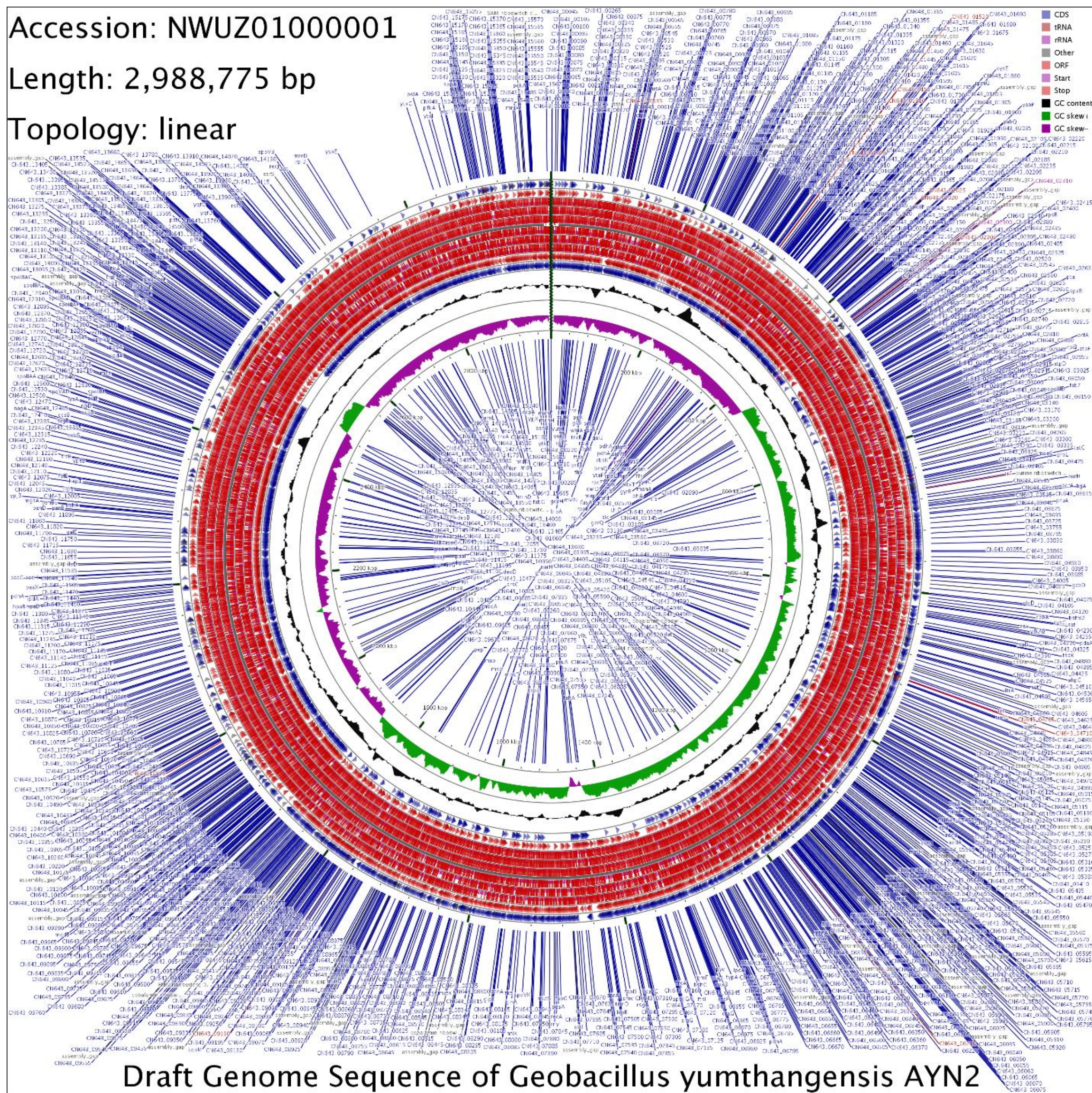

Fig.12a. Circular representation of complete genome of *Geobacillus yumthangensis* AYN2 nov. sp.. Labeling from the outside to inside circle: CDS on the forward strand (colored blue) with blast hits by reading frame, ORFs on the forward and reverse strands (colored by red), RNA genes (tRNAs maroon, rRNAs purple, other RNAs grey), (G+C) content (peaks out/inside the circle indicate values higher or lower than average (G+C) content, respectively, colored black),GC skew (calculated as (G-C)/(G+C), green/purple peaks out/inside the circle indicates values higher or lower than 1, respectively)

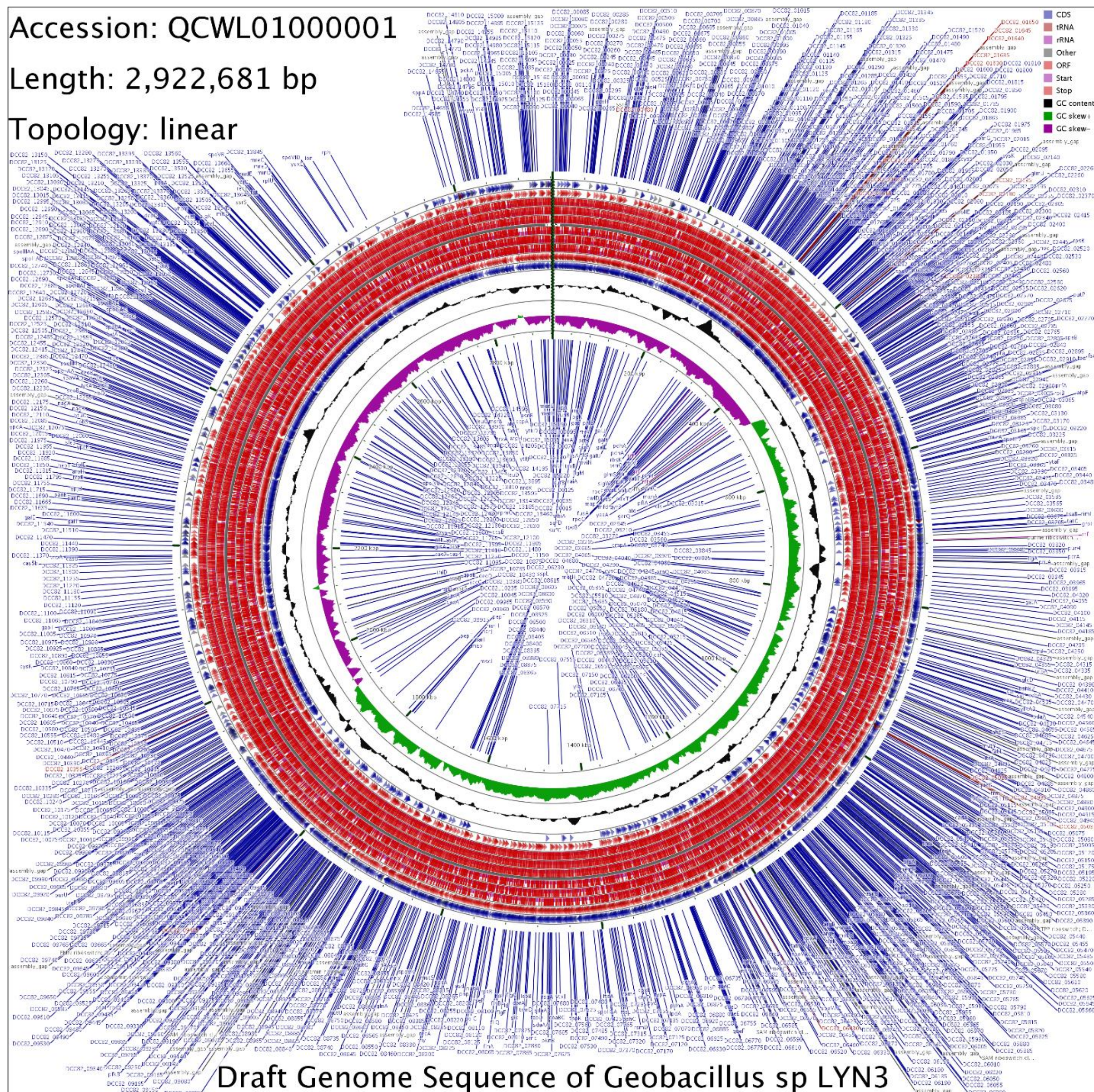

Fig.12b. Circular representation of complete genome of *Geobacillus* sp. LYN3. Labeling from the outside to inside circle: CDS on the forward strand (colored blue) with blast hits by reading frame, ORFs on the forward and reverse strands (colored by red), RNA genes (tRNAs maroon, rRNAs purple, other RNAs grey), (G+C) content (peaks out/inside the circle indicate values higher or lower than average (G+C) content, respectively, colored black),GC skew (calculated as (G-C)/(G+C), green/purple peaks out/inside the circle indicates values higher or lower than 1, respectively)

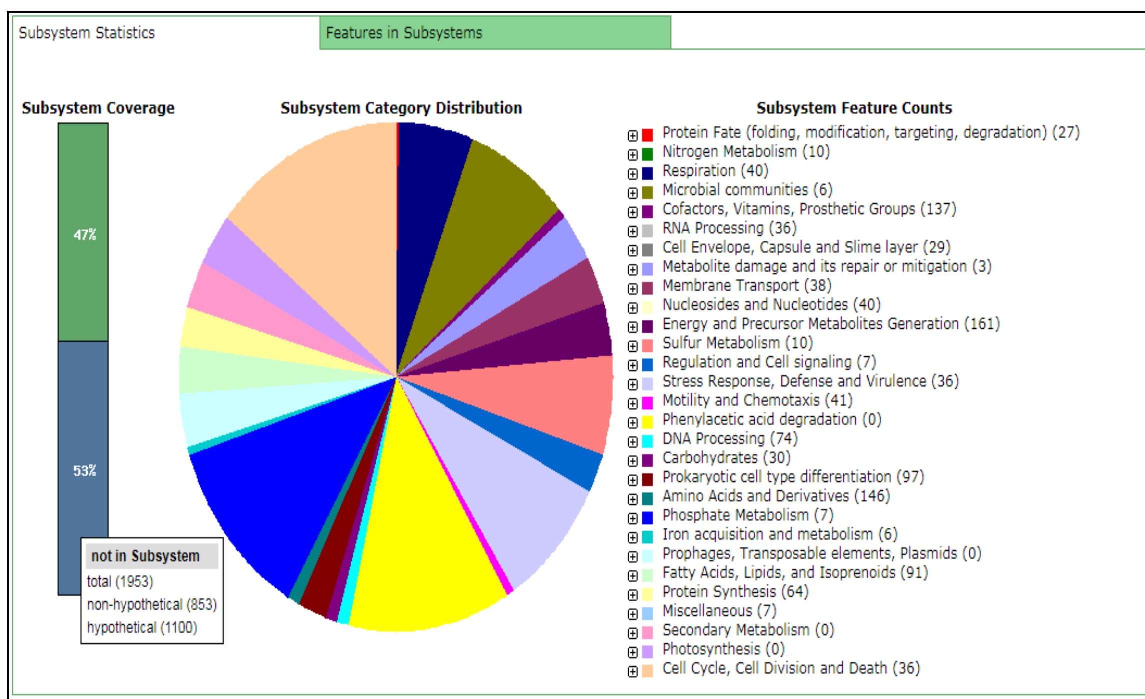

Fig13a. Subsystem features of stain AYN2.

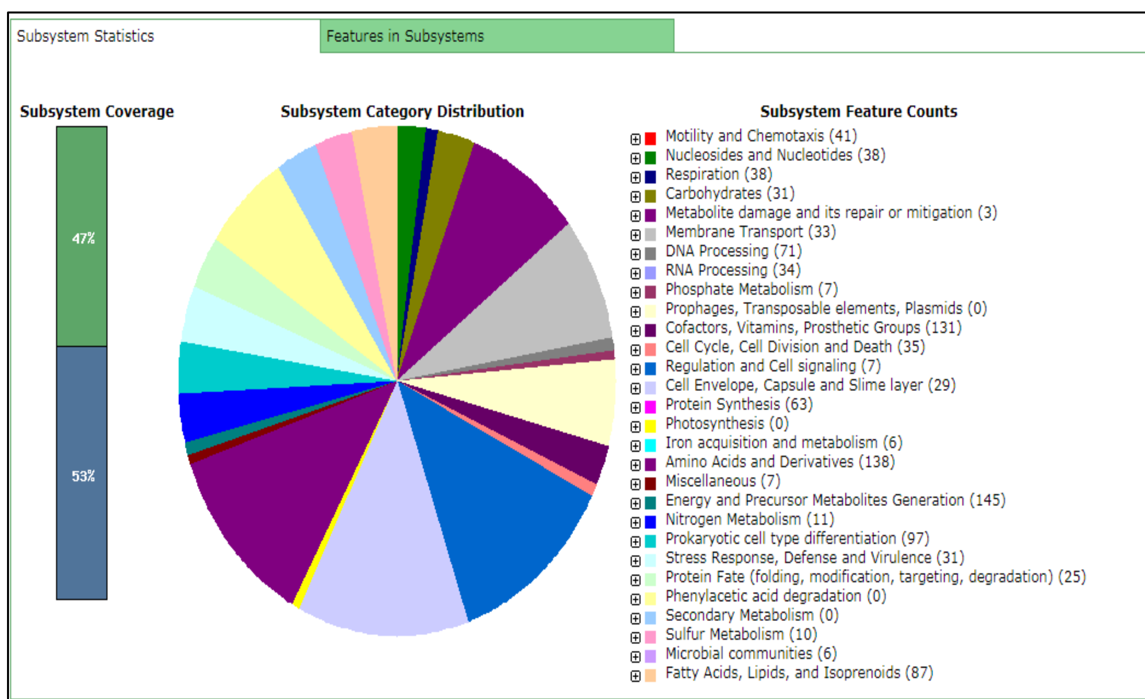

Fig13b. Subsystem features of stain AYN2.

6.5.3. Fatty acid methyl-ester Analysis

Fatty acid methyl-ester Analysis (FAME) analysis of three thermophilic bacterial isolates i.e., AYN2, LYN3 and CTRL6 was carried out. The results have shown that the major fatty acids present in AYN2 and LYN3 isolates were similar however, their concentrations vary considerably Table.10. It was found that the major and distinct fatty acids in these two isolates were iso-$C_{15:0}$, iso-$C_{16:0}$, and Iso-$C_{17:0}$. For example the fatty acid iso-$C_{15:0}$ was abundant in both the two isolates however, the percentage abundance varies considerably such that 12.8% was present in AYN2, and 21.9% in LYN3. However in case of strain CTRL6 the major fatty acids were a-$C_{15:0}$, $C_{16:0}$ and a-$C_{17:0}$ as shown in **Table.10.** These results represent the distinctive nature of these isolates which has been also confirmed by their SI (Sim Index) values. By analyzing the results using RTSBA6 Sherlock libraries, it was found that in case of strain AYN2 no matches were found with (SimIndex-0.00). The strain LYN3 was showing similarity with *Geobacillus stearothermophilus* possessing Sim Index of 0.201. And lastly the strain CTRL6 was showing similarity with *Paenibacillus macerans* having Sim Index of (SI-0.385).

Table.10. FAME analysis of three bacterial isolates.

	Fatty acid methyl ester analysis (FAME)											
Isolates	iso-C14:0	C14:0	iso-C15:0	a-C15:0	iso-C16:0	C16:0	Iso-C17:0	a-C17:0	C-17:0	Iso-C18:0	C18:1	C18:0
Geobacillus sp. AYN2	-	3.38	12.8	-	13.9	6.9	13.7	3.25	4.7	2.34	3.25	2.6
Geobacillus sp. LYN3	0.4	1.8	21.9	1.17	11.6	4.8	19.4	5.27	2.05	1.7	1.98	2.29
Geobacillus sp. CTRL6	2.23	0.85	15.2	33.91	6.04	9.6	4.29	8.34	-	-	1	3.72

6.6. Identification of novel thermophilic bacteria

16s rRNA sequencing results suggested that many of the isolates have a distinct percentage of identity from <95% and thus these isolates can be novel which also supported by whole genome sequence analysis and FAME analysis. In an order to confirm it we have carried polyphasic characterization of one of the isolates, i.e., AYN2 as guidelines given by "The International Committee on Systematic Bacteriology (ICSB)" (Logan *et al.* 2009). The bacterial strain AYN2 was isolated from Yumthang hot spring on Thermus medium incubated at 60°C for 24 hours.

6.6.1. Tentative identification by microscopy and biochemical test.

The strain AYN2 is off white in color, flat, circular and with opaque density. The cells are aerobic, rod shaped and motile. AYN2 is Gram-positive and able to form endospores. The SEM analysis showed that the size of bacteria was 2.5-5µm long and 0.4-0.6µm wide **Fig.14.** The growth at various physical parameters such as temperature (ranging from 40-

70°C), pH (ranging from 6-10) and NaCl concentration (ranging from 0-<4) was checked as per the above given protocols in material and methods. The results have shown that the strain AYN2 can grow between 40-70°C with the optimal temperature of 60°C and in the pH ranges from 6-10 with the optimum pH of 7.5-8.0 at the optimum growth temperature. The NaCl concentration for the growth of AYN2 was found to be in the range between 0-<4, with the optimum NaCl concentration of 1% at the optimum pH and temperature as shown in **Fig.15a, b and c.** Various biochemical parameters were checked such as carbohydrate fermentation, enzymatic tests such as amylase, protease, catalase, oxidase, nitrate reductase, methyl red test etc as per the procedures given above. All of the biochemical tests were also performed by BIOLOG system as per the guidelines given by manufacturer (Miller 1991). The results have shown that the cells are catalase positive, oxidase, nitrate reductase and methyl red test negative. The cells were able to utilize lactate, formate and acetate. Acids are produced from cellobiose, lactose, galactose, sorbitol, glucose but not from arabinose, ribose or xylose as shown in **Table.11.**

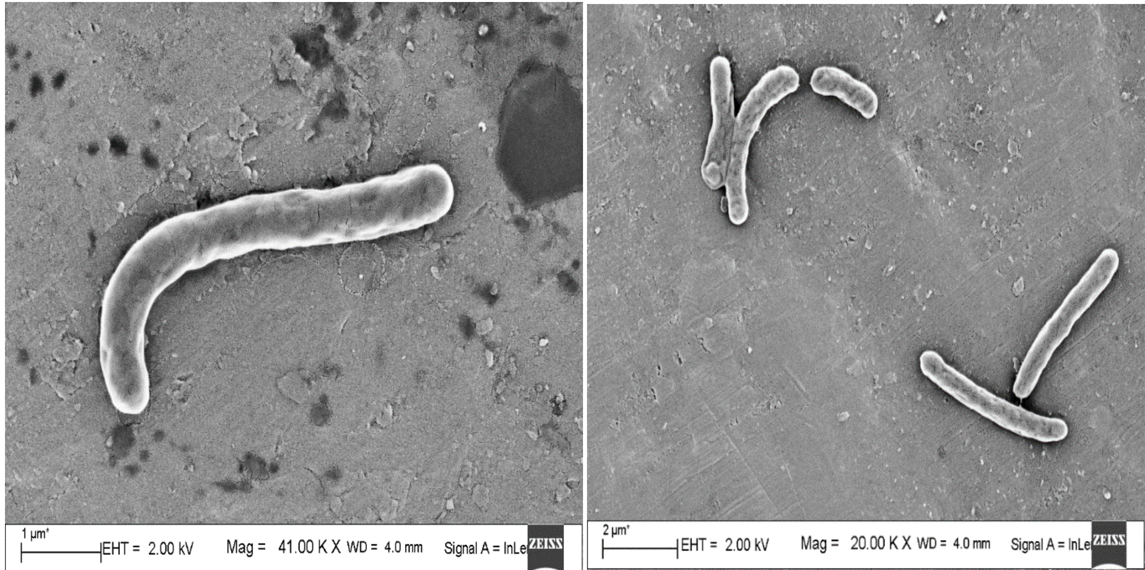

Fig.14. Scanning Electron Microscopy (SEM) of cells of strain AYN2

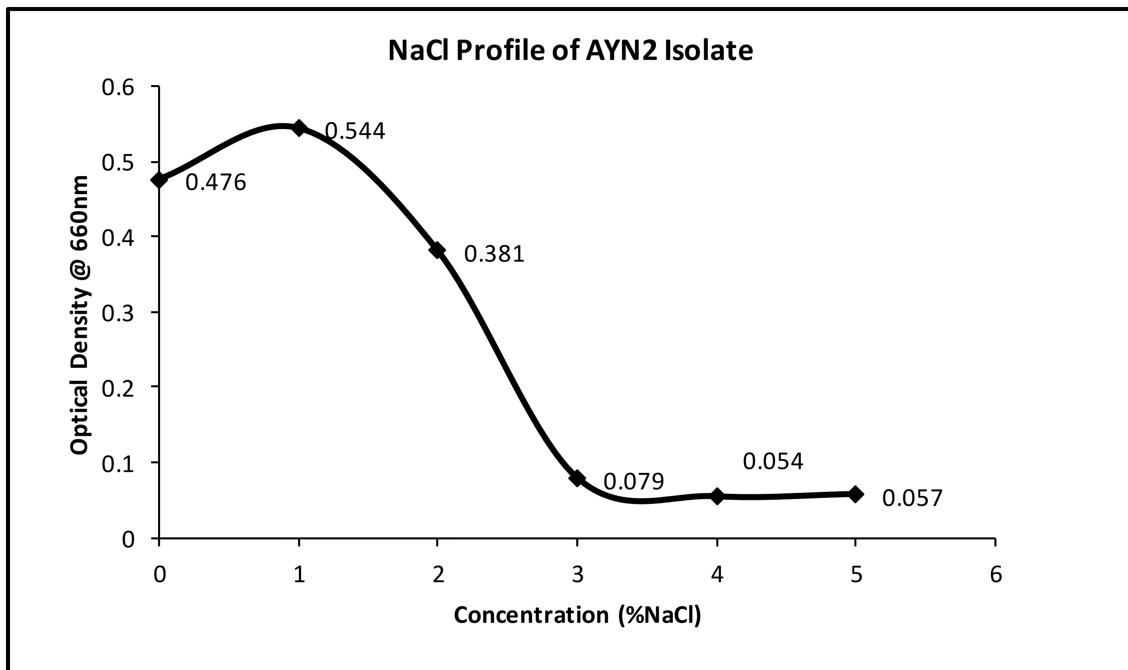

Fig 15.a. Growth profile of strain AYN2 at various NaCl concentrations. The optimum NaCl concentration is shown to be 1%.

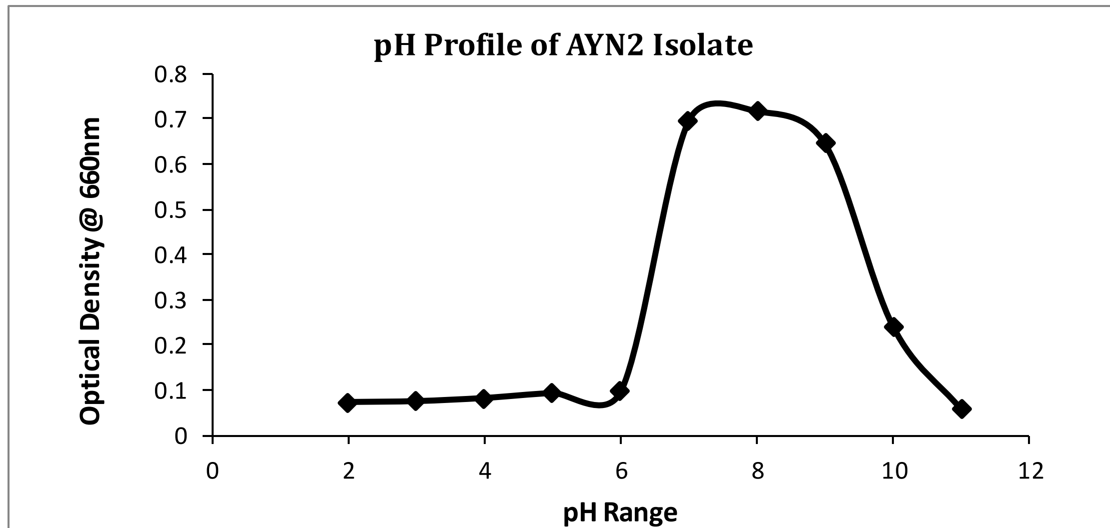

Fig 15.b. Growth profile at various pH and it was shown that optimal pH of strain AYN2 is 7.5-8.

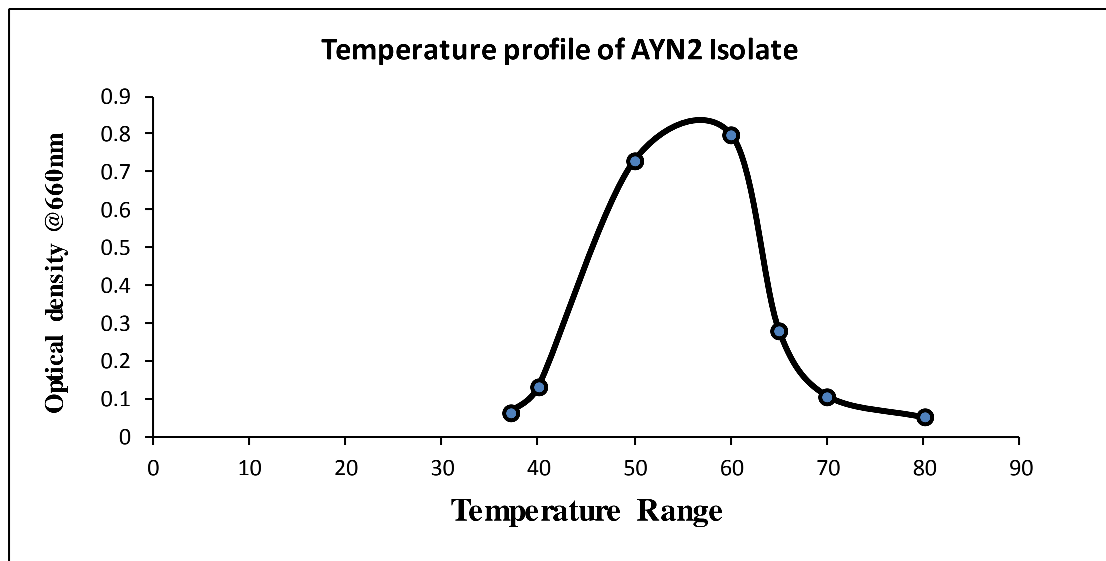

Fig 15.c. Growth profile of stain AYN2 at different temperatures. The optimum temperature was found to be 60°C.

Table 11. Phenotypic characteristics that differentiate *Geobacillus yumthangensis* sp. nov. strain AYN2^T from its phylogenetic neighbors. Taxa are indicated as: 1, *G. yumthangensis* strain AYN2^T (Manual lab based);2, *G. yumthangensis* strain AYN2^T(BIOLOG based) 3, *G. toebii* (Sung et al, 2002); 4, *G. thermoglucosidasius* (Suzuki *et al.* 1983);5,*G. uzenensis* ;6,*G. subterraneus* (Nazina et al, 2001); 7, *G.stearothermophilus* (Burgess *et al.* 2017); 8,*G. thermocatenulatus* (Golovacheva *et al.* 1975);9,*G. thermoleovorans* (Zarilla and Perry 1987);10,*G. kaustophilus* (White, Sharp and Priest 1993); 11,*G. thermodenitrifcans* (Manachini *et al.* 2000).; '+' = positive; '-' = negative; D= 11±89% of strains positive ; ND= Not Determined.

	1	2	3	4	5	6	7	8	9	10	11
Cell width (µm)	0.4-0.6	ND	0.5-0.9	<3	0.9-1.3	0.8-1.5	0.6-1	0.5-1.2	0.9	1.5	0.5-1.0
Cell length (µm)	2.5-5	ND	2-3.5	<0.9	4.7-8	4.7-8	2-3.5	3.0-7.0	6.0-8	3.5	1.5-2.5
Motility	+	ND	+	ND	+	+	+	+	+	–	ND
Production of acids from:											
Adonitol	ND	ND	–	+	–	–	ND	–	ND	ND	ND
L-Arabinose	–	ND	–	–	+	–	D	–	–	D	+
Cellobiose	+	+	–	+	+	+	–	+	+	+	+
Galactose	–	+	–	D	+	+	–	–	+	+	+
Ribose	+	ND	–	–	+	+	ND	ND	ND	+	+
Glycerol	ND	+	–	–	+	+	+	+	+	D	+
Inositol	–	+	+	+	–	–	–	–	–	–	ND
Lactose	–	+	–	–	–	–	–	–	–	–	+
Rhamnose	–	+	–	–	–	–	–	+	–	–	–
Sorbitol	–	+	–	–	–	–	–	+	ND	–	ND
D-Xylose	–	ND	–	+	–	–	D	+	–	D	+
Hydrolysis of:											
Gelatin	–	+	–	+	+	–	D	–	–	ND	ND
Casein	–	–	+	+	–	–	D	+	ND	+	–
Starch	+	+	–	+	+	+	+	+	–	D	+
Aesculin	–	ND	–	–	+	+	ND	+	ND	ND	ND
Utilization of:											
Formate	ND	+	–	D	–	+	–	ND	ND	ND	ND
Acetate	ND	+	–	–	+	+	–	ND	ND	ND	ND
Lactate	ND	+	–	–	+	+	–	ND	ND	ND	ND
Citrate	–	–	–	+			D	D	+	ND	ND
Fermentation of glucose	+	+	–	–	–	–	D	–	+	–	ND
Methyl Red test	–	ND	–	–	–	+	D	D	ND	ND	ND
Denitrification	ND	ND	+	ND	–	+	–	–	+	ND	+
NaCl %, w/v	0-<5	0-<5	0-<5	0-<5	0-4	0-5	0-5	0-1.5	0-4	ND	0-3
pH range	6.0-10	>6.0	6.0-9.0	6.0-8.0	6.2-7.8	6.0-7.8	6.0-8.0	6.5-8.5	6.2-7.8	6.2-7.5	6.0-8.0
Temperature range (°C)	40-70	ND	45-70	37-68	45-65	45-70	37-65	42-69	35-78	40-75	45-70

6.6.2. Fatty Acid Methyl Ester (FAME) analysis

The fatty acid analysis of the novel strain was performed at 50°C. Fatty acids were extracted and analyzed following the instructions of the Microbial Identification System (MIDI). The results have shown that the predominant fatty acids were iso-$C_{15:0}$ (12.8%), iso-$C_{16:0}$ (13.9%) and iso-$C_{17:0}$ (13.7%). The comparison of various fatty acids of strain AYN2^T and its closest relatives are shown in **Table.12.** By analyzing the results using RTSBA6 Sherlock libraries, it was found that in case of strain AYN2 no matches were found with (SimIndex-0.00).

6.6.3. Identification by 16S rDNA and *rpoB*, *dnak, dnaJ* gene sequence analysis.

The 16S rRNA sequence was amplified by using universal primers (27F and 1492R) as described by Hugenholtz P, *et al*, 1998 (Hugenholtz *et al.* 1998) The strain AYN2^T was showing only 96% identity with the *Geobacillus toebii* strain R-35642. The 16S rRNA sequence of strain AYN2^T was aligned with representative 16S rRNA sequences of related taxa using Clustal W software (Thompson, Higgins and Gibson 1994). A phylogenetic tree was constructed using the neighbor joining method (Saitou and Nei 1987)and the software package MEGA 7 to demonstrate the relationship of strain AYN2^T to other members of the *Geobacillus* family (Fig.16). Also, three more conserved genes, i.e., *rpoB, DnaK* and *dnaJ* genes DNA sequence was also investigated. After performing BLAST searches for *rpoB* gene, the results showed 88% identity with *Geobacillus thermoglucosidasius.* The heat shock chaperone genes, i.e., *dnaK* and *dnaJ* showed 89% and 90% identity with *Geobacillus thermoglucosidasius* and *Bacillus thermoglucosidasius* respectively.

Table.12. Fatty acid composition of *G. yumthangensis* sp. nov. strain AYN2^T and type strains of species of the *Geobacillus* with validly published names. Strains: *G. yumthangensis strain* AYN2^T; *G. toebii* (Sung *et al.* 2002)***;*** *G. thermodenitrificans* (Nazina *et al.* 2001)*; G. thermoleovorans* (Nazina *et al.* 2001)***;*** *G. thermocatenulatus* (Nazina *et al.* 2001)*; G. stearothermophilus,* (Kämpfer 1994)*;*

	iso-C14:0	**C14:0**	**iso-$C_{15:0}$**	**a-$C_{15:0}$**	**$C_{15:0}$**	**iso-$C_{16:0}$**	**$C_{16:0}$**	**Iso-$C_{17:0}$**	**a-$C_{17:0}$**	**C-$_{17:0}$**	**Iso-$C_{18:0}$**	**$C_{18:1}$**	**$C_{18:0}$**	**References**
***G. yumthangensis strain* AYN2^T**		3.3	**12.8**			**13.9**	7.0	**13.7**	3.2	4.7	2.3	3.2	2.6	[Najar *et al.* 2018b]
G. toebii			34.0			17.0		34.0						[Sung *et al.* 2002]
G. thermodenitrificans	0.4	1.8	33.6	1.8	2.3	9.5	11.0	26.6	7.3	2.9	0.2	1.3	1.3	[Nazina *et al.* 2001]
G.thermoleovorans	1.0	1.4	22.6	1.3	2.1	21.0	11.2	18.5	4.6	1.3	0.9	1.2	3.4	[Nazina *et al.* 2001]
G.thermocatenulatus	1.3	0.6	25.5	0.6	1.3	31.8	8.3	21.0	3.1	2.3	1.3	0.7	2.2	[Nazina *et al.* 2001]
G.stearothermophilus	0.1	1.5	39.8	6.4	0.5	6.2	9.2	17.1	13.3					[Kämpfer 1994]
G.thermoglucosidasius		0.6	22.0	1.6		10.4	11.6	30.3	16.6	0.8				[Kämpfer 1994]

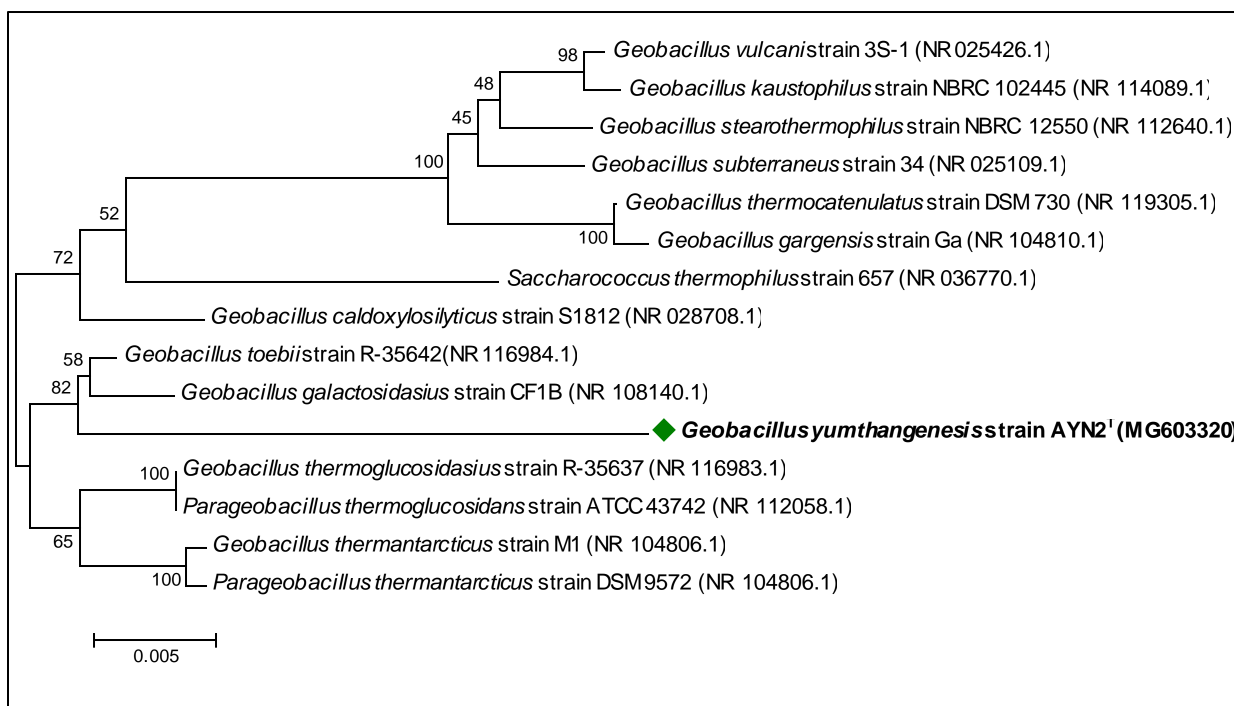

Fig.16. Phylogenetic tree showing the position of strain AYN2^T among *Geobacillus* species and related taxa. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA7.

6.6.4. G+C Content and DNA–DNA hybridization

With the help of whole genome analysis the G+C content of AYN2 genome was found to be 42.1mol%. The comparison of strain AYN2 and other related taxa is shown in **Table.13.** The original methods of determining average nucleotide identity, and average nucleotide identity of orthologous genes were calculated using the Orthologous Average Nucleotide Identity Tool version 0.93(Lee *et al.* 2016). The original average nucleotide identity, and the average nucleotide identity of orthologous genes between strain AYN2^T and the closest species *Geobacillus toebii* was 97.6% and 97.8%. Digital DNA–DNA hybridization values were determined using the Genome-to-Genome Distance Calculator (GGDC)version

2.1(Meier-Kolthoff *et al.* 2013). The digital DNA–DNA hybridization values were 69.10%. However, the average nucleotide identity (ANI) and Digital DNA–DNA hybridization values were little higher or comparable to the cut off values of ~96% and <70% respectively.

Table.13. Comparison of G+C content among various *Geobacillus* species

Species	**G+C Content (mol%)**	**Reference**
Geobacillus thermoleovorans	**53.7**	Nazina et al. (2001)
Geobacillus thermoglucosidasius	**43.9**	(Brumm, Land and Mead 2015)
Geobacillus toebii	**43.9**	Sung et al. (2002)
***Geobacillus sp.* WCH70**	**43.0**	(Brumm, Land and Mead 2016)
Geobacillus kaustophilus	**52.0-58.0**	Nazina et al. (2001)
Geobacillus stearothermophilus	**51.9**	Nazina et al. (2001)
***Geobacillus yumthangensis strain* AYN2^{T}**	**42.1**	Najar et al.(2018b)

6.6.5. Deposition and Accession numbers

The strain AYN2 has been deposited in MTCC (Microbial Type Culture Collection, India), KCTC (Korean Collection for Type Cultures) and JCM (Japan Collection of Microorganisms). The genomic sequence and the sequences dnaK and dnaJ has been submitted to NCBI. The various accession numbers are given in **Table.14.**

Table.14. Whole genome sequence and culture collection centre accession numbers.

NCBI BIOPROJECT ID	PRJNA407404
NCBI BIOSAMPLE ID	SAMN07653191
NCBI GENOME ACCESSION NUMBER	NWUZ00000000
MTCC ACCESSION NUMBER	MTCC 12749
JCM ACCESSION NUMBER	JCM 32596
KCTC ACCESSION NUMBER	KCTC 33950
16S rRNA NCBI ACCESSION NUMBER	MG603320
DnaK ACCESSION NUMBER	MG656999
DnaJ ACCESSION NUMBER	MG657000

6.7. Analysis of bacterial diversity by culture independent techniques

6.7.1. Phospholipid Fatty Acid Analysis (PLFA)

Phospholipids are crucial constituents of microbial membranes (Powl, East and Lee 2007) and it has been reported that they vary between different species among prokaryotes (Joergensen and Wichern 2008) which makes it an important chemotaxonomic marker. PLFA analysis of four hot springs of Sikkim suggested that the major fatty acids significantly varied among the four hot springs. It was found that the Branched chain fatty acids and polyunsaturated fatty acids (PUFA) were abundant and similar in all the four hot springs with branched chain highest in case of Yumthang (33.1) whereas PUFA were highest in case of Polok (60.46). However, the straight chain fatty acids were relatively higher in case of Yumthang (22.74) and Borong (11.28) as shown in **Table.15**. The comparisons of these fatty acids are also shown in matrix diagram **Fig.17.**

Table.15. Abundance of various Fatty Acids in four hot springs.

Fatty Acids	RESHI	YUMTHANG	BORONG	POLOK
Straight	7.91	22.74	11.28	3.34
Branched	28.9	33.1	26.79	27.07
MUFA	4.13	5.8	3.04	4.75
PUFA	55.21	36.62	55.32	60.46
DMA	0.93	0.66	0.54	0.94
18:1 w9c	1.22	0.19	1.25	1.44
18:1 w6,9c	1.7	0.16	1.79	1.98

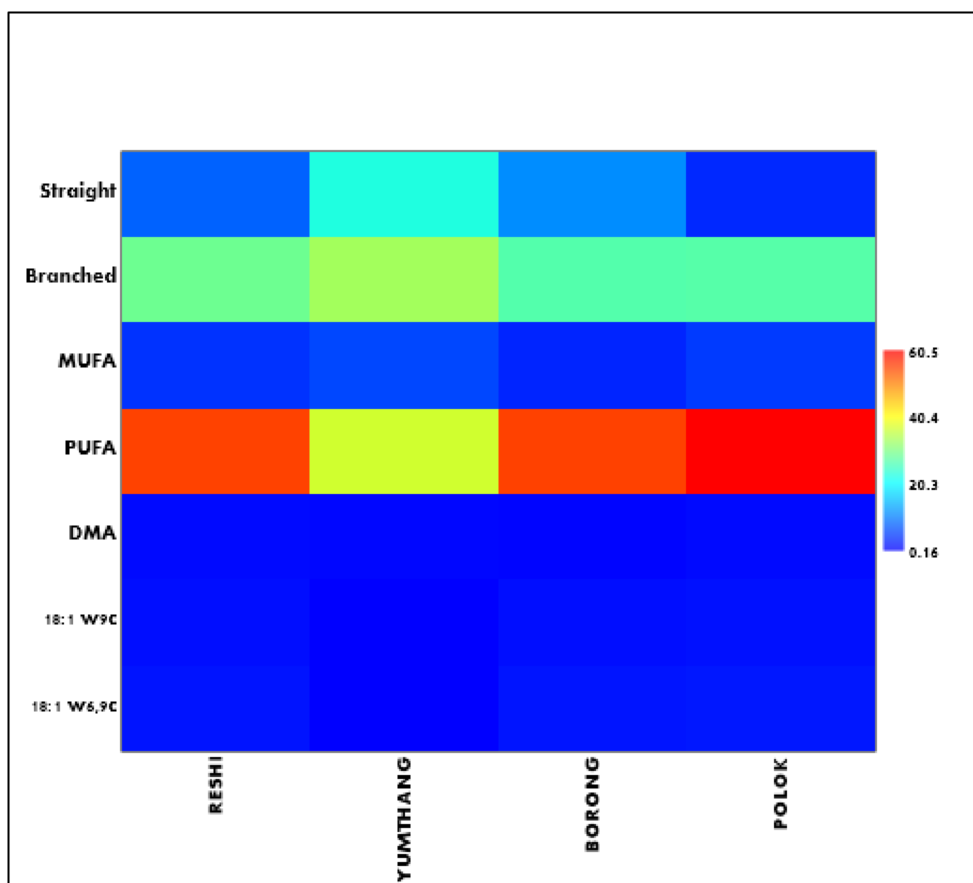

Fig.17. Matrix diagram comparing various fatty acids in four hot springs.

The PLFA results showed that the four hot springs were considerably different with respect to their biomass content. However, the biomass content of Borong hot spring was higher (1044.939 nmoles g^{-1}) followed by Reshi (905.0nmoles g^{-1}) and Polok hot spring (838.859 nmoles g^{-1}) (**Table.16.** and **Fig.18**. Fatty acid marker analysis with Sherlock PLFA tool defined the community structure of hot springs, i.e., the abundance of Gram-positive bacteria, Gram-negative bacteria, anaerobic bacteria, fungi, and eukaryotes. The results showed that Gram-positive bacteria were relatively higher in Yumthang (42.74%) followed by Borong hot spring (30.03%) and the Polok hot spring (27.94%), while Gram-negative bacteria were higher and similar in Yumthang (7.77%) and Polok (7.17%) than the Borong (4.85%). The percentage of Fungi and Eukaryotes were similar in all the hot Springs as shown in **Table.16** and **Fig.19a,b**. However, eukaryotes were slightly lower in case of Yumthang (47.48%). The abundance of various fatty acid types was also investigated and it was found that there was no much distinction between major fatty acids present in three of the hot springs such as Reshi, Polok and Borong which are located in the similar region that is in south district of Sikkim. However there was distinct fatty acid compositions in Yumthang hot spring which is located in North Sikkim. The abundant fatty acid found in the springs were 12:00, 15:3 ω3c, 15:0 anteiso, 17:1 iso ω9c, 17:0 anteiso, 18:2 ω6c, 18:1 ω9c, 19:3 ω3c and 21:3 ω3c**.** However, in case of Yumthang hot spring the abundant fatty acids were 11:0 iso, 12:00, 13:0 iso, 15:0 iso, 19:3 ω3c and 21:3 ω3c. Also it was shown that the fatty acids such as 11:0 iso, 13:0 iso, and 15:0 iso were only present in Yumthang hot spring. The abundance of various fatty acids was represented by plot matrix as shown in **Fig.20.**

Table.16. Community structure of hot springs based on PLFA studies.

Hot Spring	Gram Positive	Gram Negative	Anaerobe	Actinomycetes	AM Fungi	Fungi	Methanobacter	Eukaryote	Biomass content(nmoles/gm)
Reshi	31.22	5.82	1.01	---	---	1.86	---	60.09	905.076
Yumthang	42.74	7.77	0.86	0.95	---	0.2	---	47.48	178.48
Borong	30.03	4.85	0.61	---	---	2.03	---	62.41	1044.9
Polok	27.94	7.17	0.43	---	---	2.05	---	62.41	838.8

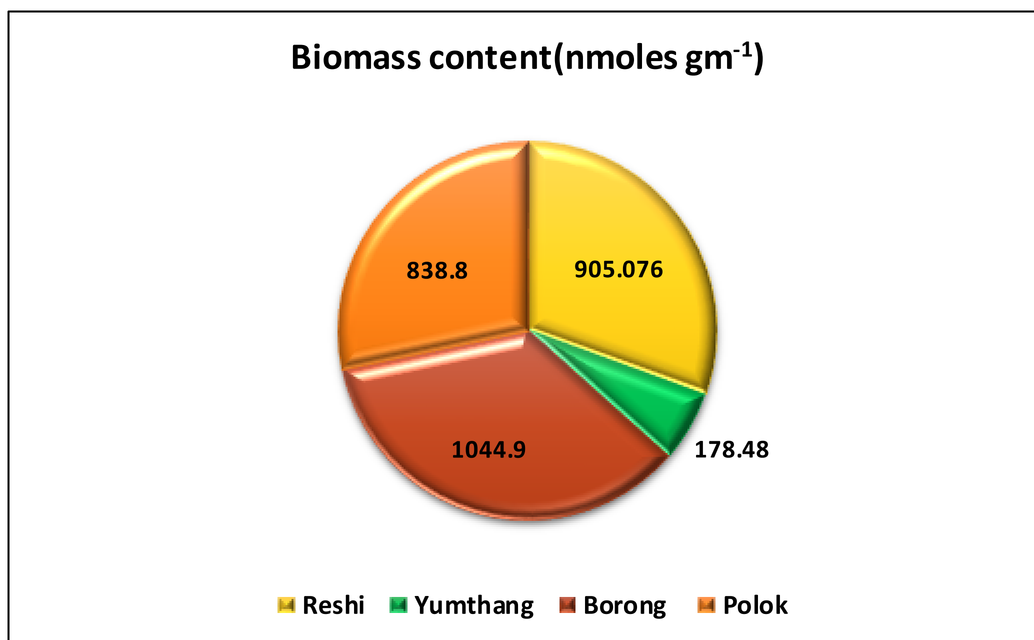

Fig.18. Biomass content (nmoles g^{-1}) in four hot springs of Sikkim

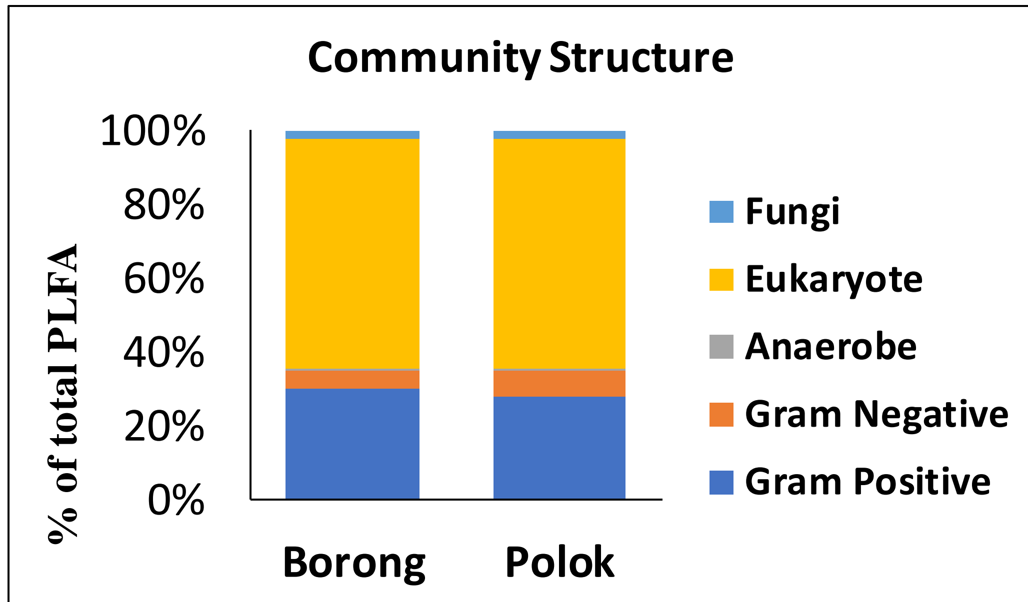

Fig.19a. Community structure of Polok and Borong hot springs based on PLFA studies

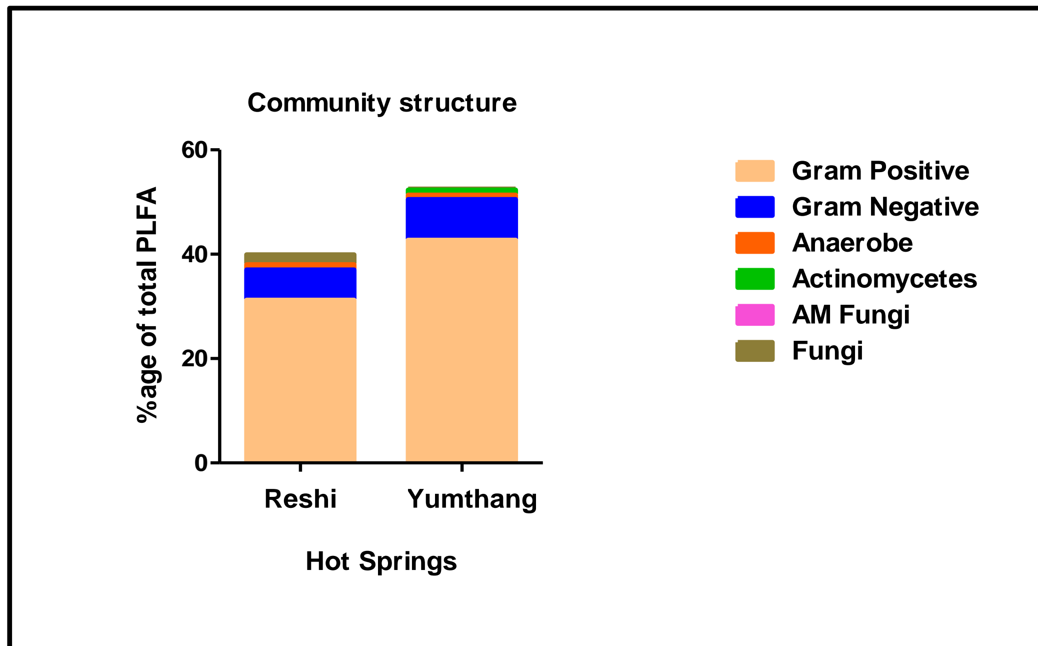

Fig.19b. Community structure of Reshi and Yumthang hot springs based on PLFA studies

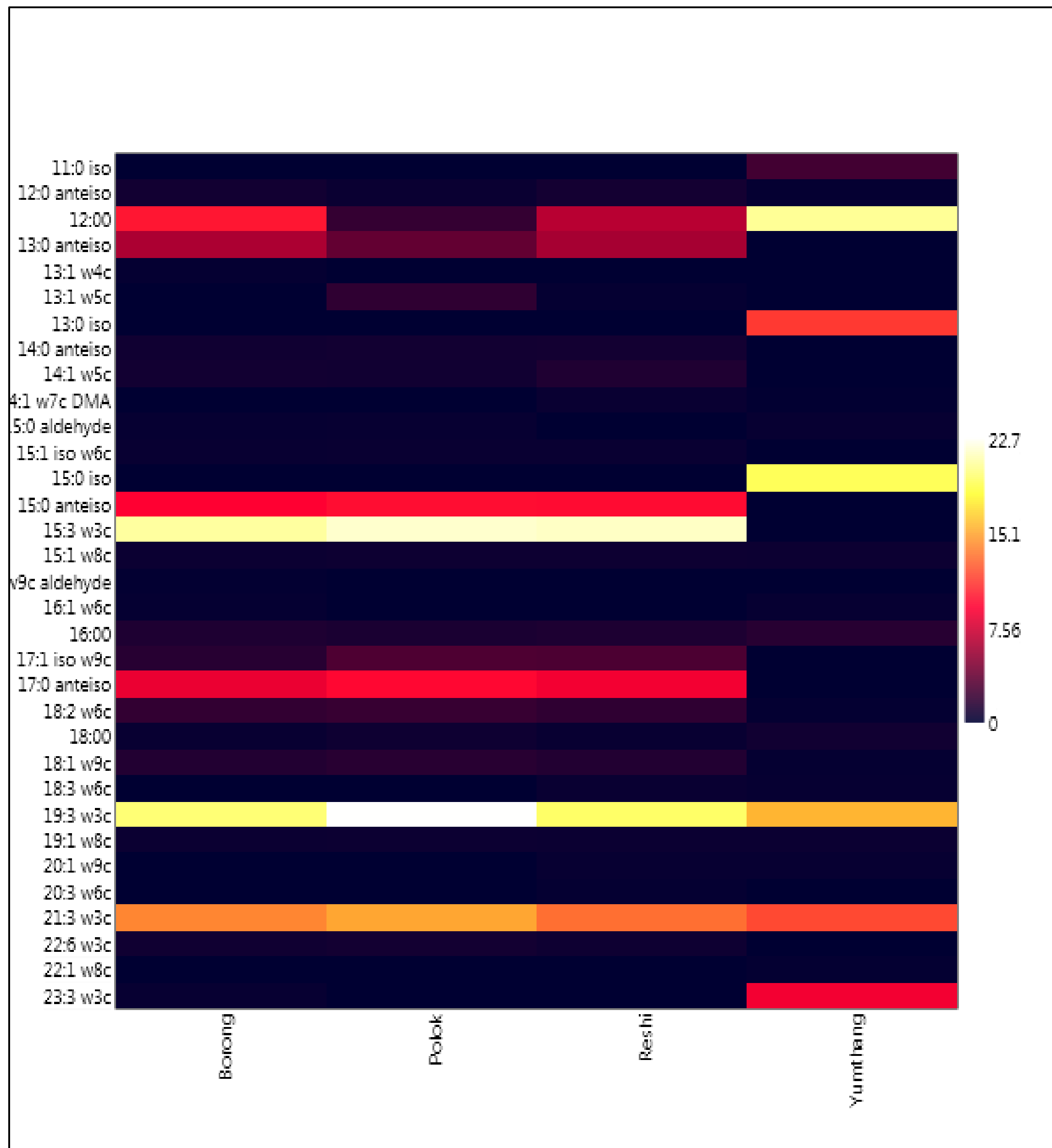

Fig.20. Matrix plot showing abundance of various fatty acids in four hot springs.

6.7.1.1. Statistical Analysis

The correlation between fatty acids with respect to different studied hot springs was carried out using Principal component analysis (PCA) **Fig.21.** The F1 component of the principal component analysis (PCA) of the fatty acids showed the relatively significant picture of the correlation between various fatty acids with respect to four hot springs. The F1 component possessing (77.44%) variability with Eigen value of 3.09 as shown in **Table.17.** There was significant Pearson (n) correlation in three hot springs such as Polok, Borong, and Reshi with significant p-value<0.05. However, Yumthang was significantly correlated to Borong hot spring only as shown in **Table.18.** The results have shown the positive correlation among 13:0 antiiso, 15:3 ω3c, 15:0 antiiso, and 17:0 anteiso to the Polok, Borong and Reshi hot springs whereas the other remaining fatty acids were less positive correlated. However, in case of Yumthang hot spring the specific fatty acids such as 12:0, 19:3 ω3c, 21:3 ω3c, 15:0 iso, 13:0 iso, 23:3 ω3c and 11:0 iso were positively correlated **Fig.21.**

Similarly correlation among microbial diversity based on PLFA and physicochemical parameters were carried out by principal component analysis. The results have shown that the PC1/F1 represents the maximum variability of (96.85%) as shown in **Table.19.** There was significant Pearson (n) correlation in all the four hot springs such as Polok, Borong, Reshi and Yumthang with significant p-value<0.05 as shown in **Table.20.** It has been shown that the Polok and Yumthang are positively correlated with each other and with respect to parameters such as temperature, Gram-positive bacteria and Chloride, whereas Reshi and Borong are positively correlated with respect to other remaining parameters such as total alkalinity, biomass, magnesium etc. as shown in **Fig.21**

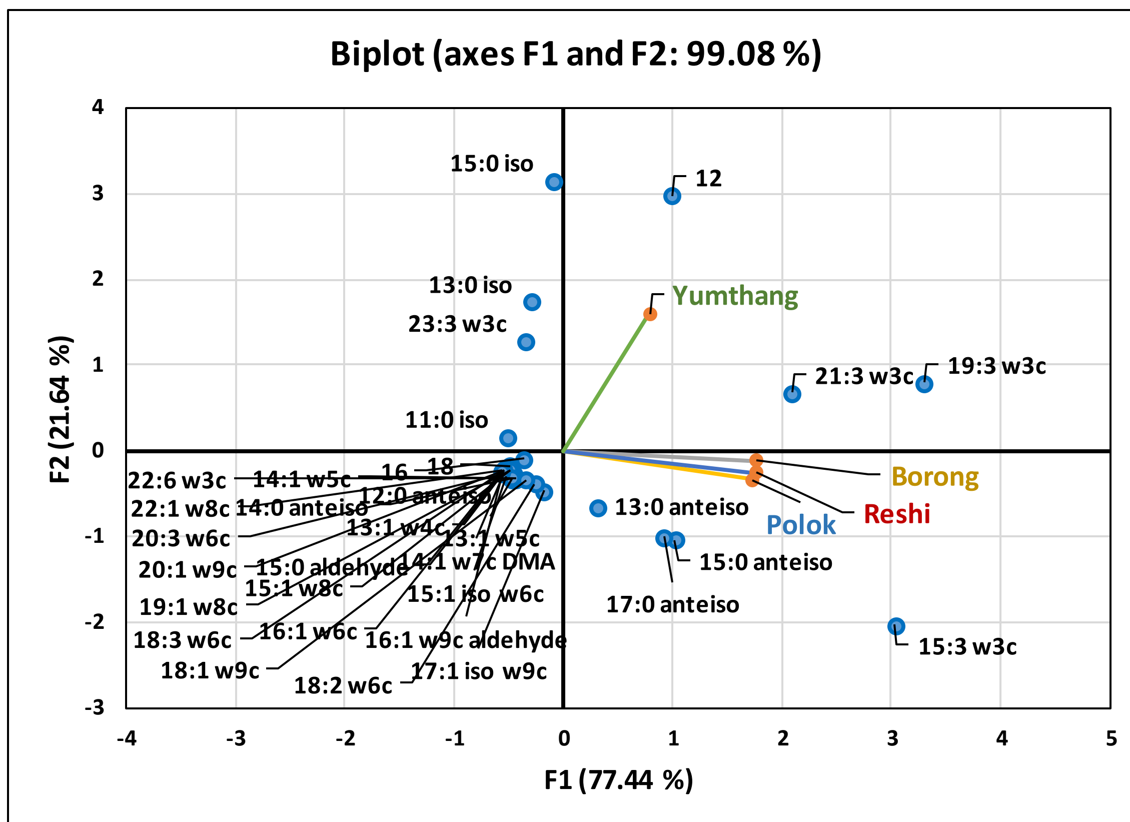

Fig.21. Principal Component Analysis showing correlation between fatty acids with respect to different studied hot springs.

Table.17. Principal Component Analysis (Eigenvalues

	F1	F2	F3	F4
Eigenvalue	3.0976	0.8657	0.0346	0.0022
Variability (%)	77.4392	21.6423	0.8645	0.0539
Cumulative %	77.4392	99.0815	99.9461	100.0000

Table.18. Correlation matrix (Pearson (n)):

Variables	Borong	Polok	Reshi	Yumthang
Borong	**1**	**0.9603**	**0.9924**	**0.3765**
Polok	**0.9603**	**1**	**0.9809**	0.2635
Reshi	**0.9924**	**0.9809**	**1**	0.3038
Yumthang	**0.3765**	0.2635	0.3038	**1**

Values in bold are different from 0 with a significance level alpha=0.05

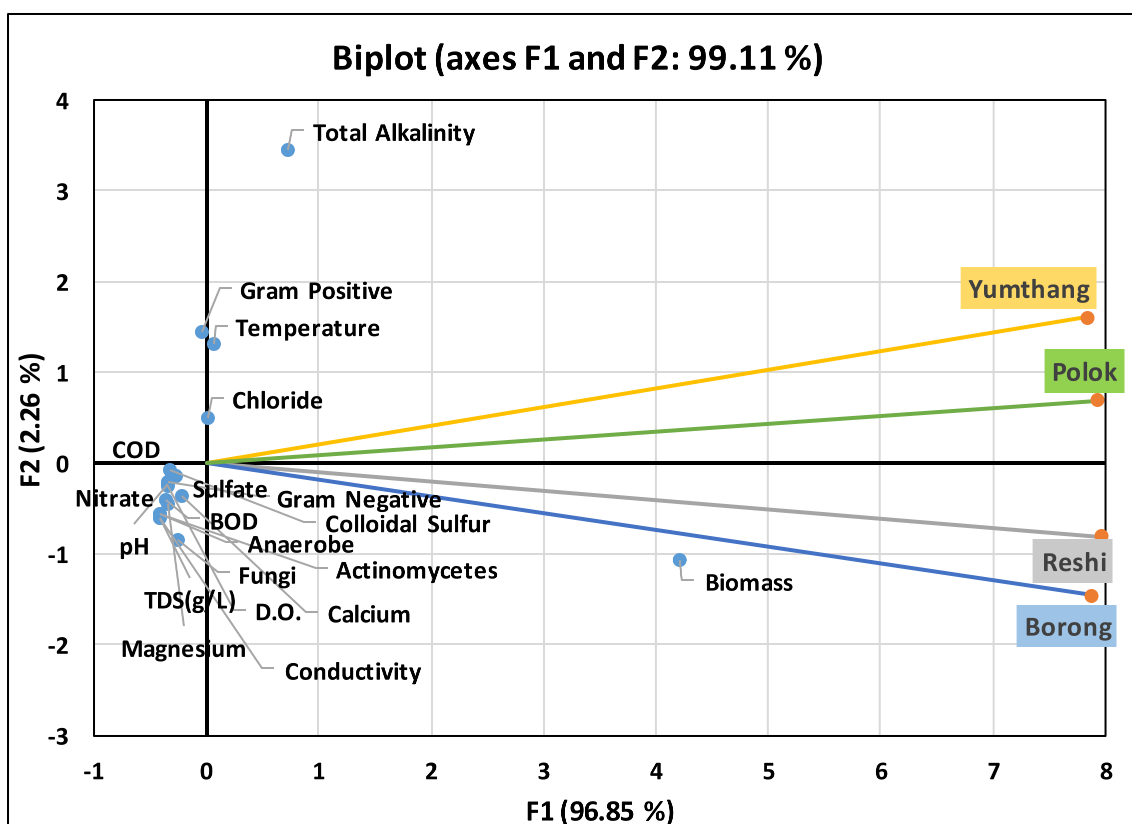

Fig.22. Principal Component analysis showing the correlation between biodiversity based on PLFA and physicochemical parameters among four hot springs of Sikkim.

Table.19. Principal Component Analysis the correlation between biodiversity based on PLFA and physicochemical parameters (Eigenvalues):

	F1	F2	F3	F4
Eigenvalue	3.8740	0.0903	0.0263	0.0093
Variability (%)	96.8498	2.2580	0.6586	0.2336
Cumulative %	96.8498	99.1078	99.7664	100.0000

Table.20. Correlation matrix (Pearson (n)) between biodiversity based on PLFA and physicochemical parameters

Variables	Reshi	Yumthang	Borong	Polok
Reshi	**1**	**0.9492**	**0.9875**	**0.9664**
Yumthang	**0.9492**	**1**	**0.9238**	**0.9693**
Borong	**0.9875**	**0.9238**	**1**	**0.9515**
Polok	**0.9664**	**0.9693**	**0.9515**	**1**

Values in bold are different from 0 with a significance level alpha=0.05

6.7.2. Metagenomic Analysis

The 16 s rRNA gene library of Borong was comprised of 3, 72,480 reads with average sequence length of 301 bp. The average GC content was 53%. While, 3, 98,782 reads were obtained from 16 s rRNA gene library of Polok hot spring with an average sequence length of 301 bp. The mean GC content was estimated to be 54%. A total of 409 OTUs were clustered using UClust. Approximately, 104 OTUs were obtained from Borong hot spring and 360 OTUs were obtained from Polok hot spring. The gene library of Reshi was comprised of 61898482 reads whereas the gene library of Yumthang was 37338796 reads with an average sequence length of 150bps in case of both the hot springs. Data quality was checked for base call quality distribution, % bases above Q20 and Q30. It was shown that

the quality data was satisfactory at both the Q20 and Q30 levels getting a higher percentage as shown in **Table.21.** The average G+C content was 61.86% and 49.2% in case of both Reshi and Yumthang respectively.

6.7.2.1. Diversity index and rarefaction curve

The diversity indices such as Shannon H, Fisher Alpha, and Chao1 were estimated using PAST software packages. The results have shown that the Polok is more diverse than Borong and Reshi hot springs. Whereas, Yumthang is having least diversity. The Shannon index was 3.54, 2.78 and 0.626 for Polok, Borong and Reshi hot springs respectively. However, the Yumthang hot spring possessed less Shannon H index of 0.167 **Table.21.** The Chao1 index was also higher in case of Polok followed by Borong hot spring **Table.21.** Rarefaction allows the calculation of species richness in a sample. The curve is a plot of a total number of species annotated as a function of the number of sequences sample (Das et al., 2017). The steep slope at the beginning on the left side signifies most common species have been identified and the plateau at the right side signifies further intensified sampling could lead to the identification of few rarest species **Fig. 23a,b**.

Table.21. Diversity indices of hot spring microbial communities.

Diversity indices of hot spring microbial communities							
Hot Springs	**Total number of Reads**	**G+C Content**	**Average Sequence Length**	**Total number of OTUs**	**Shannon H index**	**Fisher Alpha**	**Chao1**
Borong	372480	53	301	104	2.78	11.46	104
Polok	398782	54	301	360	3.54	44.5	360
RESHI	61898482	61.86	150	-	0.62	0.21	4
YUMTHANG	37338796	49.2	150	-	0.16	0.28	5

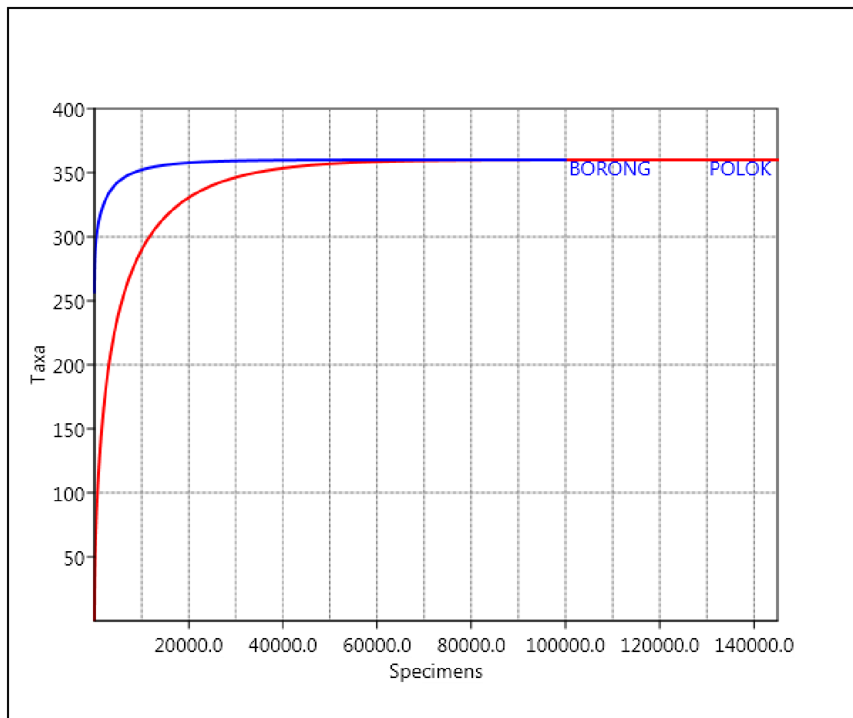

Fig.23a. Rarefaction curve, Red curve shows species richness in Polok Hot Spring whereas Blue line represents Borong Hot Spring.

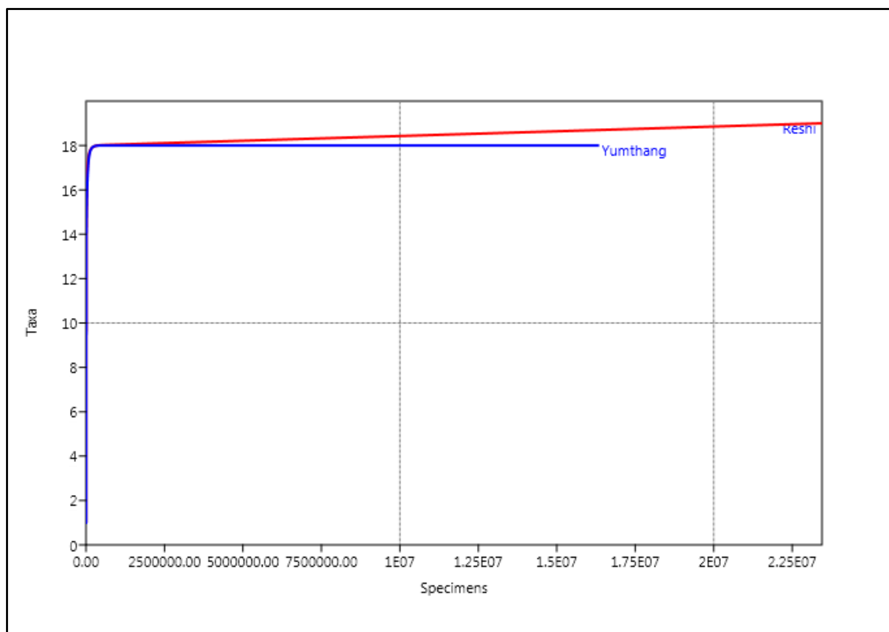

Fig.23b. Rarefaction curve, Red curve shows species richness in Reshi Hot Spring whereas Blue line represents Yumthang Hot Spring.

6.7.2.2. Diversity Analysis at Phylum, Genus and Species level.

The bacterial community showed significant variation between the springs. The phylum wise diversity showed the dominance of *Proteobacteria* (62.50%), *Bacteroidetes* (15.38%), *Acidobacteria* (3.85%), *Nitrospirae* (3.85%) and *Firmicutes* (2.88%) in Borong hot spring whereas Polok hot spring was dominated by *Proteobacteria* (47.22%), *Bacteroidetes* (3.61%), *Firmicutes* (3.06%), *Parcubacteria* (3.06%) and *Spirochaetes* (2.50%) **(Figs.24a,b).** However, the phylum wise diversity showed the dominance of *Actinobacteria* (98.1), *Proteobacteria* (1.7%), *Firmicutes* (0.01%) and *Bacteroidetes* (0.003%) in Yumthang hot spring whereas in Reshi hot spring the dominant phyla were *Proteobacteria* (75.92%), *Actinobacteria* (22.68%), *Firmicutes* (1.14%), and *cyanobacteria* (0.03%) as shown in **Fig24c,d.**

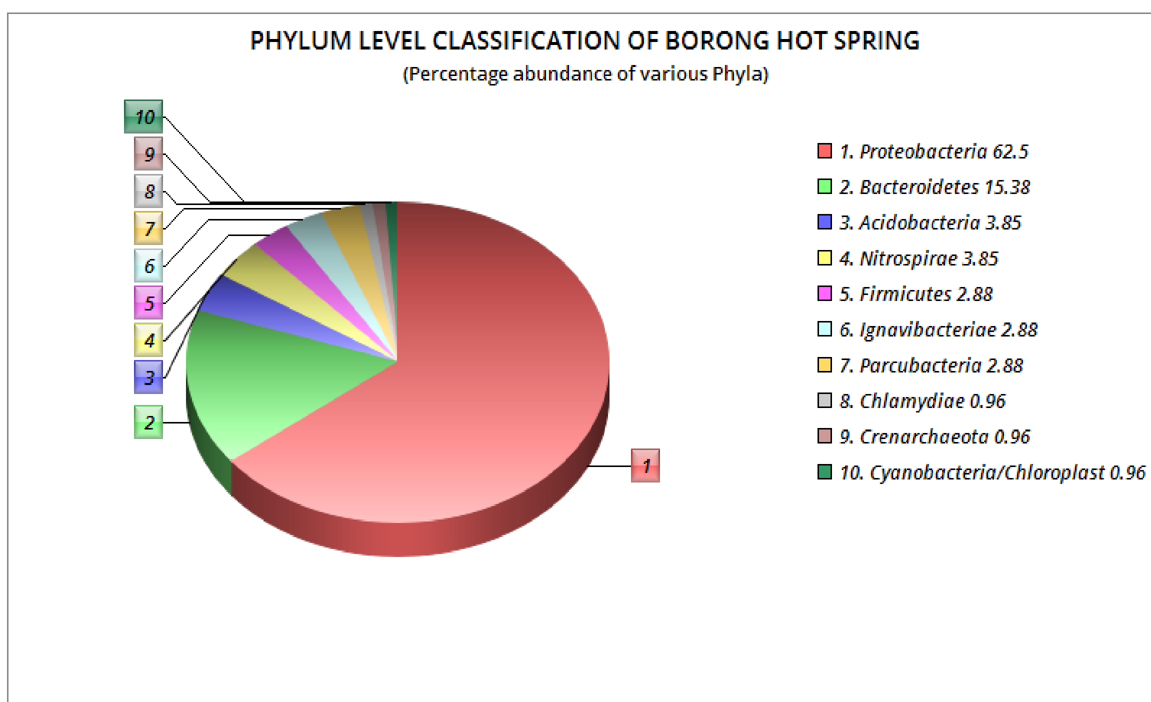

Fig.24a. Phylum level classification of Borong hot spring.

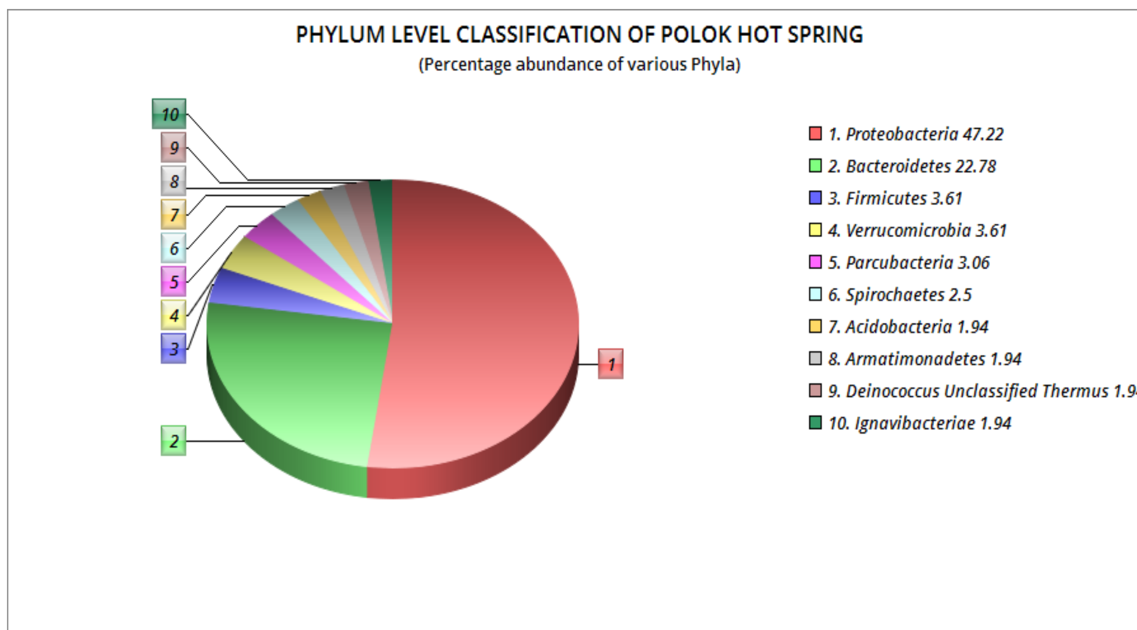

Fig.24b. Phylum level classification of Polok hot spring.

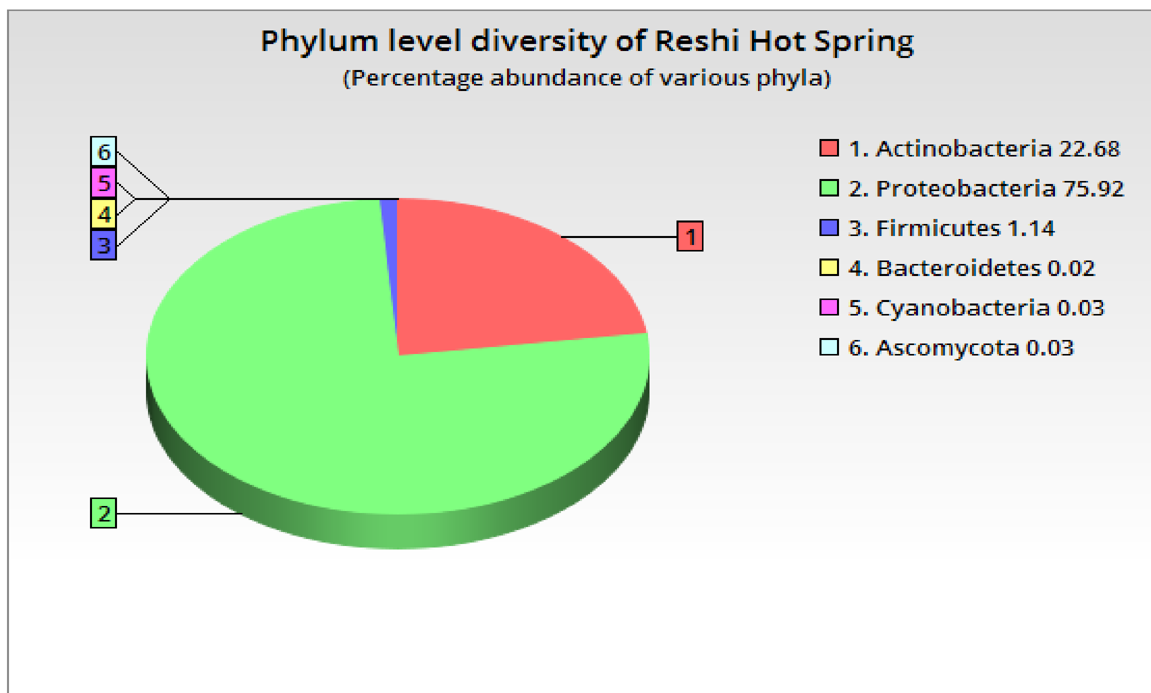

Fig.24c. Phylum level classification of Reshi hot spring.

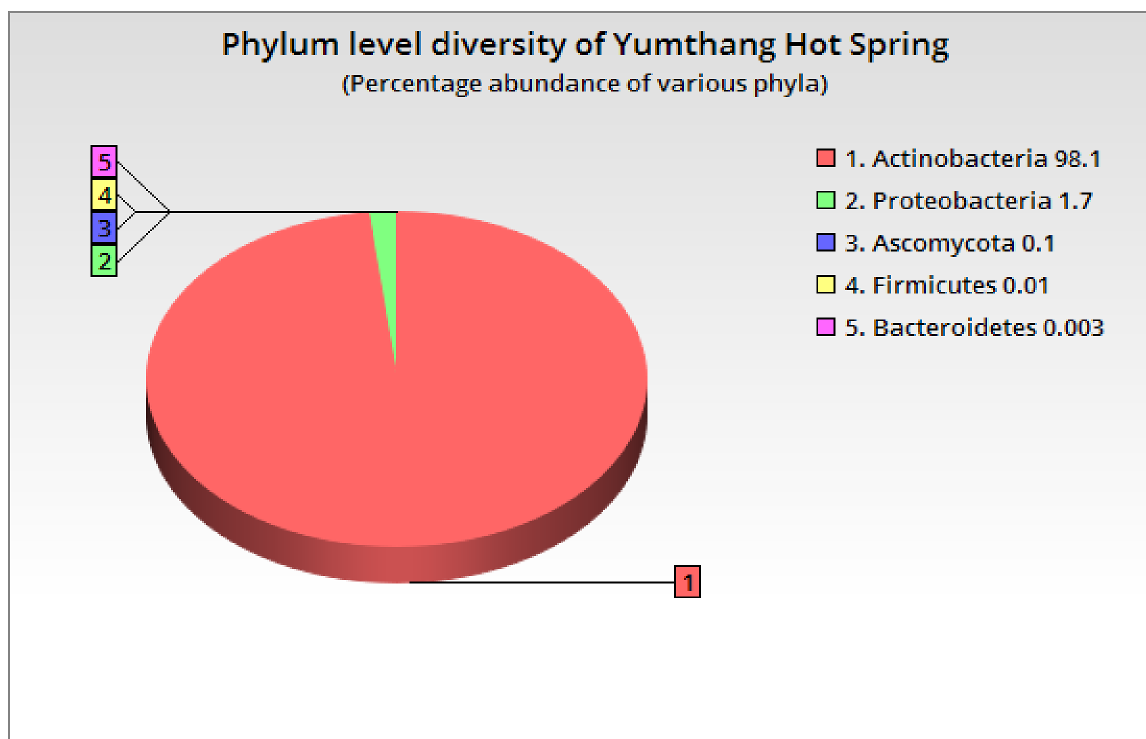

Fig.24d. Phylum level classification of Yumthang hot spring.

At genus level there was a distinct variation in hot springs. The major genus present in Borong hot spring were *Acinetobacter* (7.69%), *Flavobacterium* (3.85%), *Vogesella* (3.85%), *Ignavibacterium* (2.88%), *Sediminibacterium* (2.88%), *Thermodesulfovibrio* (2.88%) and *Acidovorax* (1.92%) **Fig25a.** While the major genera in Polok hot spring were *Flavobacterium* (3.33%), *Parcubacteria genera Incertae sedis* (3.06%), *Sediminibacterium* (2.78%), *Pseudomonas* (1.67%), *Treponema* (1.68%) and *Opitutus* (1.39%) **Fig25b.** In case of Yumthang hot spring the dominant genus were *Rhodococcus* (97.6%), E. coli (0.73%), *Serratia* (0.49%), *Nocardiopsis* (0.47%), *Brevundimons* (0.21%) and *Acinetobacter* (0.15%). However in case of Reshi hot spring the dominant genus were *Pseudomonas* (85.29%), *Rhodococcus* (4.28%), *Dietzia* (3.8%), *Arthobacter* (3.6%), *Staphylococcus* (1.03%) and *Paracoccus* (0.28%) as shown in **Fig25c,d.**

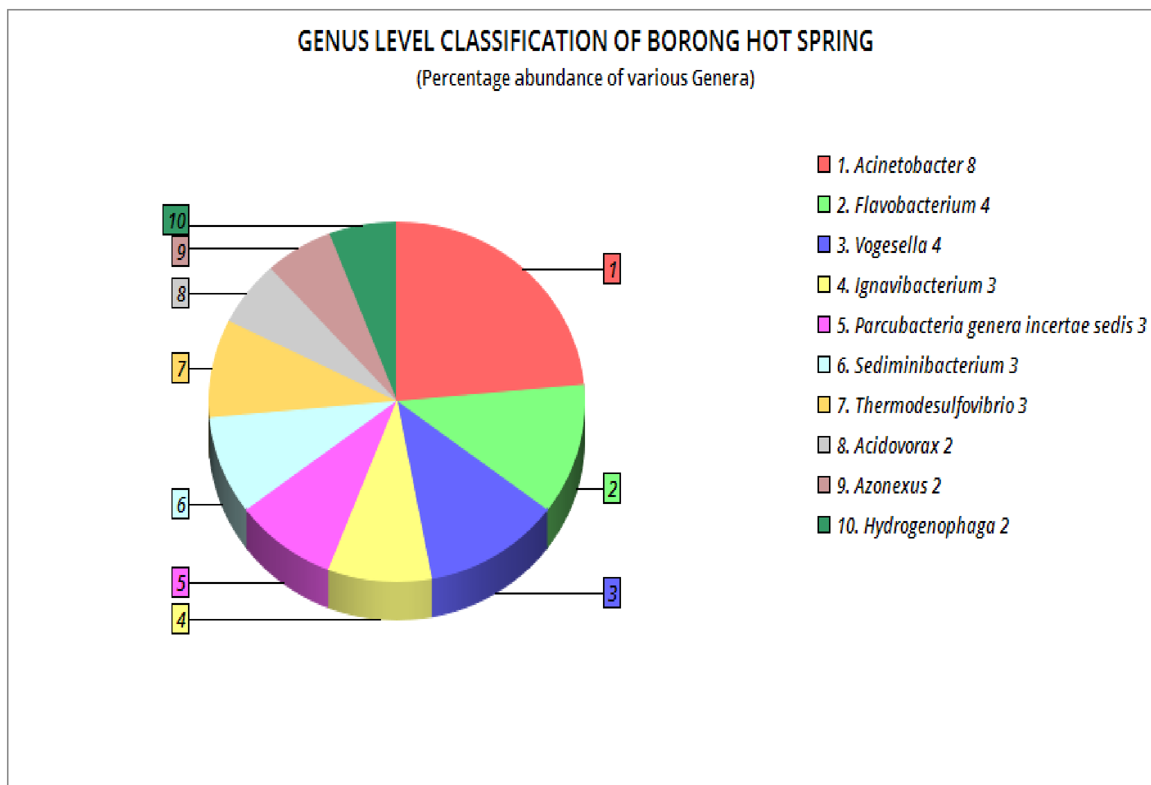

Fig.25a. Genus level diversity of Borong Hot Spring.

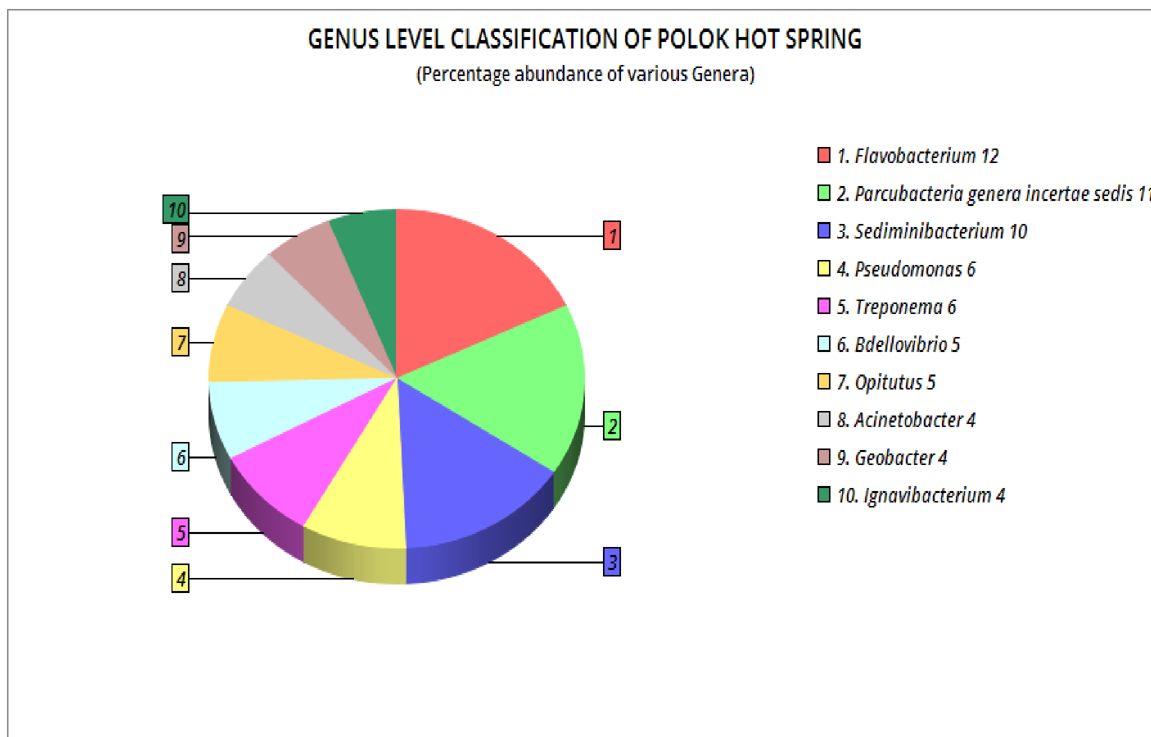

Fig.25b. Genus level diversity of Polok Hot Spring.

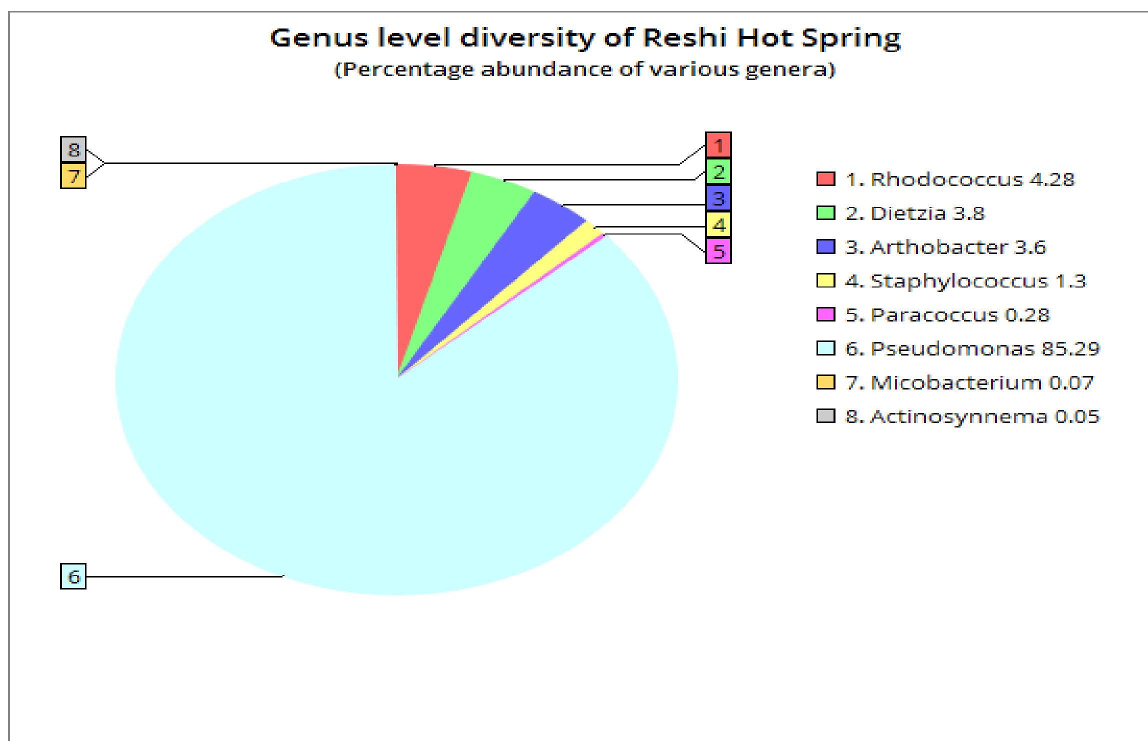

Fig.25c. Genus level diversity of Reshi Hot Spring.

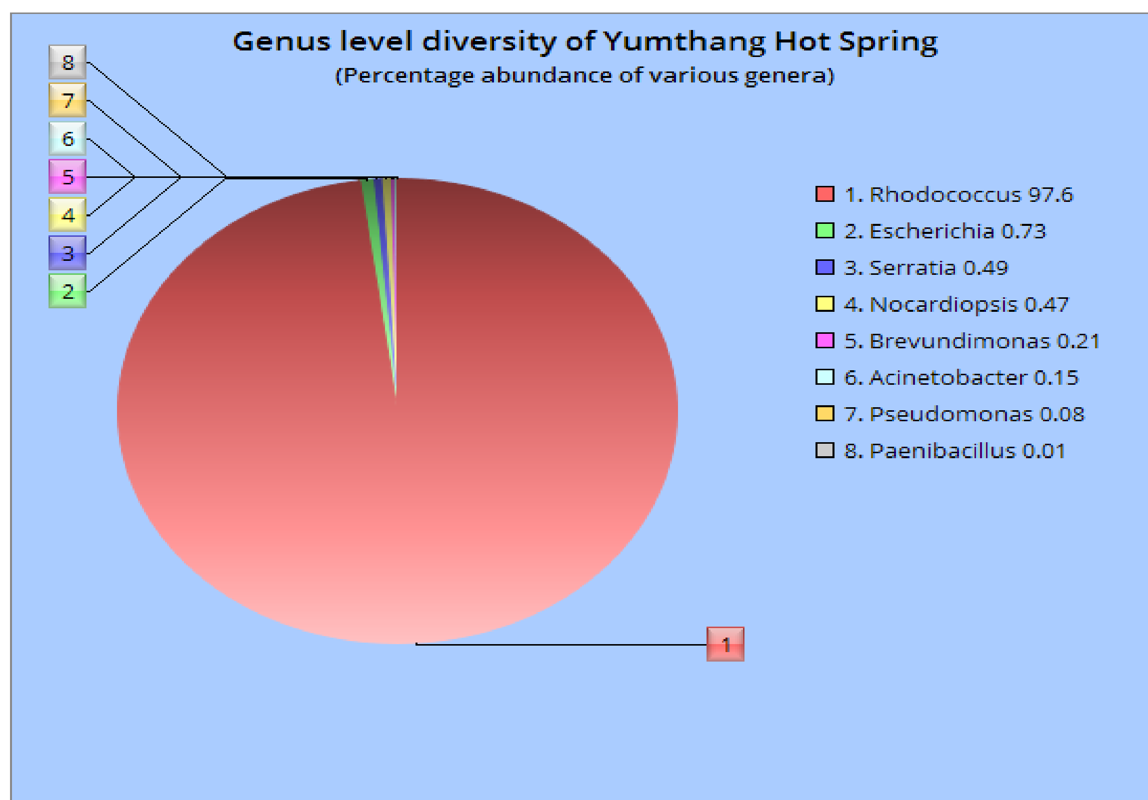

Fig.25d. Genus level diversity of Yumthang Hot Spring.

The diversity at species level varies significantly in all the four hot springs. In case of Polok and Borong hot springs the major bacterial flora were uncultured with 55% in Polok and 17% in Borong. In all the hot springs both Gram-positive and Gram-negative bacteria were found, however the majority of them were Gram-negative bacteria. In Polok and Borong hot springs some thermophilic bacteria were present however in Reshi and Yumthang hot springs showed predominance of mesophilic bacteria. In case of Polok hot spring the major species found were *Sediminibacterium goheungense, Opitutus terrae, Treponema caldarium, Ignavibacterium album, Desulfobulbus mediterraneus and Thermodesulfovibrio yellowstonii.* However, some other thermophilic bacteria were also present in smaller numbers such as *Hydrogenobacter thermophiles, Thermoanaerobacter uzonensis, Thermoanaerobaculum aquaticum, Thermodesulfovibrio hydrogeniphilus, Thermolithobacter ferrireducens, Thermus arciformis, and Thermus caliditerrae* etc **Fig.26a.** In case of Borong hot spring the major species were *Ignavibacterium album*, *Thermodesulfovibrio yellowstonii*, *Flavobacterium cheonhonense*, *Rheinheimera aquatic*, *Thiovirga sulfuroxydans* and *Meiothermus hypogaeus* etc **Fig.26b.** The results have shown that in case of Reshi and Yumthang only few species were showing dominance for example in case of Reshi *Microbacterium species* (66.7%), *Arthrobacter phenanthrenivorans* (2.7%) *and Rhodococcus erythropolis* (2%) were abundant. However in case of Yumthang hot spring only *Rhodococcus ruber* (97.6) followed by *Escherichia coli* (0.7) was abundant **Fig26c,d.**

Annotation with reference library showed, the Polok and Borong hot spring possesses lesser amount of archaeal communities (<1% but they are significantly distinct). Borong hot spring consisted of *Crenarchaeota* (0.96%), whereas Polok had *Euryarchaeota* (0.56%). The order

and genus under *Crenarchaeota* were identified as *Desulfurococcales* and *Desulfurococcus* respectively and in case of *Euryarchaeota*, the order and genus were identified as *Methanomicrobiales* and *Methanospirillum*. However, results showed that the Reshi and Yumthang hot springs does not possesses any archaeal communities. However, among both the hot springs only Yumthang hot spring possess a relatively very less percentage (0.1%) of *Eukaryotes*. Among *Eukaryotes*, the only phylum *Ascomycota* with an order *Actinomycetales* was found and the genus was identified as *Aspergillus.*

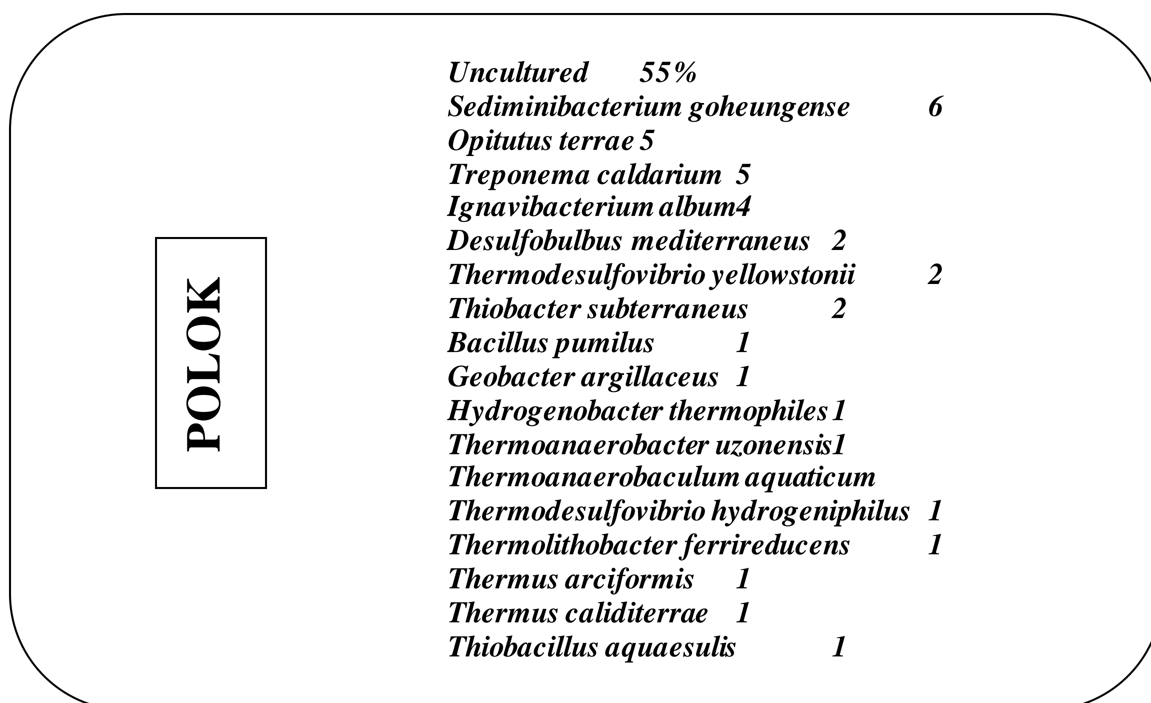

Fig26a. Species level diversity of Polok Hot Spring with abundance in percent.

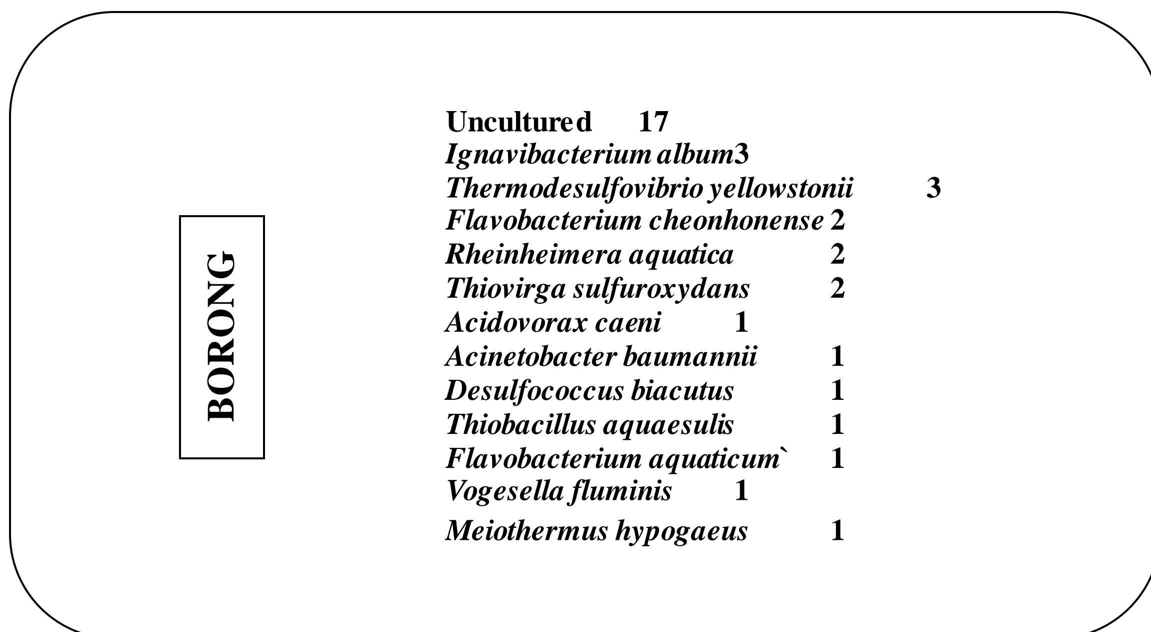

Fig26b. Species level diversity of Borong Hot Spring with abundance in percent.

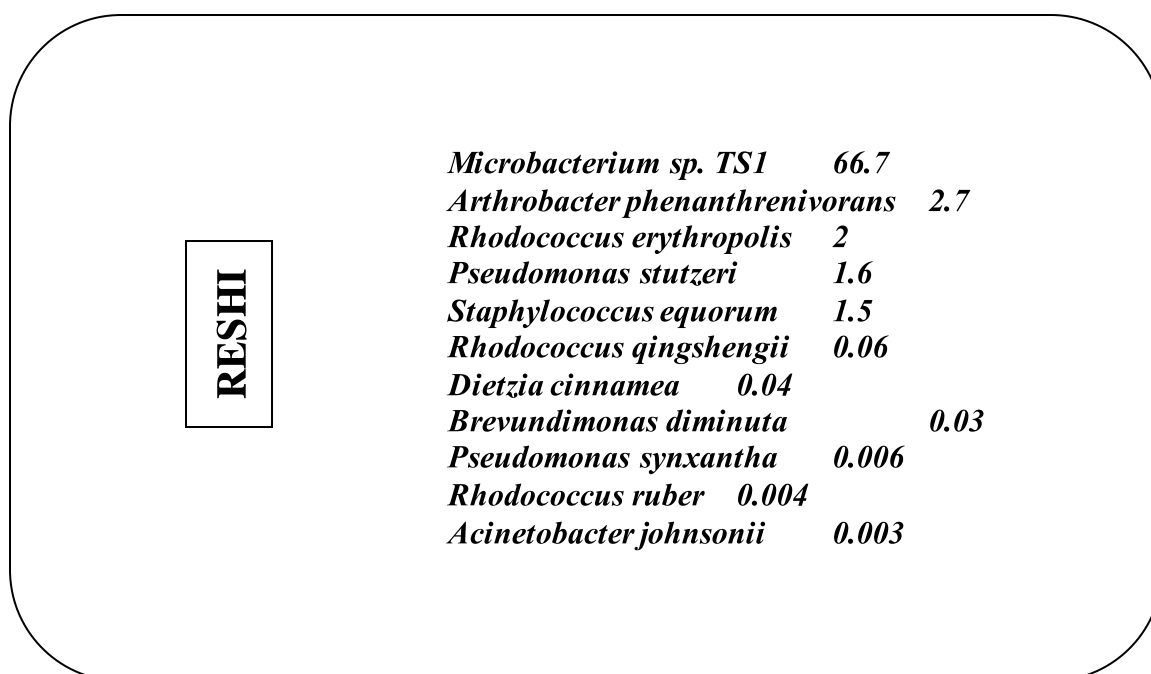

Fig.26c. Species level diversity of Reshi Hot Spring with abundance in percent.

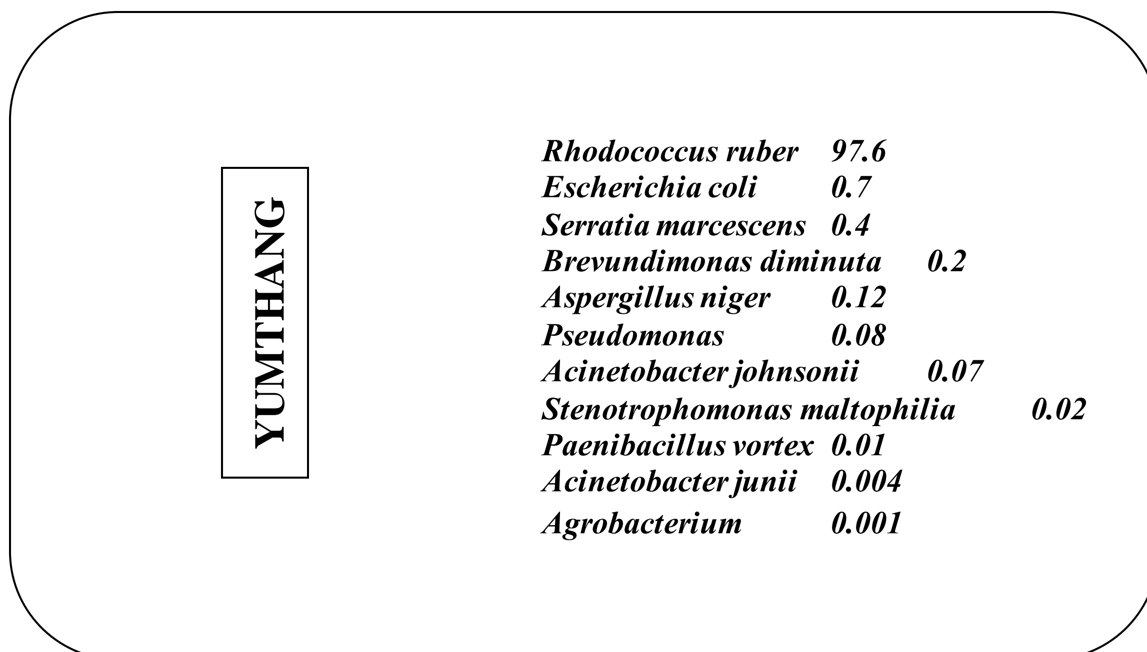

Fig.26d. Species level diversity of Yumthang Hot Spring with abundance in percent.

6.7.2.3. Correlation of bacterial diversity and physicochemical parameters

Principle component analysis (PCA) was done to check the correlation between bacterial diversity at phylum level and physicochemical parameters with respect to four hot springs of Sikkim. The PC1 (Principle component 1) or F1 represents the highest correlation between bacterial diversity and various physicochemical parameters with F1 component possessing (72.38%) variability with Eigen value of 2.89 as shown in **Table.22.** There was significant Pearson (n) correlation in three hot springs such as Polok, Borong, and Reshi with significant p-value<0.05. However, Yumthang and Borong were not significantly correlated as shown in in **Table.23.** It was shown in the biplot that Yumthang hot spring was positively correlated to Actinobacteria, temperature, colloidal sulphur, pH, nitrate and dissolved oxygen. However, other three hot springs were correlated to each other and to rest of the parameters like total alkalinity, calcium, magnesium etc. The phylum Proteobacteria

was positively more correlated to the three hot springs such as Polok, Borong and Reshi as shown in **Fig.27.**

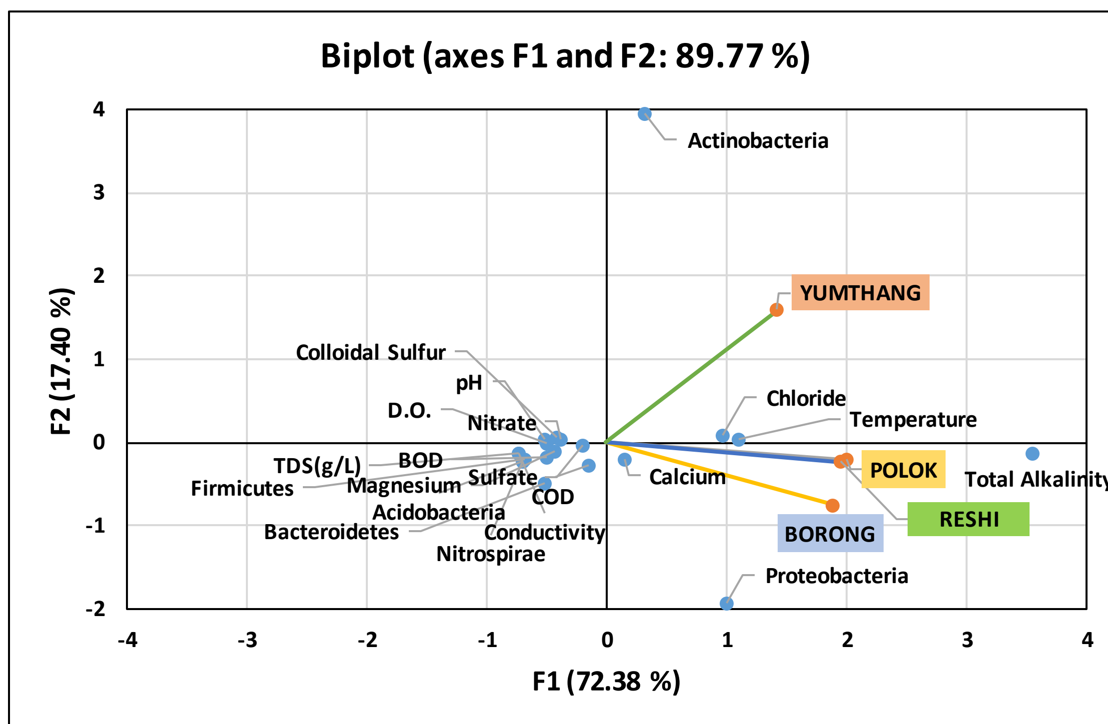

Fig.27. Principle Component Analysis showing the correlation between bacterial diversity based on metagenomic analysis and physicochemical parameters with respect to four hot springs.

Table.22. PCA (Eigenvalues): between bacterial diversity based on metagenomic analysis and physicochemical parameters

	F1	F2	F3	F4
Eigenvalue	2.8951	0.6959	0.2392	0.1699
Variability (%)	72.3766	17.3967	5.9801	4.2466
Cumulative %	72.3766	89.7733	95.7534	100.0000

Table.23. Correlation matrix (Pearson (n)): between bacterial diversity based on metagenomic analysis and physicochemical parameters

Variables	POLOK	BORONG	RESHI	YUMTHANG
POLOK	**1**	**0.8012**	**0.7930**	**0.5281**
BORONG	**0.8012**	**1**	**0.7459**	0.3524
RESHI	**0.7930**	**0.7459**	**1**	**0.4957**
YUMTHANG	**0.5281**	0.3524	**0.4957**	**1**

Values in bold are different from 0 with a significance level alpha=0.05

6.7.2.4. Comparison of microbial diversity

Microbial community structure of hot springs reported earlier from different regions of northeast India (Panda *et al.* 2015) were compared with our studies using heatmap plot with Bray Curtis dissimilarity method. Phylum level diversity comparison showed that the community structure of Borong and Polok were correlative with the Yumthang hot spring of Sikkim (Panda *et al.* 2015) as earlier reported. Whereas, Jarkem hot spring of Meghalaya showed comparatively different microbial community structure. The major phylum of hot springs Polok and Borong are Proteobacteria and Bacteroidetes while major phylum of Jarkem is *Firmicutes* and *Chloroflexi*. This difference indicates possible role of geographical location in shaping the microbial community **Fig.28.**

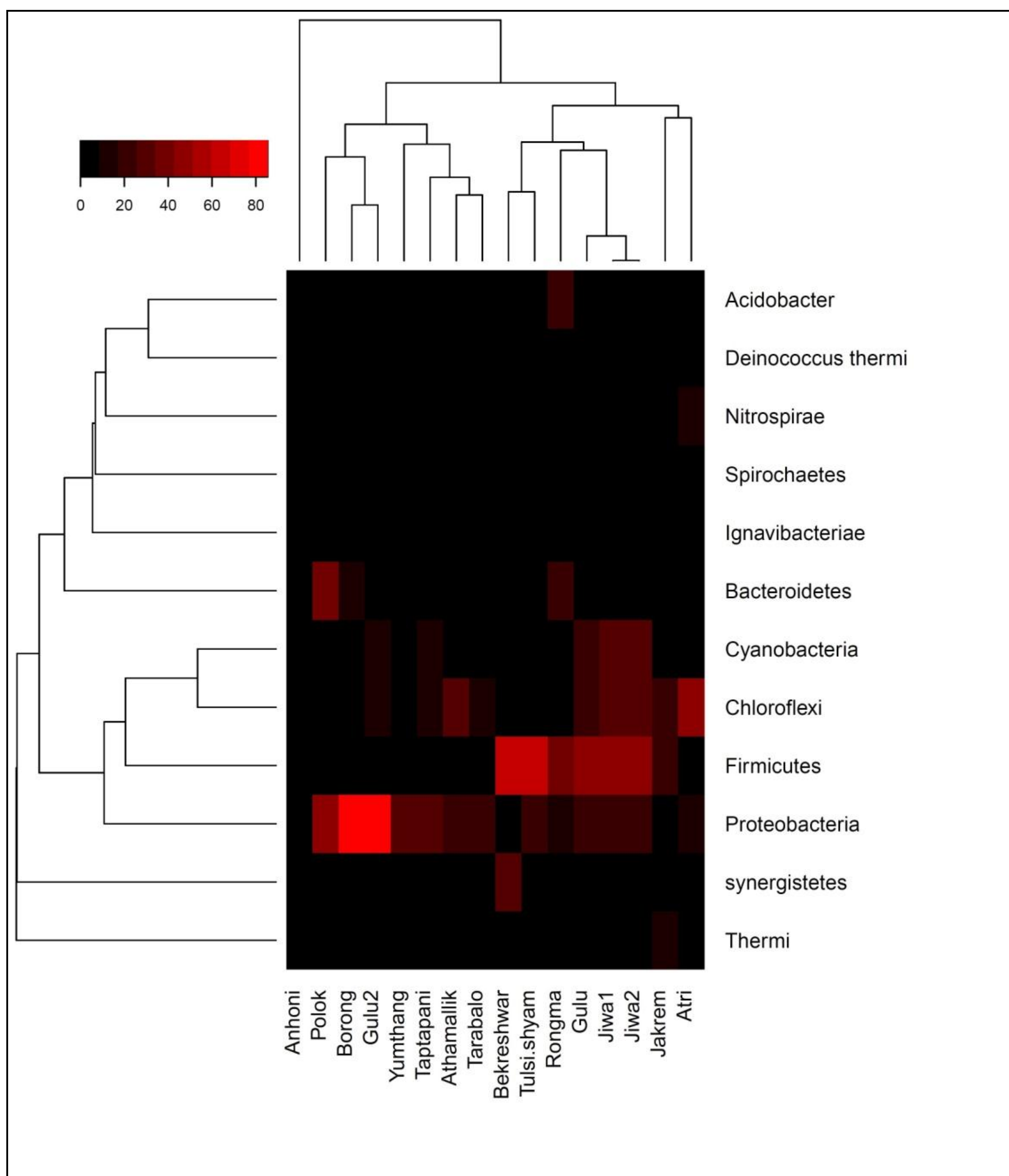

Fig.28. Heat map (Comparative analysis of top phylums among various Hot Springs of central India, North-east India and Tibet)

6.8. Antibiotic Sensitivity of Isolates.

Antibiotic sensitivity profiles of bacterial isolates were performed. 10 different antibiotics such as penicillin, methicillin, amoxicillin, ampicillin, erythromycin, chloramphenicol, gentamycin, clindamycin, norfloxacin, and ciprofloxacin were checked. The results have shown the susceptible nature of all the isolates. Almost all the isolates were having zone of inhibition >20mm as shown in the **Table.24a,b.** According to Kristjansson, et al., 1994 (kristjansson, 1994), the zone of inhibition >20 mm in diameter represents the susceptible nature of thermophilic bacterial isolates. The results were also compared with the zone size of Gram-positive bacteria such as *Staphylococcus aureus* as per CLSI guide lines. It also suggested the susceptible nature of all the bacterial isolates obtained from all the four hot springs of Sikkim. We have also checked the antibiotic susceptibility of two standard *Geobacillus* species, i.e., *Geobacillus stearothermophilus* (MTCC37) and *Geobacillus thermoleovorans* (MTCC4219). Interestingly, we found them susceptible too as shown in **Table.24c.**

Table.24a. Antibiotic Susceptibility of Yumthang and Reshi isolates.

	Antibiotic Susceptibility (mm)								
ISOLATES	Erythromycin 15mcg	Methicillin 10mcg	Penicillin 10U	Clindamycin 2mcg	Gentamycin 10mcg	Chloramphenicol 30mcg	Norfloxacin 10mcg	Ciprofloxacin 10mcg	Amoxicillin 10mcg
TY1	28	19	35	32	27	23	31	26	37
TY2	23	20	31	30	23	23	27	22	39
TY3	27	21	35	30	26	21	30	25	36
TY4	32	20	37	33	26	26	31	25	34
TY5	35	23	38	35	31	29	32	27	37
TY6	30	23	37	33	26	26	28	29	38
TY7	31	21	35	32	26	23	30	24	39
TY8	30	28	36	32	27	27	27	23	39
TY9	32	27	37	30	29	26	29	25	36
TY10	34	25	35	30	26	25	36	27	38
TYNT4	29	39	32	29	24	23	33	24	34
TYNT6	33	23	32	30	23	24	23	21	36
TYNT 10	25	28	29	29	26	24	32	25	34
LYNT1	30	39	35	32	27	23	32	25	38
LYNT2	26	40	34	32	27	24	27	24	38
LYNT3	27	36	30	28	22	21	30	23	28
LYNT5	31	37	33	29	25	23	32	28	32
LYNT9	34	35	35	32	25	25	30	30	39
LYNT10	28	35	35	28	27	24	24	29	34
AYN2	26	18	31	31	25	22	22	23	28
XTR1	29	34	33	29	26	26	33	25	38
XTR2	33	38	32	34	23	24	29	33	40
XTR3	34	36	34	27	24	22	31	28	37
XTR4	32	38	34	30	27	25	35	33	40
XTR5	27	39	29	29	20	28	31	32	34
XTR6	32	32	33	30	25	30	33	25	38
XTR7	32	37	34	27	22	26	30	31	36
XTR8	29	36	32	27	23	25	33	27	37
XTR9	31	33	31	26	26	28	33	34	36
XTR10	30	34	40	26	24	28	38	32	31
XTR11	31	36	32	26	22	23	32	32	39
XTR12	33	31	34	29	21	23	36	27	40

XTR13	30	34	30	27	22	27	32	33	40
XTR14	32	38	34	27	25	24	30	27	38
XTR15	31	32	26	29	21	26	27	25	34
XTR16	33	33	32	31	24	27	36	33	40
XTR17	32	37	32	31	25	28	36	35	39
XTR18	36	40	30	32	26	26	38	33	30
XTR19	32	34	32	28	24	29	31	31	38
XTR20	32	36	32	30	26	22	32	29	37
XTR21	31	37	33	32	25	30	34	34	40
XTR22	29	32	39	31	28	34	37	38	36
XTR23	25	33	29	30	26	30	37	38	40
XTR24	33	31	37	28	22	29	34	33	40
XTR25	29	34	31	32	32	25	35	37	40
XTR26	28	38	25	27	23	28	32	35	38
XTR27	29	32	28	27	23	24	35	33	39
XTR28	28	33	27	27	22	29	31	32	38
XTR29	28	37	27	28	23	28	31	32	37
XTR30	28	40	29	29	26	25	35	35	40
XTR31	28	34	27	30	23	26	34	36	40
XTR32	30	36	29	29	23	27	38	37	40
XTR33	35	37	29	31	23	27	34	34	40
XTR34	31	32	30	34	23	32	33	31	40
XTR35	29	33	32	32	23	28	39	38	40
XTR36	29	35	28	28	27	32	35	35	40
XTR37	35	31	35	32	29	31	36	36	40
XTR38	31	29	29	28	22	25	32	38	39

Table.24b. Antibiotic Susceptibility of Polok and Borong isolates.

ISOLATES	Antibiotic Susceptibility								
	Erythromycin 15mcg	Methicillin 10mcg	Penicillin 10U	Clindamycin 2mcg	Gentamycin 10mcg	Chloramphenicol 30mcg	Norfloxacin 10mcg	Ciprofloxacin 10mcg	Ampicillin 10mcg
TP1	27	27	28	29	27	34	22	32	36
TP2	27	30	30	29	29	22	30	30	39
TP3	25	25	26	31	29	28	39	34	26
TP4	25	26	27	22	25	24	26	28	31
TP5	26	28	26	25	24	26	27	28	36
TP6	28	27	28	28	27	31	31	26	27
TP7	26	26	24	26	28	27	27	28	25
TP8	31	25	29	31	26	27	28	29	25
TP9	27	30	28	30	24	27	29	31	33
TP10	31	30	25	23	29	28	25	29	31
TP11	26	27	27	29	24	26	31	32	36
TP12	26	24	26	23	29	24	26	27	30
TP13	24	28	28	27	26	27	29	28	40
BPP1	27	26	26	30	26	25	28	27	33
BPP2	23	24	37	28	18	26	27	30	34
BPP3	32	31	32	30	35	27	35	29	35
BPP4	37	28	18	28	27	30	26	29	24
BPP5	25	29	24	25	28	36	24	36	28
TB1	29	32	34	27	27	27	34	34	36
TB2	38	34	30	27	28	27	28	28	36
TB3	25	29	29	29	24	26	28	28	29
TB4	25	30	25	29	24	27	35	29	35
TB5	30	30	31	28	27	27	31	30	39
TB6	22	27	27	27	22	23	25	29	34
TB7	27	30	25	29	24	25	26	28	37
TB8	27	27	28	27	20	25	28	27	30
TB9	25	27	26	28	22	22	25	28	36
TB10	27	29	28	31	24	27	25	28	34
TB11	27	26	29	24	30	28	27	25	29
TB12	22	24	36	28	25	27	27	24	26
BPB1	28	29	25	28	24	24	24	29	31
BPB2	28	30	26	29	24	24	25	27	33
BPB3	27	25	24	36	28	31	26	37	35
BPB4	24	26	28	28	23	25	27	26	35

Table.24c. Antibiotic susceptibility pattern of *Geobacillus thermoleovorans* (MTCC4219) *and Geobacillus stearothermophilus* (MTCC37)

Antibiotic susceptibility of two known *Geobacillus* species		
Antibiotics	***Geobacillus thermoleovorans***	***Geobacillus stearothermophilus***
Erythromycin	29	33
Methicillin	33	32
Clindamycin	35	30
Ampicillin	>40	35
Chloramphenicol	29	26
Penicillin	>40	40

6.8.1. Minimum Inhibitory Concentration (MIC)

The MIC of penicillin, vancomycin, erythromycin, chloramphenicol and methicillin was checked. Various dilutions from stock antibiotic solution were made such as 0.015, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16mg/l. The results have shown that the minimum inhibitory concentration values for almost all the antibiotics checked are very less. In case of gentamycin, vancomycin, erythromycin, and chloramphenicol the MIC values were around 0.5 µg/ml, 2 µg/ml, 2 µg/ml, and 8 µg/ml respectively. The least MIC of 0.25 µg/ml was shown in case of penicillin G and oxacillin as shown in **Table.25a,b,c,d** and **Fig.29.** The MIC was defined as the lowest concentration producing no visible growth. No internationally accepted criteria for susceptibility testing or breakpoints for susceptible or resistant isolates are available for thermophilic bacteria. However, the two thermophilic Campylobacter species were investigated and their breakpoints has been established (Guévremont *et al.* 2006). Briefly, the breakpoint values of the MIC for resistance were as follows: for ciprofloxacin, clindamycin, and enrofloxacin, ≥4 µg mL^{-1}; for erythromycin,

≥8 μg mL^{-1}; for gentamicin and tetracycline, ≥16 μg mL^{-1}; for ampicillin and chloramphenicol, ≥32 μg mL^{-1}; for streptomycin, ≥ 64 μg mL^{-1}; and for sulfamethoxazole, ≥512 μg mL^{-1}(Guévremont *et al.* 2006). So as per Guévremont et al, 2005, the MIC values of various antibiotics against our isolates is thus very less and it supports our antibiotic susceptibility test which indicated that all the isolates are antibiotic susceptible in nature or very sensitive to the above mentioned tested antibiotics.

Table.25a. Minimum inhibitory concentration of erythromycin.

	MIC ERYTHROMYCIN (μg ml^{-1})					
SNO.	**0.25**	**0.5**	**1**	**2**	**4**	**8**
AYS8	G	NG	NG	NG	NG	NG
AYS10	G	G	G	NG	NG	NG
AYN2	G	G	G	NG	NG	NG
SY1	G	G	NG	NG	NG	NG
SY3	G	G	G	NG	NG	NG
TY1	G	G	G	NG	NG	NG
XTR9	G	G	G	NG	NG	NG
XTR10	G	G	G	NG	NG	NG
XTR4	G	G	G	NG	NG	NG
XTR3	G	G	G	NG	NG	NG
XTR1	G	G	G	NG	NG	NG
XTR15	G	G	G	NG	NG	NG
TP1	G	G	G	NG	NG	NG
BPP1	G	G	G	NG	NG	NG
BPP2	G	G	NG	NG	NG	NG
TB2	G	G	NG	NG	NG	NG
TB3	G	G	G	NG	NG	NG
TB9	G	G	G	NG	NG	NG

G=growth, NG=No Growth.

Table.25b. Minimum inhibitory concentration of vancomycin.

	MIC VANCOMYCIN (µg ml^{-1})					
SNO.	**0.25**	**0.5**	**1**	**2**	**4**	**8**
AYS8	G	G	G	NG	NG	NG
AYS10	G	G	NG	NG	NG	NG
AYN2	G	G	NG	NG	NG	NG
SY1	G	G	NG	NG	NG	NG
SY3	G	G	NG	NG	NG	NG
TY1	G	G	NG	NG	NG	NG
XTR9	G	G	NG	NG	NG	NG
XTR10	G	G	NG	NG	NG	NG
XTR4	G	G	G	NG	NG	NG
XTR3	G	G	NG	NG	NG	NG
XTR1	G	G	G	NG	NG	NG
XTR15	G	G	NG	NG	NG	NG
TP1	G	G	NG	NG	NG	NG
BPP1	G	G	NG	NG	NG	NG
BPP2	G	G	G	NG	NG	NG
TB2	G	G	G	NG	NG	NG
TB3	G	G	G	NG	NG	NG
TB9	G	G	NG	NG	NG	NG

Table.25c. Minimum inhibitory concentration of chloramphenicol.

	MIC CHLORAMPHENECOL (µg ml^{-1})					
SNO.	**0.25**	**0.5**	**1**	**2**	**4**	**8**
AYS8	G	G	G	G	G	NG
AYS10	G	G	G	G	G	NG
AYN2	G	G	G	G	G	NG
SY1	G	G	G	G	G	NG
SY3	G	G	G	G	G	NG
TY1	G	G	G	G	NG	NG
XTR9	G	G	G	G	G	NG
XTR10	G	G	G	G	G	NG
XTR4	G	G	G	G	G	NG
XTR3	G	G	G	G	G	NG
XTR1	G	G	G	G	NG	NG
XTR15	G	G	G	G	G	NG
TP1	G	G	G	G	G	NG
BPP1	G	G	G	G	G	NG
BPP2	G	G	G	G	NG	NG
TB2	G	G	G	G	NG	NG
TB3	G	G	G	G	NG	NG
TB9	G	G	G	G	G	NG

Table.25d. Minimum inhibitory concentration of penicillin.

	MIC PENICILLIN (µg ml^{-1})					
SNO.	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**
AYS8	NG	NG	NG	NG	NG	NG
AYS10	NG	NG	NG	NG	NG	NG
AYN2	G	NG	NG	NG	NG	NG
SY1	G	NG	NG	NG	NG	NG
SY3	G	NG	NG	NG	NG	NG
TY1	G	NG	NG	NG	NG	NG
XTR9	G	NG	NG	NG	NG	NG
XTR10	G	NG	NG	NG	NG	NG
XTR4	G	NG	NG	NG	NG	NG
XTR3	NG	NG	NG	NG	NG	NG
XTR1	G	NG	NG	NG	NG	NG
XTR15	G	NG	NG	NG	NG	NG
TP1	G	NG	NG	NG	NG	NG
BPP1	NG	NG	NG	NG	NG	NG
BPP2	G	NG	NG	NG	NG	NG
TB2	G	NG	NG	NG	NG	NG
TB3	G	NG	NG	NG	NG	NG
TB9	G	NG	NG	NG	NG	NG

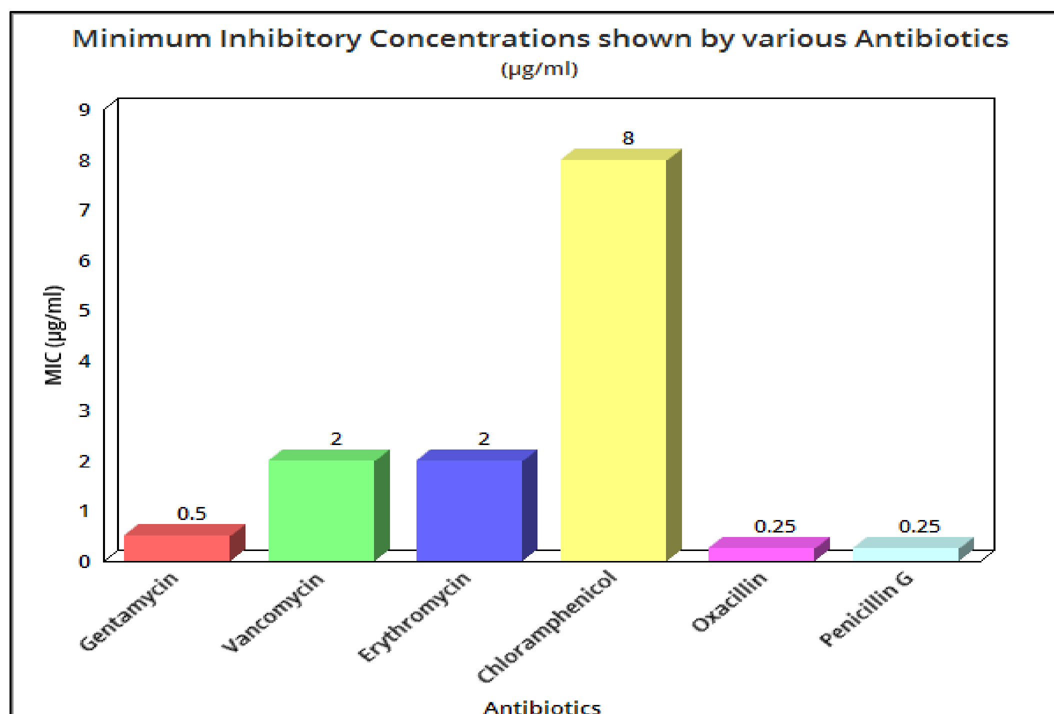

Fig.29. Minimum inhibitory concentration shown by various antibiotics in µg ml^{-1}.

6.8.2. Detection of plasmid

It is commonly observed that antibiotic resistant genes are present on the plasmids of bacteria. Therefore, isolation of plasmids from 50 bacterial isolates were carried out by using kit methods as per the guidelines of manufacturer. The results have shown that none of the isolates were possessing any plasmid as no plasmid DNA was detected by agarose gel electrophoresis on other hand plasmid DNA was detected for positive control shown in **Fig.30.** The results were also supported by whole genome sequencing of AYN2 and LYN3 isolates, as no plasmids sequences were found in that also. **Fig.32a,b.**

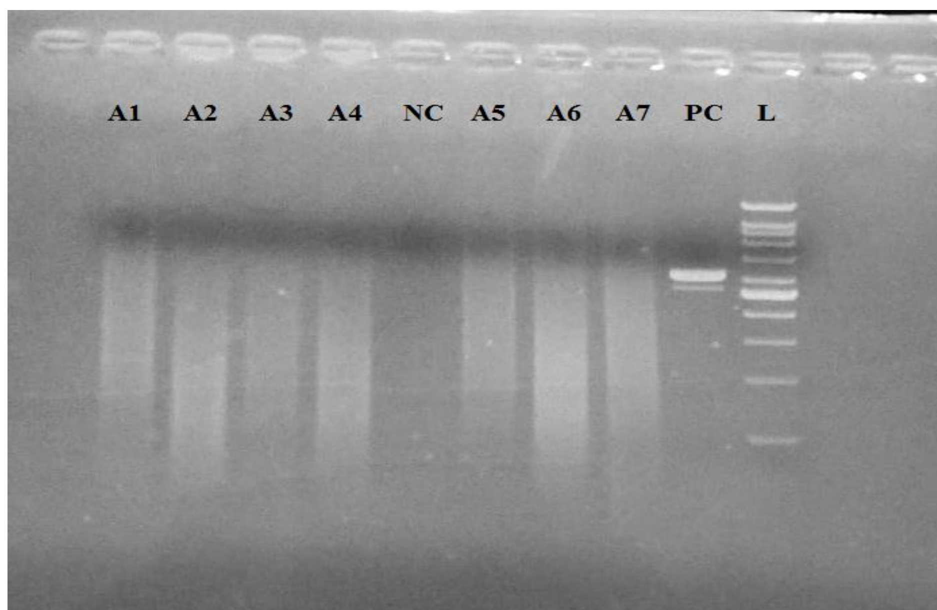

Fig.30. Detection of plasmid by kit method
A1-A7- Isolates (no bands found), PC- *E- Coli* positive control-containing plasmid, L- 1kb Ladder, NC- Negative control.

6.8.3. Detection of antibiotic resistant genes by PCR amplification and whole genome sequencing.

The antibiotic susceptible nature of our bacterial isolates could be due to absence of antibiotic resistant genes or due to inhibition of antibiotic resistant genes expression. Therefore, initially we checked the presence of some common antibiotic resistant genes among our isolates. These were belonging to class β-lactams. The detection of resistance causing genes were carried out for various antibiotics such as ampC genes for ampicillin, *PBP1A, PBP2A* genes for penicillin, and mecA for methicillin. However, the results have shown the absence of such genes in cultured isolates as no PCR products obtained from these isolates on the other hand the PCR products for positive control were obtained **Fig.31a,b,c**. These results suggest the absence of some common resistant genes in thermophilic bacteria isolated from four hot springs of Sikkim. It could be possible that these common resistant genes are absent, however other antibiotic resistant genes might be present in these bacterial isolates. Therefore, to detect antibiotic resistant genes whole genome sequencing of two isolates were carried as mentioned earlier. Whole Genome Sequencing was performed for two isolates AYN2 and LYN3 which were found to be closely identical to *Geobacillus toebii*. Applying RAST it was shown that none of the isolates AYN2 and LYN3 were possessing any antibiotic resistant genes as shown in **Fig.32a,b.** The figures were directly downloaded from the RAST server. Also with the help of whole genome analysis it was found that none of the isolates sequenced that is AYN2 and LYN3 were possessing any plasmid sequences. All the above mentioned experiments including plasmid isolation, detection of common antibiotic resistant genes by PCR and whole genome sequencing proves that the thermophilic bacteria isolated from four hot

springs of Sikkim were antibiotic susceptible in nature and were not carrying any antibiotic resistant genes. However, due to the uncultivated status of the major taxa in hot springs, culture-dependent analysis led to the identification of few distinct genera from the hot springs of Sikkim and therefore the detection of antibiotic resistant genes by functional metagenomic approach was needed and thus we extended research and then performed metagenomic analysis of antibiotic resistance genes.

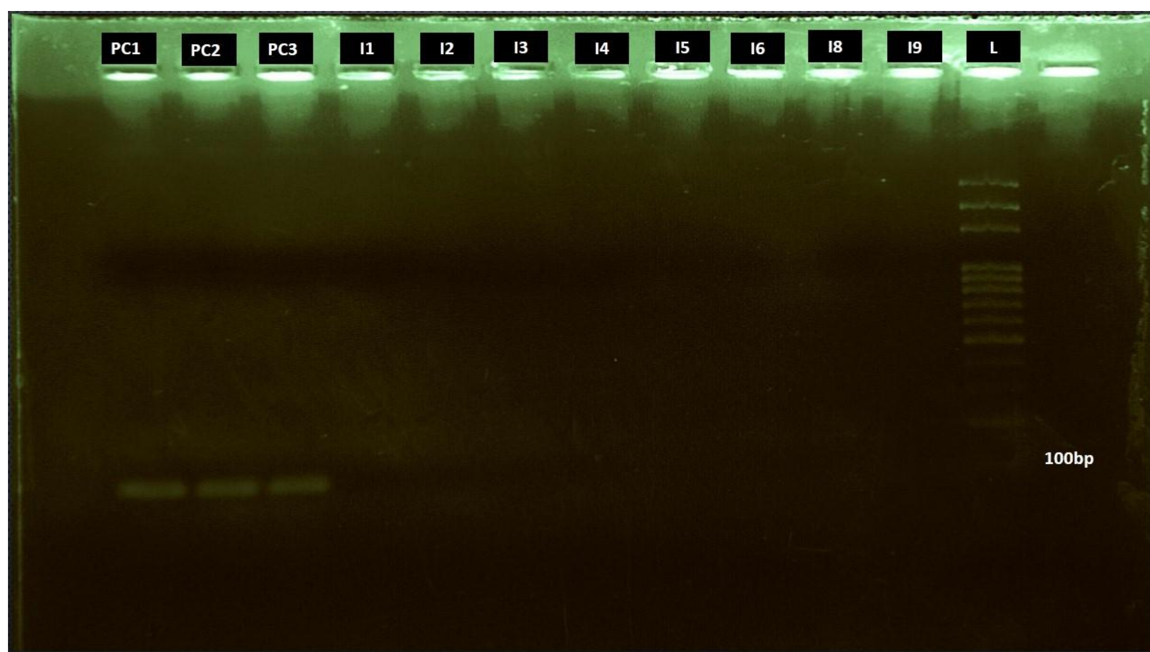

Fig.31a. ampC gene (100bp), PC1/2- Positive Control, L= 100bp Ladder, I= Isolates, I1-XTR1, I2-17R2, I3-XTR10, I4-YTPR1, I5-TY1, I6-AYN2, I7-TP12, I8-BPP1, I9-TB9.

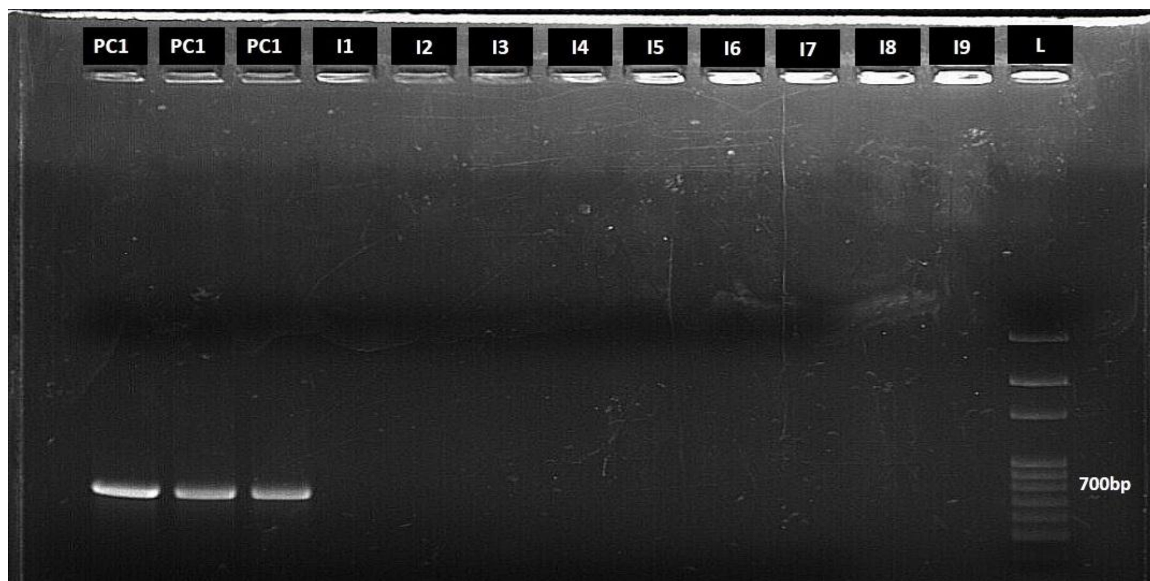

Fig.31b. Pbp1- gene (686bp), PC1/2/3- Positive Control, L= 100bp Ladder, I= Isolates, I1-XTR1, I2-17R2, I3-XTR10, I4-YTPR1, I5-TY1, I6-AYN2, I7-TP12, I8-BPP1, I9-TB9.

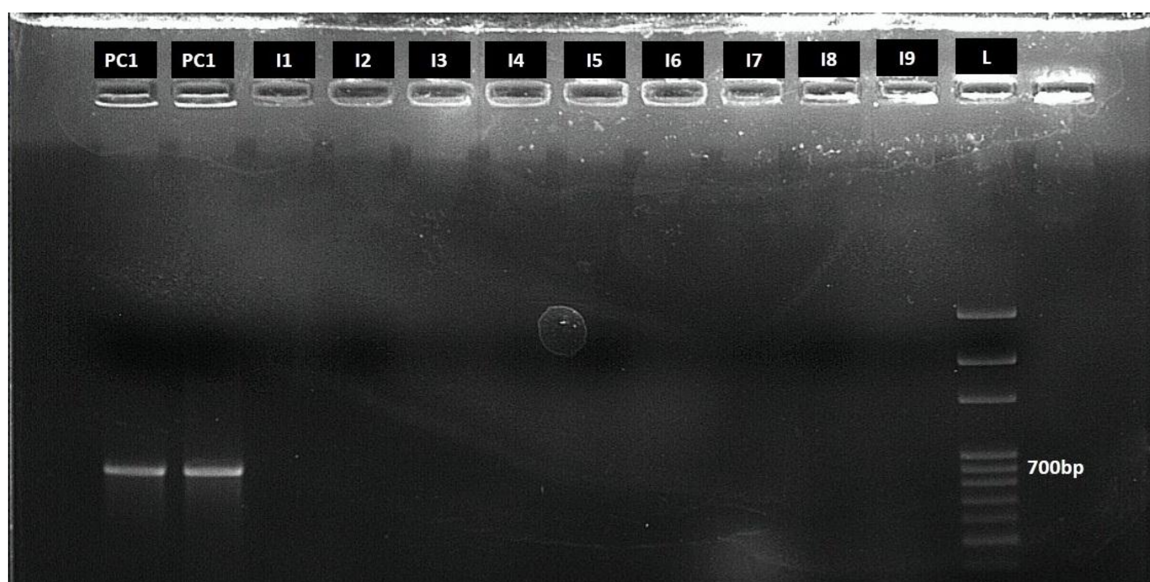

Fig.31c. mecA gene (679bp), PC1/2- Positive Control, L= 100bp Ladder, I= Isolates, I1-XTR1, I2-17R2, I3-XTR10, I4-YTPR1, I5-TY1, I6-AYN2, I7-TP12, I8-BPP1, I9-TB9.

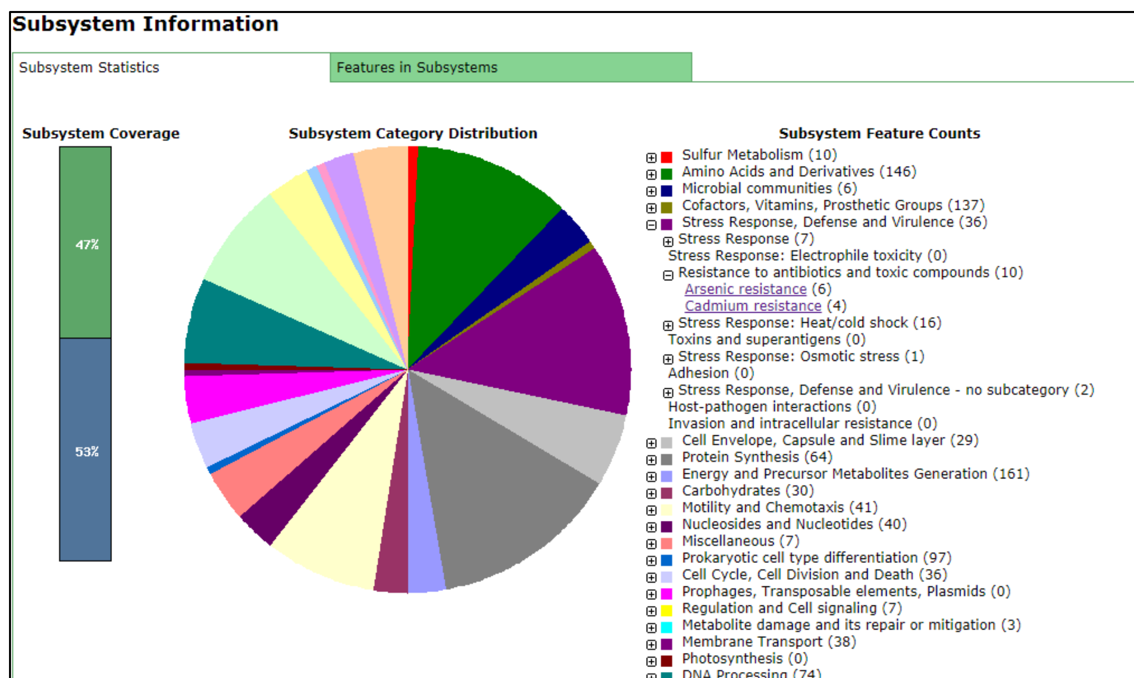

Fig.32a. Subsystem information of *Geobacillus yumthangensis* AYN2 showing absence of Antibiotic resistance and Plasmids.

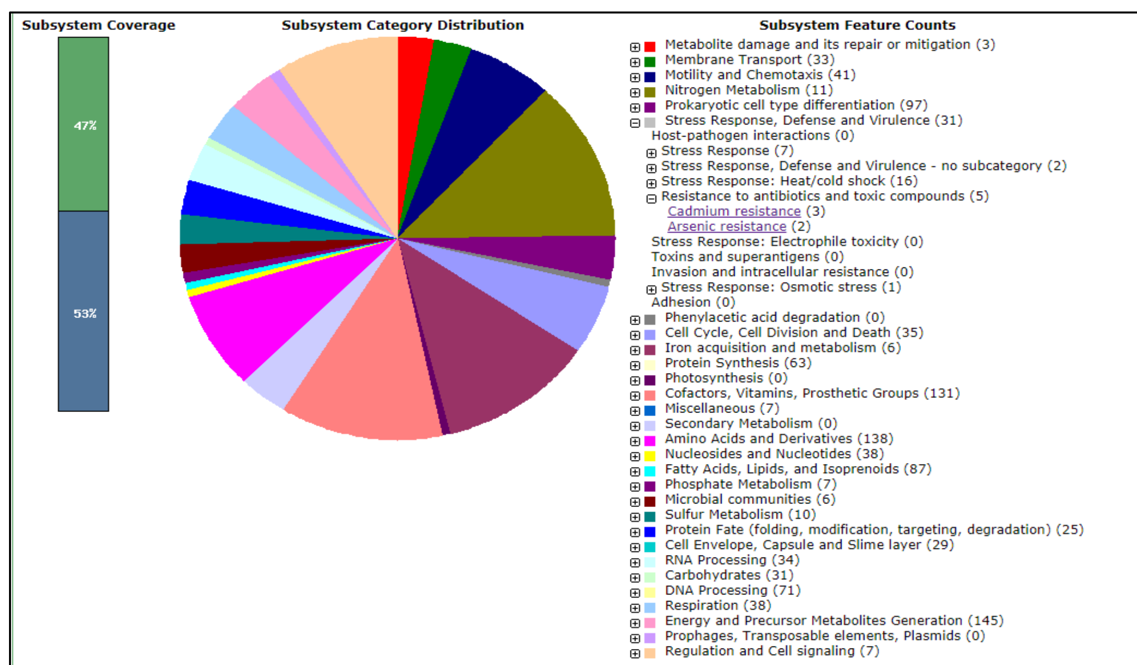

Fig.32b. Subsystem information of *Geobacillus* sp. LYN3 showing absence of Antibiotic resistance and Plasmids.

6.8.4. Metagenomic studies of antibiotic resistance genes (ARGs)

Metagenomic sequence data was generated using Illumina HiSeq and study of antibiotic resistance genes (ARGs) were carried out using ardbAnno V.1.0. For the functional metagenomic analysis two hot springs, i.e., Reshi and Yumthang were selected as these two hot springs are located in different region of Sikkim. The results have shown the different resistance genes in both the hot springs. In Reshi hot spring the resistance genes were belonging to class aminoglycosides, chloramphenicol, and tetracycline. The aminoglycoside gene was showing closest identity (99%) with *Acinetobacter baumanni* (genes for *aminoglycoside nucleotidal-transferase ant (2)-1a*), chloramphenicol gene was showing (100%) identity with *Listeria monocytogenes* LM2 *catA* gene. The tetracycline resistance gene was showing (99%) identity with *Staphylococcus epidermidis* strain K264 (*norA*) gene. Similarly in Yumthang hot spring the resistance genes were belonging to class aminoglycosides, chloramphenicol, macrolide, β-lactams, polymyxin and tetracycline. In this case aminoglycoside gene was showing closest identity (99%) with *Acinetobacter baumanni* (genes for aminoglycoside nucleotidal-transferase *ant* (2)-1a) and other aminoglycoside gene with *Serratia marcescens* w2.2 aa (6') gene for *aminoglycoside 6'-N-acetyltransferase*. The chloramphenicol gene was showing (100%) identity with *E-coli.* The tetracycline resistance gene was showing (91%) identity with *Serratia marcescens* FMC 1-23-O-tet (41) gene. The β-lactam gene was showing (100%) identity with *E- coli* strain RM14721 and other gene from class C- β-lactamase-cephalosporin was showing (97%) identity with *Serratia* sp YD25. The macrolide gene *Mac B* also shows (99%) identity with *Serratia* sp YD25 and the polymyxin resistance gene was showing (100%) identity with *E-coli* strain RM14721. Besides these results, based on COG (cluster of orthologous groups)

classification by blastx, various resistance genes were predicted corresponding to each antibiotic class discussed above as shown in **Fig.33.** The antibiotic resistant genes were diverse in Yumthang hot spring than that of Reshi hot spring. However, the genes were abundant in Reshi hot spring. Few of the antibiotic classes such as β-lactam with COG predicted genes such as *ampC, blh, CphA, blab, bla, blaZ, hcpA* and *penPC*; Polymyxin with COG predicted genes *arnA*; macrolide with genes *macA, macB* and bicyclomycin with predicted gene such as *bcr* were only detected in Yumthang hot spring. Although metagenomic analysis showed the presence of certain antibiotic resistance genes, however, all the genes were showing identity with mesophilic bacteria and not with any thermophilic bacteria. Thus this may be concluded that these genes might be the contaminations of mesophilic bacteria residing in surrounding soil layers.

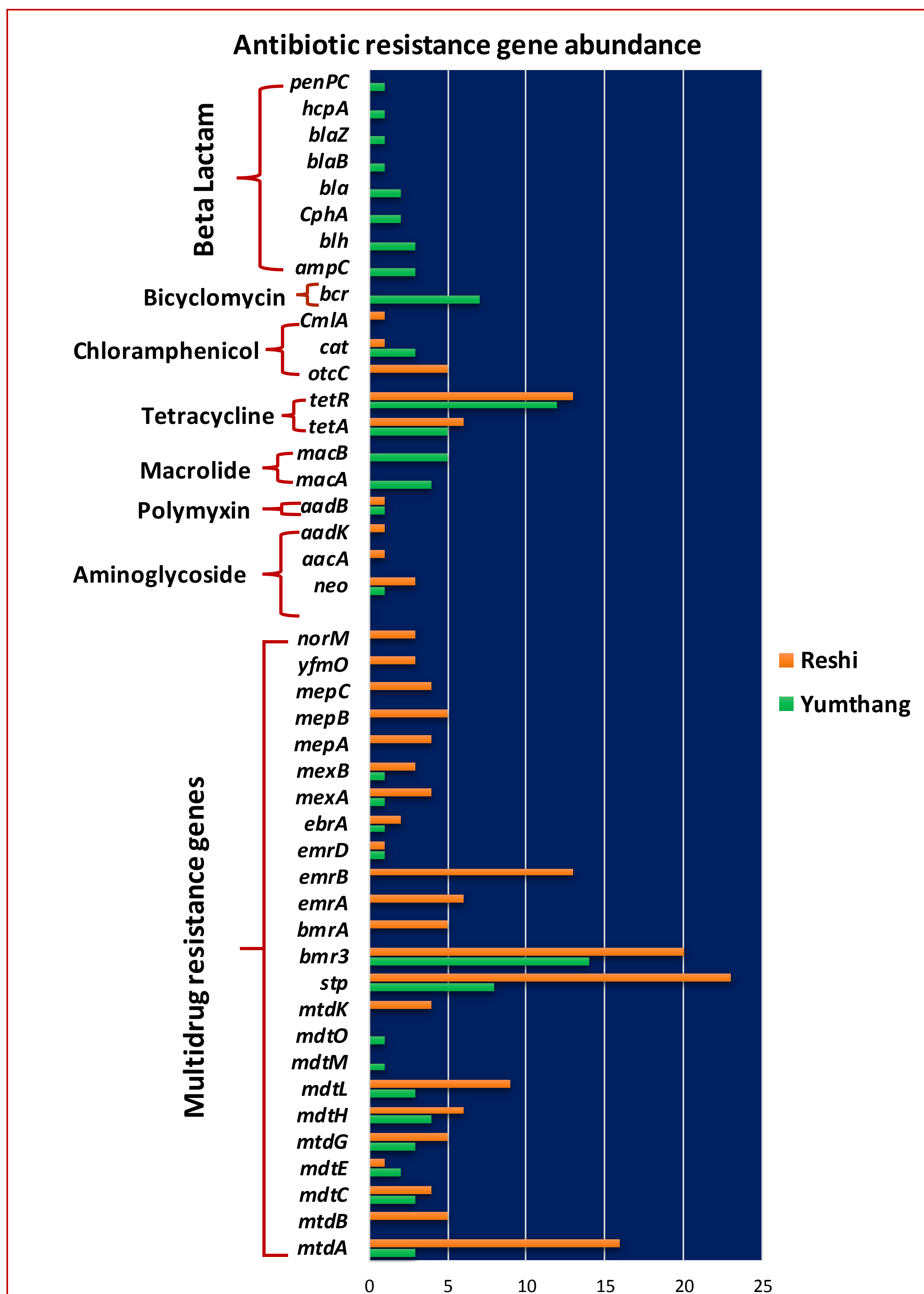

Fig.33. Antibiotic resistance gene abundance predicted by COG analysis.

6.9. Heavy metal tolerance to isolates

6.9.1. Screening and assessment of metal toxicity

Prior to introduction of antibiotics for treatment diseases, heavy metals were used for disease treatment for centuries and close nexus has been reported by many researchers between the antibiotic resistant phenomena and heavy metal resistant phenomena. Therefore, heavy metal tolerance were checked for our thermophilic bacterial isolates. Five different heavy metals such as $CuSO_4$, $MnSO_4$, $ZnCl_2$, $HgCl_2$ and $CoCl_2$ were screened and assessed for metal toxicity. Tube dilution method was used to check the minimum inhibitory concentrations. Experiments in liquid culture were conducted to determine the precise concentrations of metals at which the isolates could grow. All the results obtained were expressed in MIC **Table 26**. The EC_{50} value was determined and the results are shown in **Table 27.** The MIC data of the results were statistically significant with p-value <0.05 for all the isolates.

Copper toxicity

Most of the isolates studied tolerated more than 1.5mM concentration of $CuSO_4.5H_2O$. It was observed that 5 isolates were having 20mM MIC. However, the most tolerant strains were XTR9, XTR15, XTR25 and 17R5 with MIC of 25mM (Table 26 and Figure 34). These results were supported by high EC_{50} values for these isolates as XTR9 (EC_{50}= 4.886), XTR25 (EC_{50}= 38.52) and 17R5 (EC_{50}=63.93) respectively as they were highest as compared to others as shown in Table 27.

Manganese toxicity

Many of the isolates were having tolerance at 3mM (MIC 4mM) concentration of $MnSO_4$. However, the isolates AYS2, AYS1, YTPR1 and SY6 were more tolerant strains at 10mM concentrations. Moreover, the most tolerant strains were XTR1, TRR2, and XTR25 etc (MIC 15mM) as compared to others (Table 26 and Figure 34). According to EC50 results the isolates with higher tolerance to manganese were AYS2, AYS1, SY6, XTR1, TRR2 and YTPR1 with the EC50 values of 5.360, 4.250, 2.791, 6.66, 4.72 and 4.7 respectively as shown in Table 27.

Cobalt toxicity

The level of tolerance among isolates against cobalt was 1mM concentration. However, many of the isolates were tolerant such as SY5, SY6, XTR15 and AYS10 at a concentration of 2mM (MIC 5mM). Also few of the isolates were highly tolerant with (MIC 10mM) such as XTR1, TRR2, XTR10 etc (Table 26 and Figure 34). However, the EC_{50} values was higher in case of SY5, SY6, AYS10 and XTR15 with values 11.79, 6.7, 4.882 and 9.2. Thus these isolates show considerable tolerance to cobalt as shown in Table 27.

Zinc toxicity

All the isolates were tolerant to zinc at a concentration of 1.5mM however, 9 strains were tolerant at a concentration of 5Mm (MIC=5.5mM) such as strain AYS2, AYS3, AYS4, AYS6, AYS10, AYS11 and AYS13. Some of the isolates such as XTR10, XTR25, YTPR1 and 17R5 were tolerant to concentrations above 5.5mM with MIC of 10mM. However, few of the strains such as SY17, AYS1 and AYS7 were highly tolerant to the concentration of 10mM (MIC=15mM) (Table 26 and Figure 34). The EC_{50} results were in agreement with

the tolerance results as above. The strain XTR10, XTR25, 17R5, YTPR1 and SY17 possessed the highest EC_{50} value of 5.01, 7.07, 4.1, 2.1 and 3.594 respectively as shown in Table 27.

Mercury toxicity

The mercury was highly toxic to all the strains as only less concentration <0.1mM of $HgCl_2$ was tolerable. However, many strains were tolerant to concentration of 0.1mM (MIC=0.2mM) such as strain SY14, SY15, AYS1, AYS4, AYS7 and AYS10 (Table 26 and Figure 34). Also few other isolates such as XTR1, XTR9, 17R2 and XTR25 etc were tolerant to 0.2mM (MIC=0.5mM). The most tolerant isolates with (MIC=2.5mM) were YTPR1, 17R4 and 17R5. However EC_{50} results of strains SY14, SY15, 17R2 and XTR25 were in accordance with the tolerance result as these strains were showing higher EC_{50} values of 3.637, 10.73, 2.97 and 13.6 respectively. This showed that besides these four strains, mercury was highly toxic to all the other strains.

The number of isolates showing higher tolerance to various heavy metals with higher minimum inhibitory concentration values are represented in Fig. such as 10, 12, 7, 7, and 3 isolates were showing higher MIC values for $CuSO_4$, $MnSO_4$, $CoCl_2$, $ZnCl_2$, and $HgCl_2$ respectively. Metal tolerance was also checked in order to find some interesting thermopilic isolates tolerent to various heavy metals that can be expolited in bioremediation and also to check any co-occurance of heavy metal resistance to antibiotic resistance. The results here were interesting. It was shown that many of the isolates were tolerant to heavy metals, showing higher MICs than that of *E. coli* (Nies 1999). Also when compared with the other two *Geobacillus* species such as, *G. thermoleovorans* and *G. thermantarcticus* (Poli *et al.* 2009), it was found that our isolates are showing much higher resistance as shown in **Fig.35.**

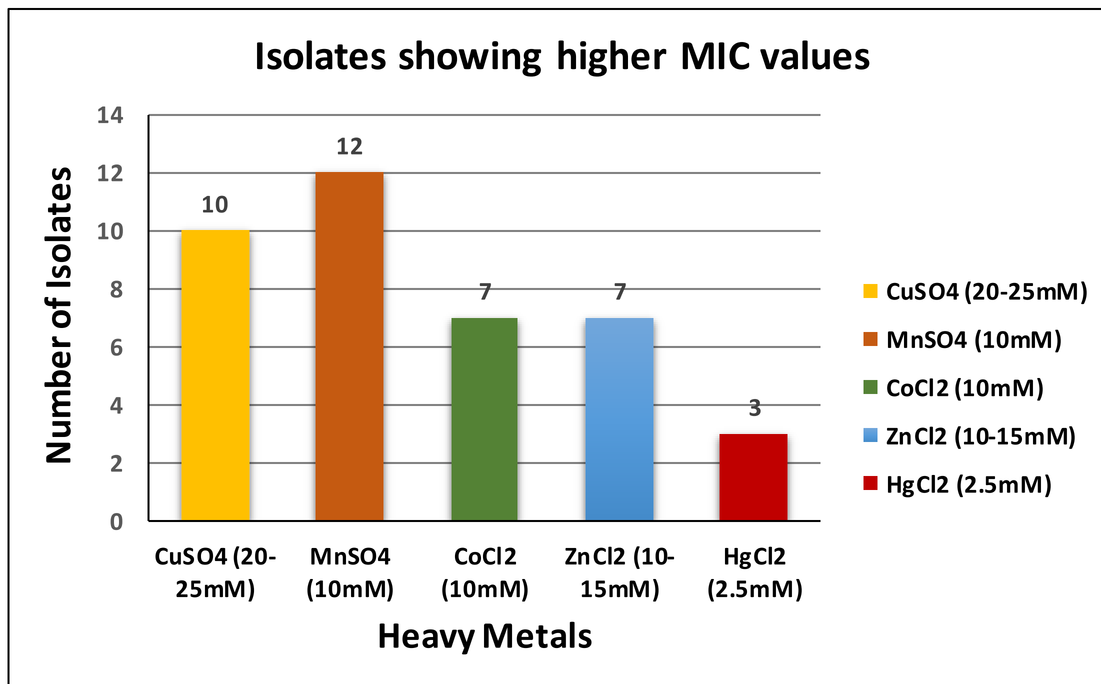

Fig.34. Number of isolates showing highest MIC values with in each heavy metal.

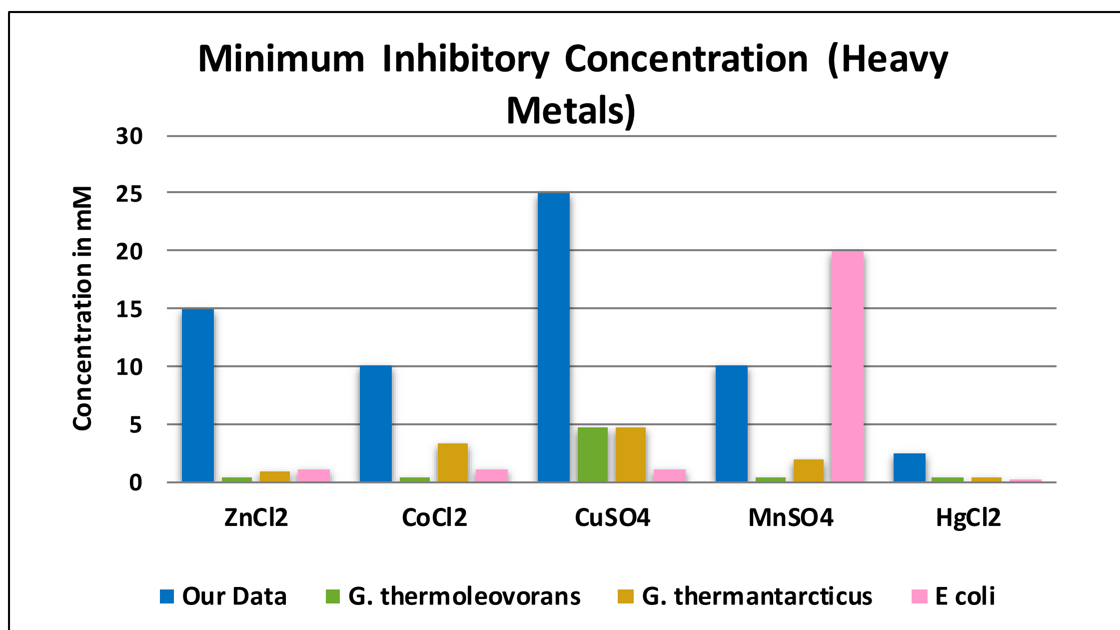

Fig.35. Comparison of minimum inhibitory concentrations among studied isolates and two known *Geobacillus* species and an *E Coli.*

Table.26. Minimum Inhibitory Concentration of heavy metals (in liquid media).

Minimum Inhibitory Concentration (in liquid media)							
Isolates	**$CuSO_4$**	**$MnSO_4$**	**$CoCl_2$**	**$ZnCl_2$**	**$HgCl_2$**	**R^2**	**P-value**
SY1	1.5	4	2	1.5	0.1	0.6	<0.0001
SY3	1.5	4	5	1.5	0.1		
SY4	1	4	2	1.5	0.1		
SY5	1.5	4	5	2	0.1		
SY6	1.5	10	5	2.5	0.1		
SY8	1.5	4	2	2.5	0.1		
SY12	1.5	4	2	2.5	0.1		
SY14	1.5	4	2	2.5	0.2		
SY15	1.5	4	2	2.5	0.2		
SY17	1.5	4	2	15	0.1		
AYS1	5	10	2	15	0.2		
AYS2	1.5	10	2	5.5	0.1		
AYS3	1.5	10	2	5.5	0.1		
AYS4	5	3	2	5.5	0.2		
AYS6	1.5	3	2	5.5	0.1		
AYS7	1.5	3	5	15	0.2		

AYS8	1.5	3	2	5.5	0.2		
AYS10	1.5	3	5	5.5	0.2		
AYS11	1.5	3	2	5.5	0.1		
AYS13	1.5	3	2	5.5	0.1		
XTR1	20	10	10	2.5	0.5		
XTR9	25	10	5	2.5	0.5		
17R2	25	10	5	2.5	0.5		
TRR2	20	15	10	2.5	0.5		
XTR15	25	10	5	2.5	0.5		
XTR10	20	10	10	10	0.5		
XTR25	25	15	10	10	0.5		
YTPR1	20	10	10	10	2.5		
17R4	20	10	10	5	2.5		
17R5	25	10	10	10	2.5		

Table.27. Estimated EC50 values for bacterial isolates.

Estimated EC50 values for strains					
Strains	**$CuSO_4$**	**$MnSO_4$**	**$CoCl_2$**	**$ZnCl_2$**	**$HgCl_2$**
SY1	1.7	0.5334	0.117	0.2825	-0.8797
SY3	1.999	0.7855	0.107	-0.4687	-0.799
SY4	1.471	2.996	2.141	-0.2371	-0.9054
SY5	2.656	2.653	**11.79**	0.1253	-0.7718
SY6	2.681	2.791	**6.732**	0.1877	-0.6622
SY8	4.599	3.415	-0.1815	0.1149	-0.9025
SY12	**4.681**	3.187	**10.41**	0.08492	-0.5866
SY14	4.576	2.878	-0.2153	0.146	3.637
SY15	4.476	0.522	-0.08118	0.1338	**10.73**
SY17	1.997	2.564	0.00216	**3.594**	-0.9898
AYS1	**4.989**	**4.251**	-0.2131	0.5598	-0.8004
AYS2	4.146	**5.36**	-0.1864	0.4729	-0.9201
AYS3	2.299	0.7118	**11.21**	0.388	-0.3906
AYS4	**4.479**	3.345	-0.1997	0.5097	**9.823**
AYS6	4.401	**4.336**	-0.1939	0.5528	-0.9132
AYS7	2.266	3.335	0.07729	0.5512	-0.01096
AYS8	4.047	3.844	-0.05464	0.5139	-0.6595
AYS10	4.404	2.921	**4.882**	0.5389	-0.565
AYS11	4.032	2.291	**6.553**	0.5586	**5.679**
AYS13	**4.59**	2.068	-0.07472	0.4642	-0.678
XTR1	3.005	**6.66**	0.4827	8.77E-05	0.2805
XTR9	**4.886**	1.181	7.100e-001	6.80E-05	0.06013

17R2	0.1368	-4.453	0.000107	7.08E-05	**2.97**
TRR2	0.4644	**4.72**	0.4168	6.69E-05	0.7259
XTR15	2.03	3.06E-06	**9.245**	6.75E-05	0.2938
XTR10	**5.091**	1.018	0.1862	**5.01**	0.2903
XTR25	**38.52**	0.7225	0.5996	**7.078**	**13.63**
YTPR1	1.375	**4.727**	0.001713	2.107	0.951
17R4	**10.2**	2.989	8.26E-05	1.134	0.1367
17R5	**63.93**	7.36E-05	0.05073	**4.185**	0.1729

6.9.2. Statistical significance of heavy metal tolerance through principal component analysis (PCA)

PCA of heavy metals was calculated and correlated with the isolates. The first two Eigen values among the five principal components were having >1 values such as 2.0710, and 1.1150, respectively as shown in **Table 28**. The total variance of the five principal components was 100%. The Bartlett's sphericity test was performed which represents the test for null hypothesis that the correlation matrix has an identity matrix. Taking this into consideration, these tests provide the minimum standard to proceed for Factor Analysis. The results shown by this test with p-value 0.0097 (threshold value of <0.05) thus represents the factor analysis is valid and significant as shown in **Table.29.** PCA results were similar to results obtained from MIC and EC_{50} analysis i.e., PCA shows positive correlation among individual heavy metals to those isolates which were having higher MIC and EC50 values with respect to that particular heavy metal. **Fig 36**. Through PCA it can be inferred that the two principal components F1 and F2 shows the significant correlation among the heavy metals and isolates studied. There was positive correlation between $CuSO_4$, $ZnCl_2$ and $HgCl_2$. However, $MnSO_4$ and $CoCl_2$ were distantly correlated. With respect to the isolated strains, it has been shown that there was a positive correlation between individual heavy metals and the strains highly tolerant with respect to their corresponding heavy metals. The

highly tolerant strains were present in the vicinity of concerned heavy metal, for example, SY17, XTR10and XTR25 are present in the vicinity of Zn, is positively correlated, i.e., these isolates are highly tolerant to Zn. Similarly, isolates ASY1, AYS2, TRR2, YTPR1 and XTR1 are present in the vicinity of Mn. Thus these isolates are positively correlated to Mn which indicate that these isolates are highly tolerant to Mn. Similar is the case with other variables such as Cu, Co and Hg studied as shown in **Fig 36**. From our study, it can be inferred that the isolates showed highest tolerance to Cu>Zn>Mn>Co>Hg on the basis of MIC results in liquid media.

Table.28. Principal Component Analysis (correlation between heavy metals and isolates): Eigenvalues

	F1	**F2**	**F3**	**F4**	**F5**
Eigenvalue	2.0710	1.1150	0.8174	0.6670	0.3296
Variability (%)	41.4206	22.3003	16.3476	13.3404	6.5911
Cumulative %	41.4206	63.7209	80.0685	93.4089	100.0000

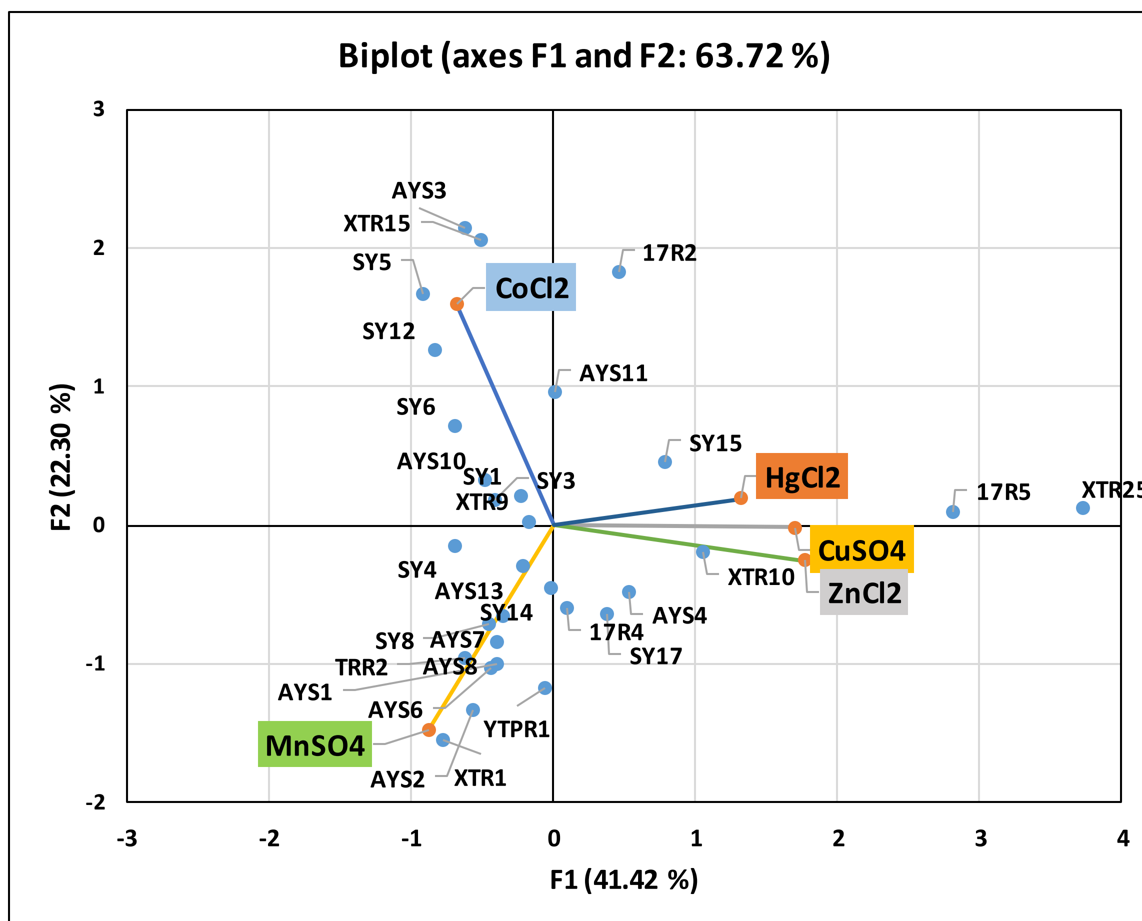

Fig.36. Principal Component Analysis showing correlation between heavy metals and various studied isolates).

Table.29. Bartlett's sphericity test:

Chi-square (Observed value)	**23.3116**
Chi-square (Critical value)	18.3070
DF	10
p-value	**0.0097**
alpha	0.05

6.9.3. Detection of various metal toxic genes by Whole Genome Sequencing.

It has been already mentioned above that whole genome analysis of two isolates AYN2 and LYN3 were carried out. Using RAST it was shown that both the isolate AYN2 and LYN3 were possessing metal tolerance genes. The genes related to copper homeostasis were found in both the isolates with the main function as *copper translocating P-type ATPase* or *copper/silver efflux P-type ATPase.* However, other than strain LYN3, the strain AYN2 possess other gene responsible for encoding copper tolerance protein. Besides copper homeostasis, both the isolates possess cobalt-zinc-cadmium resistance genes with the feature counts of 9 and 6 for AYN2 and LYN3 respectively. The genes responsible for this resistance have been known to code for *Cobalt-zinc-cadmium resistance protein CzcD; Probable Co/Zn/Cd efflux system membrane fusion protein; Transcriptional regulator, MerR family; Heavy metal resistance transcriptional regulator HmrR, and Cadmium-transporting ATPase* (EC 3.6.3.3). The strain AYN2 possess arsenic resistance genes which may code for *arsenical resistance operon repressor, arsenic efflux pump protein, arsenic reductase and arsenic resistance protein ACR3.* However, only *arsenic efflux pump protein, arsenic reductase* were found strain LYN3. Similarly, both the isolates possess cadmium resistance genes that were found to code for *cadmium transporting ATPase* (EC.3.6.3.3) *and cadmium efflux system accessory protein*. Apart from these, the strain AYN2 also possess mercury tolerance gene which is found to be absent in strain LYN3. The mercury resistance genes were found to code for *mercuric resistance operon regulatory protein* and *mercuric ion reductase* (EC 1.16.1.1).

6.9.4. Metagenomic studies of metal resistance genes (MRGs)

Metagenomic study of metal resistance genes (MRGs) were carried out using BacMetScan V.1.0. The results have shown the different resistance genes in both the hot springs. However, it was shown that the Reshi hot spring possess higher diversity of resistance genes than the Yumthang hot spring. The resistance genes responsible were belonging to various metals such as cadmium, copper, zinc, mercury and arsenic. In case of Yumthang hot spring only zing resistance genes were found rest all the other genes responsible for resistance in copper, cadmium, mercury and arsenic were found in Reshi hot spring. The copper resistance gene (copper resistance protein C and D) was showing closest identity (100%) with *Pseudomonas fluorescens* strain TSS Cop RSCD gene cluster. The cadmium resistance gene (for Cadmium resistance transcriptional regulatory protein Cad) shows 99% identity with *staphylococcus aureus* plasmid pl258 cadmium resistance (*cad A*) gene. Similarly mercuric resistance gene coding for mercuric reductase was showing 99% identity with *psychrobacter* sp. Strain ANT H52 Mer A (*Mer A*) gene. Arsenic resistance genes for arsenical pump membrane protein was showing 82% identity with *staphylococcus xylosus* arsenic efflux pump protein (*ars B*), arsenate reductase (*ars C*) and operon regulatory protein (*ars R*) genes. Apart from these genes the efflux pump membrane transporter Bep E was also found in Reshi hot spring which was showing 97% identity with *Pseudomonas fluorescens* strain MFN1032 putative cation efflux protein (*mex F*) gene. However in case of Yumthang only zinc resistance was found, the zinc resistance gene for zinc uptake regulatory protein was showing 100% identity with *E. coli* HKUOPY1 chromosome. Based on COG (cluster of orthologous groups) classification, various metal resistance genes were predicted corresponding to each metal resistance class discussed above as shown in **Fig.37.**

However, the metal resistance genes were diverse in Reshi hot spring than that of Yumthang hot spring. Few of the metal resistance classes such as Cadmium with COG predicted genes such as *czzA, czcB, czcC, and cadC;* Mercury with genes *merA, merR* and *merR1*; and Arsenic with genes *arsR1, arsR2* and *arsB* genes were only detected in Reshi hot spring. However, in case of Yumthang hot spring only the Zinc resistance genes were found such as *Zur, ZupT, ZnuA, ZnuB, ZnuC* and *ZntB.*

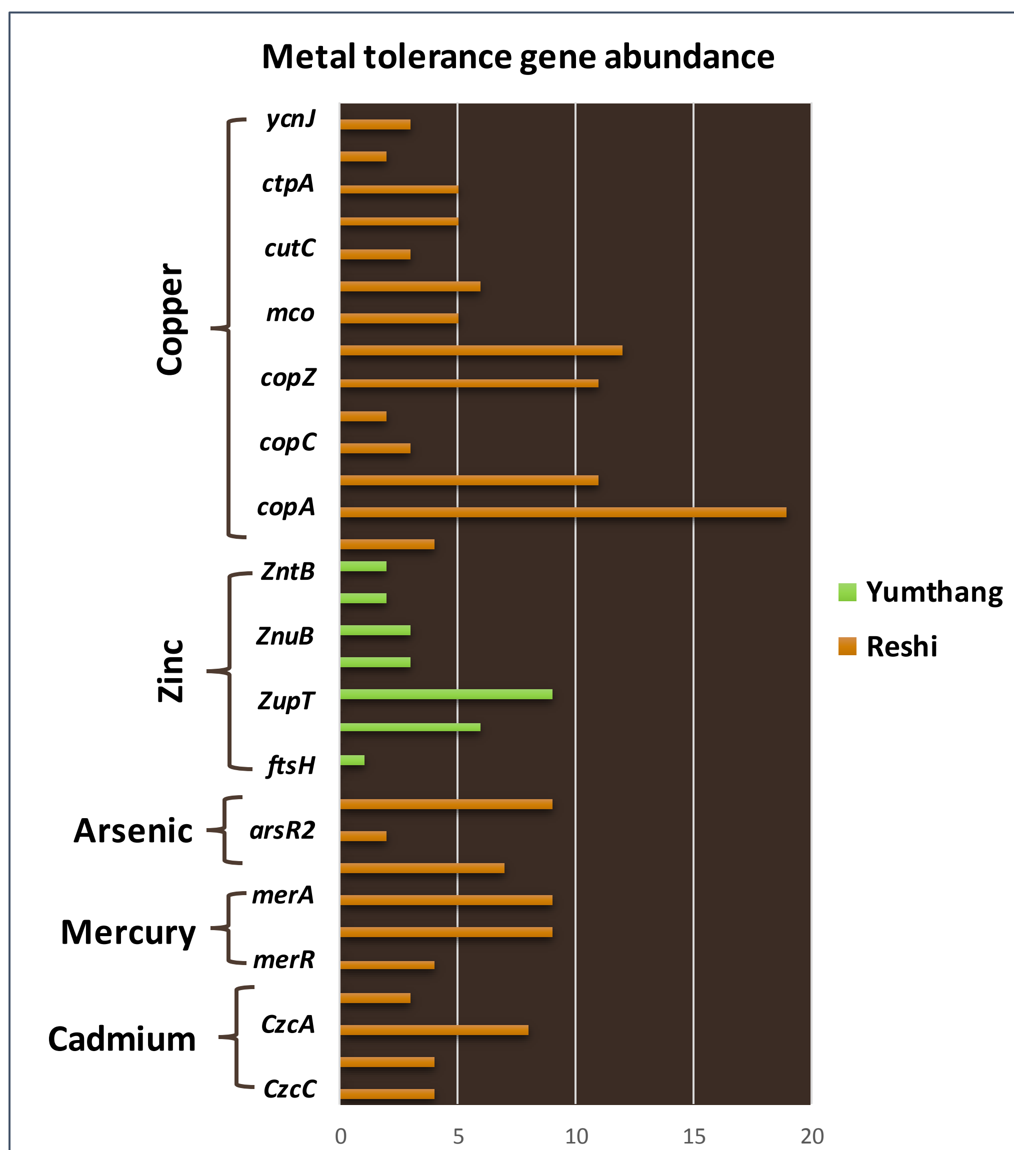

Fig.37. Metal tolerant gene abundance predicted by COG analysis.

7. DISCUSSION

The systems with a continuous circulation of heat and fluid, where fluid enters the reservoir from the recharge zones and leaves through discharge constitute geothermal fields. The unique spots within these fields are mainly hot springs, which are present all over the world. Although the hot springs are not merely the ponds where hot water oozes out, these thermal springs have been in use for religious and/or medicinal tenacities before 2000 BC in India and for hundreds of years in China, Egypt, Japan, Turkey and in many European and Middle-Eastern countries as shown by archaeological marks (Olivier and Jonker 2013). Many thermal springs urbanized into thriving centers of religion, culture, and health, such as those at Bath in England, Vichy in France and Baden-Baden in Germany (Olivier, Venter and Jonker 2011).

The main importance of these hot springs is the microbiota present in them. The microorganisms present in such hot springs can thrive under extreme temperatures. These microorganisms are known as thermophiles, which can grow into the temperatures above 45ºC (Stetter 1999). The biodiversity of several hot environments such as hot springs, oil reservoirs, composter were studied using shotgun metagenomics sequencing (Kotlar *et al.* 2011; Martins *et al.* 2013; Mehetre *et al.* 2016b). These new molecular methodologies have enhanced the microbial ecology studies by aiding in the analysis of comprehensive microbial community structure of an environment. Geothermal craters in earth crust are naturally diverse in microbial community structure. However, due to the uncultivated status of the major taxa in hot springs, culture-dependent analysis led to the identification of few distinct genera from the hot springs of Sikkim. Thus the main aim of the study was to explore the microbial diversity mainly bacteria and to correlate these with the

physicochemical parameters and to check the antibiotic resistance patterns present in the microbes of such isolated environments.

In 1973, the Geological Survey of India began the geothermal investigation in India and it was discovered that in excess of 350 hot springs with temperatures extending from 30° to 98°C are known to happen all through the nation. Krishnaswami (1975) gave a comprehensive record of the hot spring events and separated them into two groups based on their structural setting: an orogenic clutch, spoken to by warm springs in the Himalayas and a non-orogenic cluster, spoken to by the warm springs in peninsular India. In orogenic cluster, numerous hot springs are arranged in Sikkim which is topographically situated somewhere in the range of 27°04'46" and 28°07'48" North scope and 88°00'58" and 88°55'25" East longitude on the Eastern Himalayas. The abiotic and biotic issues decide the attributes of any hot springs and additionally its reasonableness for human utilize. Therefore several physical and chemical parameters of water from these hot springs were checked. The physicochemical analysis of four hot springs of Sikkim was suggested that besides being distinctly located these hot springs possess similar elemental concentrations. However, Reshi hot spring had few elements which are present in higher concentration compared to other three hot springs. In case of Yumthang hot spring sulphur content was higher and which can be sensed by the smell of the hot spring water. As per APHA (American Public Health Association), the elements present in these hot springs are under permissible limits thus it may also be concluded here that the water from the main source can be used for bathing and other purposes. The piper analysis shows the nature of hot springs and it has been predicted that the water of these hot springs is ground water, deep or shallow influenced by various environmental factors. The total dissolved solids are

highest in case of Reshi and Polok hot springs and the results are not surprising as these hot springs are not well constructed with no distinguished source openings and the human flux to visit these hot springs is relatively high than the other hot springs in Sikkim. These may be the reasons of high TDS mainly in case of Reshi hot spring.

Isolation of total 218 isolates were carried out and among them 152 were selected for further analysis on the basis of morphological and biochemical characterization. The morphological analysis suggested that the most of the bacteria were Gram-positive, aerobic, rod-shaped and spore forming. The carbohydrate fermentation shows the most of the isolates were able to utilize simple sugars such as dextrose, maltose, ribose, fructose, mannose etc. These results suggested that these isolates might belong to group *Bacillus*. The other biochemical characteristics such as catalase, oxidase, nitrate reductase, amylase and protease also adds to the tentative identification of group *Bacillus*. The growth profile of the bacterial isolates at various ranges of temperature, pH and NaCl concentrations were also checked. The optimum temperature of most of the isolates was 60°C, which reflect the thermophilic nature of these isolates. The optimum pH range of the isolates were found to be 7-8, thus suggesting the neutrophilic or facultative alkalophilic nature of these bacterial isolates. The optimum NaCl concentration of <4% suggested the non halophilic nature of bacterial isolates. The temperature of the four hot springs studies was also in the range of 45-75°C, thus the chances and presence of thermophilic bacteria in such hot springs are probably high. The pH of the hot springs was also in range of 7-8 thus suggesting the alkaline nature of hot springs. Therefore, it is not surprising that most of our isolates are thermo-alkalophilic and non halophilic in nature. In India most of the hot springs are alkaline in nature such as hot springs from North East India such as Jakrem (Meghalaya),

hot springs from Odisha (Atri)(Badhai, Ghosh and Das 2015), Barkeshwar West Bengal (Chaudhuri, Chowdhury and Chattopadhyay 2017), Uttrakhand (Soldhar and Ringigad) (Pandey *et al.* 2015), Gujarat (Tuwa) (Mangrola *et al.* 2015a), Ladakh (Puga) (Gupta *et al.* 2017). Pandey et al, 2015, while working on Soldhar and Ringigad hot springs of Uttarakhand, India, have shown that their isolates mainly belonging to *Bacillus* sp., can grow in the wide range of temperature, i.e., 20–80 °C. Thus they have shown that their isolates can also grow in the mesophilic range which is partially contradictory to our results as our isolates were unable to grow at temperature below 30°C. Similarly they have shown that their isolates can grow in much wider range of pH from (4–14) (Pandey *et al.* 2015) compared to our isolates which could actively survive and grow in pH range of 4–9. Many of the isolates of Borong hot spring were showed higher growth at acidic conditions compared to isolates from other hot springs of Sikkim. The reason may be the acidic nature of hot springs itself, as the pH of the Borong hot spring is little acidic (5.5).

Besides the optimal pH or temperature, the isolates were able to survive in a wide range of these environmental factors. The possible reasons could be hypothesized that this wide range of temperature and pH tolerance shown by these bacteria may be due to their presence in fluctuating surrounding environments at different geothermal heights. The hot waters present in reservoirs deep down to earth, flows out through high pressures. The physicochemical parameters may have the tendency of getting changed due to distinct regions in the plumbing system of hot springs deep down the earth. The microbes present may interact with fluctuating water substance with shifting physicochemical organization at different statures. Hence these fluctuating situations concerning particular physicochemical substance assist these microscopic organisms with becoming tolerant and

versatile to the evolving condition. It has been found that besides being distinctly located, all the hot springs possess similar kind of microbial diversity. The major phylum determined was Firmicutes. Among Firmicutes, many *Geobacillus* species and few *Bacillus* and *Anoxybacillus* species were found. The present results are in agreement with results documented by various researchers and it has generally been found that only few specific genera can be found by culture dependent techniques in the hot springs of India mainly *Firmicutes* (Sahoo 2017). Usually hot springs of India have temperature between 50°C and 70°C and have *Firmicutes* in predominance. Also globally it has been seen that the phylum *Firmicutes* were most versatile in the hot springs and could populate within a wide range of temperature gradients (Zentgraf 1992; Khalil 2011; Deep, Poddar and Das 2013; Panda and Sahu 2013; Sen and Maiti 2014; Daupan and Rivera 2015; Baltaci *et al.* 2016; Mohammad *et al.* 2017; Parul, Rituja and Vineet K 2017). Their predominance might be due to formation of spores in Gram-positive bacteria (Boetius *et al.* 2015). The genus *Geobacillus* has been earlier reported from the Himalayan geothermal provinces of Himachal Pradesh (Mehta, Kumar and Gupta 2012; Sharma, Sharma and Bhalla 2013) and Uttarakhand regions (Dheeran *et al.* 2010). But, to the best of our knowledge, it is the first report of finding the *Geobacillus* species from the hot springs of the north-eastern region of India. Apart from Himachal, there was no such known report of *Geobacillus* from other geothermal provinces in India such as Sohana geothermal province (Sareen and Mishra 2008), west coastal geothermal province (Mehetre, Paranjpe, Dastager, & Dharne, 2016), Mahanadi geothermal province (Badhai, Ghosh and Das 2015). So in our study, although the majority of isolates were identified as *Geobacillus* sp., however, the percentage similarity of many of these isolated bacteria have found to be <95% which suggest the

novelty of these isolated bacteria. However, getting such a less bacterial diversity using culture dependent approach, it was important to check the left out phyla in these hot spring, thus using PLFA and metagenomic (culture independent) approaches were much needed.

The conventional culture-dependent methods fail to give comprehensive microbial community structure of an environment due to the large unculturable status of the microbial world. The development of wide range of culture-independent methods has widened the scientific knowledge about previously unknown microorganisms and their involvement in different biological processes. Culture-independent techniques such as PLFA analysis which was a pioneer in the field was first used in 1979 to estimate the microbial biomass from marine sediments (White *et al.* 1979). This technique is important to determine viable microbial biomass, microbial community composition and metabolic activity in an environment (Rzonca and Schulze-Makuch 2003). Phospholipid fatty acids (PLFA) are an essential structural component of microbial cell membrane and each group of bacteria possess some particular phospholipid fatty acids, which makes it an important chemotaxonomic marker. PLFA analysis is widely used to estimate the total biomass and to observe the changes in community composition of the microbiota in soil and aqueous environment. In the present study the culture dependent analysis and culture independent approach that is PLFA has shown positive correlation. As in both the methods we have found Gram-positive bacteria in abundance, however in culture dependent analysis we were unable to find any Gram-negative bacteria. The results showed that Gram-positive bacteria were relatively higher in Yumthang (42.74%) followed by Borong hot spring (30.03%) than the Polok (27.94%), while Gram-negative bacteria were higher and similar in Yumthang (7.77%) and Polok (7.17%) than the Borong (4.85%). The various fatty acids

abundant in these hot springs were 15:3 ω3c, 15:0 anteiso, 16:00, 17:1 iso ω9c, 17:0 anteiso, 18:2 ω 6c, 18:1 ω 9c, 19:3 ω 3c and 21:3 ω 3c. The abundant fatty acids showed the dominance of biomarkers for Gram-positive bacteria such as 15:0 anteiso, 16:00, 17:0 anteiso, 18:0.(Willers, Jansen van Rensburg and Claassens 2015). The total biomass of Borong hot spring (1044.9nmoles gm^{-1}) was higher than that of Polok hot spring (838.8nmoles gm^{-1}). The similar studies have been performed by (Rzonca and Schulze-Makuch 2003), however, they have found an abundance of Gram-negative bacteria than that of Gram-positive bacteria. Moreover, there is very less data present on PLFA studies of hot springs. Thus it becomes little difficult to correlate the data with other data present in the scientific world.

Surprisingly by metagenomic analysis, we found the predominance of Gram-negative bacteria and limited presence of *Firmicutes* or *Geobacillus sp*. As being distinctly located, the bacterial community show significant variation between the springs. The phylum wise diversity showed the dominance of *Proteobacteria* (62.50%), *Bacteroidetes* (15.38%), *Acidobacteria* (3.85%), *Nitrospirae* (3.85%) and *Firmicutes* (2.88%) in Borong whereas Polok was dominated by *Proteobacteria* (47.22%), *Bacteroidetes* (3.61%), *Firmicutes* (3.06%), *Parcubacteria* (3.06%) and *Spirochaetes* (2.50%). The phylum wise diversity showed the dominance of *Actinobacteria* (98.1), *Proteobacteria* (1.7%), *Firmicutes* (0.01%) and *Bacteroidetes* (0.003%) in Yumthang hot spring whereas in Reshi hot spring the dominant phyla were *Proteobacteria* (75.92%), *Actinobacteria* (22.68%), *Firmicutes* (1.14%), and *cyanobacteria* (0.03%). Similarly, other hot springs located across India (Sangwan *et al.* 2015); (Panda *et al.* 2016); (Mehetre et al., 2016); (Ghelani *et al.* 2015); (Mangrola *et al.* 2015b); (Saxena *et al.* 2017a); (Badhai, Ghosh and Das 2015) and China

(Song *et al.* 2013) possess the similar kind of bacterial diversity with abundant phyla including *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Chloroflexi*, *Bacteroidetes* and *Cyanobacteria*. However, the percentage of various phyla vary among various hot springs. Surprisingly, the predominance of *Firmicutes* and *Geobacillus sp*., was reported only in Bakreshwar hot spring, West Bengal, India and with increase in temperature of these hot springs, number of *Firmicutes* and more specifically *Geobacillus sp*., numbers also increased. This variation might be due to their different sampling, preservation protocols and DNA isolation techniques (Quince *et al.* 2017). It has already been suggested that the sampling and DNA isolation protocols can affect both the quality and accuracy metagenomic results. Detection of microbial community profiles can be affected by various factors such as length of time between sample collection and the number of freeze–thaw cycles a sample undergoes (Wesolowska-Andersen *et al.* 2014; Muldoon *et al.* 2016). Metagenomic analysis also revealed the presence of very less archaeal communities in these hot springs of Sikkim. Owing to decrease in the temperature below 50°C the probability decline in the archaeal communities are higher as they require the optima of >80°C temperature to maintain their metabolic activity

To shape the microbial community of any ecological niche, geochemical parameters are often being the deciding factor. Thus various statistical analysis were needed performed to correlate geochemical parameters with the microbial diversity. Principle component analysis (PCA) was done to check the correlation between bacterial diversity at phylum level and physicochemical parameters with respect to four hot springs of Sikkim. It was shown in the PCA biplot that hot springs were positively correlated to Actinobacteria, Proteobacteria, temperature, colloidal sulphur, pH, calcium etc.

The main characteristic feature of any hot spring is temperature, thus, the abundance of a particular phylum in the hot springs has been correlated and interpreted by many researchers as a function of temperature. Subudhi et al. (2017) have shown the predominant shifting of thermophilic cyanobacteria as a function of temperature and also, have shown the abundant growth of different strains at different temperatures (Subudhi *et al.* 2018). Similarly, Sahoo et al., 2017, have correlated and linked the predominant nature of Proteobacteria in the hot springs of Odisa, India, as a function of temperature (Sahoo 2017). In our study, the phylum level diversity of three hot springs is similar to a great extent, but the relative abundance is considerably different. Borong and Reshi hot springs are having higher abundance of Proteobacteria than that of Polok and Yumthang hot spring. The three hot springs such as Polok, Borong and Reshi are located in the same area, only few miles apart and at a similar altitude and similar chemical constituents. Thus having similar geographical and geological features, this might be the reason for having similar bacterial diversity in the two hot springs. However there is a considerable difference in their temperature. The phylum Proteobacteria are known to dominate in moderate thermophilic temperatures (Wang *et al.* 2013). Since, Borong and Reshi hot springs possess the lower temperature than Polok hot spring and therefore, the function temperature may be the reason for Proteobacterial predominance in these hot springs. The other characteristic feature of phylum Proteobacteria is that they are known to tolerate higher concentration of sulfur and utilize sulfur as an electron donor during their physiological process (Bolhuis, Cretoiu and Stal 2014). Therefore, it is not surprising that Borong hot spring has higher abundance of Proteobacteria, as Borong hot spring possess high sulfur content then Polok hot spring which was confirmed by ICPMS results. This correlation of temperature and

sulfur concentration to phylum Proteobacteria has been supported by our PCA results which is in accordance with Sahoo et al., 2017 (Sahoo 2017).

Comparison with the reported microbial diversity of hot springs from different provinces of India showed a significant pattern of differentiation and correlation along the community structure. This difference in microfloral diversity may be due to the geographical and geochemical distinction among the hot springs. The heatmap constructed with Bray Curtis Dissimilarity distance showed two major clades or groups with two sub-clades each and one out-group. Four of the Tibetan hot springs (Rongma, Gulu, Jiwa1 and Jiwa2) (Huang *et al.* 2011) and two hot springs from Barkeshwar, West Bengal, India (Chaudhuri, Chowdhury and Chattopadhyay 2017), Junagarh, Gujrat, India (TulsiShyam) (Ghelani *et al.* 2015), one from Shillong, Meghalaya, India (Jakrem)(Panda *et al.* 2015) and one from Odisha (Atri) (Badhai, Ghosh and Das 2015), formed a single group, whereas the diversity of Atri and Jakrem was similar with the dominance of *Chloroflexi*. The other hot springs formed the second sub group where the dominant group is *Firmicute*s. The Rongma of Tibet formed a single branch in the second subgroup of first clade as it the only spring in first group with dominance of *Acidobactera* and *Bacteroidete*s. The second clade was made by seven hot springs including three hot springs of Sikkim (Polok, Borong and Yumthang) and one from Shimla, Himachal (Tattapani) (Mohanrao et al., 2016), two hot springs from Odisha, India (Athamallik and Tarabalo) (Badhai, Ghosh and Das 2015), and one from Tibet (Gulu 2) (Huang *et al.* 2011) respectively. The hot springs from the second clade were dominated by *Proteobacteria*. Polok and Borong in the second clade showed correlative diversity with the dominance of *Proteobacteria* and *Bacteroidetes*. Anhoni hot

spring from Madhya Pradesh, India (Saxena *et al.* 2017b) formed the out-group, where the relative abundance of the phyla was least in comparison to the other springs.

The modern microbiology is having a concept of "a pure culture is not enough". Thus the present day Microbiology has taken the challenge to explore and characterize the uncultured microbial diversity. Many distinct and isolated environments such as deep sea vents, ocean surfaces, hot springs, soil, animal rumen, and human gut were explored with respect to microbial diversity. The other challenge expounded was the functional characterization of cultured or uncultured microbial diversity. These challenges procreated the establishment of various neo techniques such as metagenomics and microbiologists are smartly exploiting these phylogenetic or functional metagenomic approaches to explore hidden, unexplored or unculturable microbial diversity. One of the most important aspects of functional metagenomic approach is the elucidation of antibiotic and metal resistance related genes and its functions. Antibiotic resistance which is threatening the mankind has become the great concern and large scale research has been carried out on this subject worldwide. Metagenomic analysis has played a significant role and has been considered a promising culture independent method of determining diversity and abundance of ARGs and MRGs in various environments such as activated sludge (Peltier *et al.* 2010), drinking water (Fernando *et al.* 2016), sediment (Yang *et al.* 2016)and soil (Knapp, Dolfing and Ehlert 2010).

It has been suggested that antibiotics have been produced for over 500 million years. The only environments that are truly "pristine" are exempted from the influence of human antibiotic use might have existed before the antibiotic era (Allen *et al.* 2010). The time before the introduction of sulphonamides, which occurred in the late 1930s, can be

considered 'antibiotic naive' era, in the sense that no industrial production of antibiotics took place (Hughes and Datta 1883; Pallecchi *et al.* 2007; Rosenblatt-Farrell 2009). However, heavy metals were used for disease treatment for centuries prior to the use of antibiotics (Hughes and Datta 1883; Rosenblatt-Farrell 2009; Allen *et al.* 2010). Before the extensive spreading of the drugs it was shown by reflective studies that the resistance genes were present in bacteria that did not yield antibiotics and possibly the use of heavy metals for disease treatment and wide occurrence heavy metals in different ecosystem, might have selected for genes encoding both heavy metal and antibiotic resistance (Allen et al 2010; Hughes and Datta 1983; Rosenblatt-Farrell 2009; Bartoloni *et al.* 2004). Thus, factors of antibiotic resistance existed naturally and were maybe subject to horizontal transfer long before the intense choice pressure that was obligatory within the antibiotic era (Davies 1998, 2009). This predisposition for the genetic exchange of resistance elements is certain to have facilitated the rapid outgrowth of antibiotic resistance in pathogenic bacteria. ARGs proliferate mainly through two processes: vertical gene transfer attributed to the reproduction of bacterial hosts, and horizontal gene transfer attributed to the transfer of ARGs between different bacterial cells via mobile elements (Sørensen *et al.* 2005).

The present study was also aimed to check the antibiotic and metal resistance, ARG and MRG gene patterns in two hot springs of Sikkim present in the different locations. The study was carried out by using culture dependent and culture independent methods. The ARG and MRG genes were also characterized by whole genome sequencing of few isolates. The main aims were to check prevalence and abundance of resistance genes, the commencement and progression of resistance, and the co-occurrence of antibiotic and metal resistance.

The environmental microbes which neither cause disease nor closely related to antibiotic production have been ignored over the course of antibiotic era. However, they play a very important role in evolutionary development of antibiotic resistance. The evolution and proliferation of antibiotic resistance in pathogens or environmental microbes is very important and is still controversial. To address it, we checked the antibiotic resistance profile in four hot springs of Sikkim.

Antibiotic resistance was checked using 7 different classes of antibiotics such as β-lactams, aminoglycosides, macrolides, quinolones, glycopeptides, lincosamides, and chloramphenicol and we found that all the isolates were susceptible to these antibiotic classes and also their MIC values are very less. The susceptible nature of these isolates can be a local effect and therefore we checked antibiotic susceptibility of two standard *Geobacillus* species, i.e., *Geobacillus stearothermophilus* and *Geobacillus thermoleovorans* found them susceptible. It was reported that *Firmicutes* and *Actinobacteria* are not important host of ARGs and MRGs and their numbers in microbial community increased with increase in temperature of local environment. Further, unavailability of any report on antibiotic resistant *Geobacillus* species from thermophilic environment along with our results suggest that naturally or generally *Geobacillus* species are probably antibiotic susceptible. Due to their antibiotic susceptible nature, thermostability and non-pathogenic nature, *Geobacillus* might find several industrial applications. The susceptible nature of these culturable bacteria may be due to many reasons such as less anthropogenic activities or isolated nature of hot springs, high temperatures deep down the hot spring reservoirs, less diversity and less competition among microbial communities. As the thermophilic bacteria reside at higher temperatures

where antibiotics are not stable, thus these bacteria may find no need to acquire any antibiotic resistance genes. This argument has been supported by the absence of plasmids in these culturable thermophilic bacteria suggesting the absence of horizontal gene transfer.

The less cultivable bacterial diversity among these hot springs suggests the less competition among them and thus may be the less production of various antimicrobials. These results were supported by other study involving the targeting of common resistance genes such as genes encoding penicillin-binding proteins such as *PBP1A* and *PBP2A,* ampicillin resistant *ampC* genes and methicillin-resistant *mecA1* and *mecA2* genes. However, none of the isolates showed positive results which suggest the absence of these common and universally found resistance genes among these thermophilic bacteria. These results incite us to check the antibiotic resistance gene through whole genome sequencing of two of these bacteria which are chosen randomly. The whole genome sequencing shows the detection of various basic resistance genes of few classes such as β-Lactams and fluoroquinolones. However, these few genes may be putative because they didn't showed any identity with any known genes. Also, after doing BLAST, no hits were found, when checked in ARDA BLAST. Moreover, it was found after performing RAST, that these genes such as *parC, parE, gyrA*, and *gyrB* have other functions also. For instance, *ParC* apart from providing resistance to fluoroquinolones, it also functions as DNA Topoisomerase (Type II ATP dependent Topoisomerase IV subunit A). Thus these genes having other functions can be considered as putative and inactive with respect to antibiotic resistance. It has been shown by various studies that environmental organisms are the repository of these resistance genes (Dcosta *et al.* 2011; Bhullar *et al.* 2012). In contrast other studies, still controversial, depicts anthropogenic use involved in the emergence of

these genes (Knapp, Dolfing and Ehlert 2010; Thaller *et al.* 2010). Moreover, the hot springs being unique ecosystems with less anthropogenic activities and high temperature may possibly be devoid of antibiotics. Thus the lack of antibiotics may have less pressure or competition to acquire antibiotic resistance genes by these thermophilic microbes. This may be also supported by the fact that the thermophilic bacteria possess small genome size than that of mesophiles.

Metagenomic study reveals the presence of various antibiotic resistance genes belonging to class aminoglycosides, chloramphenicol, macrolide, β-lactams, polymyxin and tetracycline. However, these genes showed the maximum identity, i.e., more than 97% with Gram negative and mesophilic bacteria. For example the gene related to β-lactam was showing (100%) identity with *E- coli* strain RM14721 and other gene from class C- β-lactamase-cephalosporin was showing (97%) identity with *Serratia* sp YD25. Similarly all the other predicted genes shown in functional metagenomics results were having closest similarity with mesophilic Gram-negative bacteria. Thus it can be concluded that the genes detected through metagenomics may be the contamination of soil micro flora surrounding the top soil layers of hot springs. But our introspection of this ambiguity lead us to search for Heavy Metal Resistance Genes (MRGs) as a close nexus has been reported by many researchers between the antibiotic resistant phenomena and heavy metal resistant phenomena. Heavy metals like Cu and Zn are abundant in ecology and their sources found in environmental samples are myriad. Since, ages these heavy metals have been directly correlated and linked to the development of heavy metal tolerance in environmental microflora (Wales and Davies 2015; Poole 2017). In the environmental samples, Cu and Zn have always been found along with the concomitant presence of antibiotic resistant

microbial communities (Knapp *et al.* 2011; Becerra-Castro *et al.* 2015) The probability of transfer of mobile genetics elements (MGEs) increases with increase in the concentration of Cu, forecasting the ability of Cu-dependent ARGs as readily accessible or mobile (Hu *et al.* 2016) There has been interesting reports where it has been shown that presence of Cu at relatively low levels such as subtoxic metal positively affects certain ARGs (Knapp *et al.* 2017).

Antibiotics can form strong metallo ligand coordination complexes with metal ions and their ionic interaction can depict broad results for example, duct ion in antibiotic potency (Weinberg 1957; Niebergall *et al.* 1966). Zn inactivation of penicillin was first reported by Eisner by promoting hydrolysis of β-lactam (Eisner and Porzecanski 2018) Aminoglycosides, tetracycline, macrolides, vancomycin, quinolones, class of b-lactams can all bind with Zn (Poole 2017). Vancomyin binds Zn and induces expression of Zn limitation-inducible genes in a variety of bacteria, including E. coli, *Bacillus subtilis,* and *Streptomyces* sp., a result consistent with it withholding this needed trace metal from these organisms (Zarkan *et al.* 2016). Cu has also been shown to bind to penicillin (Cressman *et al.* 1966) and also promotes hydrolysis of this β-lactam (Eisner and Porzecanski 2018). Cu can also bind to aminoglycosides, tetracyclines, chloramphenicol, novobiocin, macrolides, isoniazid, quinolones, vancomycin, and a variety of β-lactams, including cepahalosporins and penicillins and also interferes with the activity of streptomycin and neomycin (Poole 2017). So, we initially screened the metal tolerance (if any) among our bacterial isolates from the hot springs of study.

Metal tolerance was also checked in order to find some thermophilic species tolerant to various heavy metals that can be exploited in bioremediation and also to check any co-

occurrence of heavy metal resistance to antibiotic resistance. Interestingly, it was found that many of the isolates were tolerant to heavy metals, showing higher MICs than that of *E coli* (Nies 1999) and other *Geobacillus* species such as, *G. thermoleovorans* and *G. thermantarcticus* (Poli *et al.* 2009). Whole genome sequencing results also support the metal resistance as both the isolates AYN2 and LYN3 possess the heavy metal resistance genes against copper, cobalt/zinc/cadmium and arsenic resistance. Metagenomic analysis also shows the presence of heavy metal resistance genes. However, similar to antibiotic resistance gene detection through metagenomics, the metal resistance genes showed maximum identity, i.e., more than 97% with mesophilic bacteria. For example the gene related to copper resistance (copper resistance protein C and D) was showing closest identity (100%) with *Pseudomonas fluorescens* strain TSS Cop RSCD gene cluster. Similarly, the cadmium resistance gene (for Cadmium resistance transcriptional regulatory protein Cad) shows 99% identity with *staphylococcus aureus* plasmid pI258 cadmium resistance (*cadA*) gene. The known fact that hot spring water comes out of reservoirs deep down the earth through fissures surrounded by various kinds of rocks and metal contaminants. Thus it may be assumed that the metal tolerance found in culturable bacteria may be due to various elemental concentrations in the hot springs. Although, very less *Geobacillus* or *Bacillus* were found in metagenomic studies but still metagenomics analysis found various metal resistance genes, however these genes were corresponding to Gram-negative bacteria. Thus here the results support the assumption that the antibiotic resistance detected through metagenomics may be due to the contamination of soil micro flora surrounding the top soil layers of hot springs. As the heavy metal resistance genes were found in culturable thermophiles in contrast to their antibiotic counterparts in this study

thus it may be concluded that there may be no co-occurrence or co-selection of these genes in such isolated habitats. However, this is a broad assumption which needs to get counter check in other such habitats.

8. SUMMARY

The microbial communities residing in extreme environmental niches are very important in terms of industrial and biotechnological perspectives. Among all the microbes living in such extreme conditions, thermophiles (temperature loving microbes) got widespread devotion from various researchers in recent decades. The prime advantage is the thermostable nature of these microbes and their enzymes thus this characteristic made them the choice of various industrial and biotechnological scientists (Coker 2016).. The dwelling places of these thermophiles are scattered all over the world in the form of Hot Springs, hydrothermal vents, thermal composts, marine trenches etc. (Anderson, Sogin and Baross 2014; Brumm, Land and Mead 2016; Mohammad *et al.* 2017). Among other Asian countries, India is also blessed with a lot of Hot Springs. There are almost 400 Hot springs in India (Singh *et al.* 2016) and most of them are pristine. Sikkim, one of the biodiverse states of India holds many Hot Springs in its vicinity and most of them are immaculate with respect to the microbial diversity. Various neo methodologies and approaches such as next generation sequencing (NGS) technologies have been developed to study such a microbial world. Thus there is an immediate need to explore the microbial diversity of these Hot Springs, which may lead to the understanding and accomplishing novel microbes and their enzymes for industrial and biotechnological benefits. Thus the present study was aimed to investigate the microbial diversity of these Hot Springs by culture dependent and culture independent techniques such as next generation sequencing (NGS) and PLFA approaches.

The first aim was to check the physicochemical properties of four hot springs such as Yumthang, Polok, Borong and Reshi hot springs. It was found that besides being distantly

located all the four hot springs possess similar pattern of elements. The concentration of various elements was also similar, however, Reshi hot spring was rich in several elements such as chloride, calcium and magnesium. The Reshi hot spring was followed by Polok hot spring in elemental rich ness. The sulfur concentration was found to be higher in case of Yumthang. The temperature of Polok hot spring was found to be highest followed by Borong hot spring. The pH analysis shows that all the hot springs are alkaline in nature, however, Borong hot spring is slightly acidic in nature. Statistically piper analysis shows that the deep ancient ground water nature in case of Yumthang and Reshi whereas shallow fresh ground water in case of Polok and Borong Hot spring.

The second aim was the isolation and characterization of thermophilic bacteria from these four hot springs. 218 isolates were isolated and on the basis of morphological and biochemical characterization only 152 isolates were taken to further analysis. All the isolates were Gram positive, aerobic, rod-shaped and most of them were able to form endospores. Carbohydrate fermentation analysis shows that the isolates were able to utilize simple sugars such as dextrose, maltose, ribose, fructose and mannitol, however, they were unable to utilize cellobiose, dulcitol, melezitose etc. Our results have found 47 isolates as amylase enzyme producers and 7 protease enzyme produces. Based on Morphological, Biochemical characterization and carbohydrate fermentation it can be predicted that the isolates might belong to group *Bacillus.*

The third aim was to identify the bacteria on the basis of 16S rRNA sequencing. The dominance of phylum *Firmicutes* was observed. Major genus found in the study was *Geobacillus* with a few representatives of genus *Anoxybacillus* and *Bacillus.* Also, some uncultured bacteria were reported such as Uncultured 17R2 and Uncultured TRR2. The

alignment and similarity search of 16S rRNA sequence with nr/nt database of NCBI have shown that many of the isolates have a distinct percentage of identity from <95%. These results suggested the novelty of these isolated bacteria. The identification of few bacterial isolates was also done by FAME (Fattyacid methyl ester) analysis. The three isolates AYN2, LYN3 and CTRL6 were identified. The fatty acids present in them were distinct and with variable concentrations. These results represent the unique nature of these isolates which has been also confirmed by their SI (Sim Index) values. By analyzing the results using RTSBA6 Sherlock libraries, In case of strain AYN2 no matches were found with (SimIndex-0.00). The strain LYN3 was showing similarity with *Geobacillus stearothermophilus* possessing Sim Index of 0.201. And lastly the strain CTRL6 was showing similarity with *Paenibacillus macerans* having Sim Index of (SI-0.385). The small diversity found in culturable techniques led us to extend the work and to check the bacterial diversity by culture independent techniques such as Next Generation Sequencing (NGS) and Phospholipid Fatty Acid (PLFA) analysis.

The PLFA analysis shows the abundance of Gram positive bacteria followed by Gram negative, which was thus correlating with the culture dependent diversity analysis. PUFA and branched chain fatty acids were abundant fatty acids in all the hot springs. However, apart from these major fatty acids Yumthang hot spring was possessing straight chain fatty acids also which were absent in other hot springs. Total biomass was found to be highest in Reshi followed by Borong and Reshi. Metagenomic analysis shows the prevalence of Phylum *Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria* with major species as Uncultured, *Ignavibacterium album, Sedimentibacterium, Rhodococcus ruber, Bacillus*

pumilus, Thiobacillus, Geobacter, Thermoanaerobacter, Thermus sp., Flavobacterium aquaticum and so on.

A novel bacterium was claimed after polyphasic characterization such as Biochemical Identification, Biolog, FAME analysis, Whole Genome Analysis etc, and these results have found that there is a considerable distinction between our isolate AYN2 and to that of other closely related Geobacillus species thus can be confirmed as a novel species. The strain was named as *Geobacillus Yumthangensis* AYN2 based on the hot spring where from the strain was isolated. The Gram-positive, endospore forming aerobic rods, with 2.5-5 µm long and 0.4-0.6 µm wide. The cells are able to utilize cellobiose, lactose, ribose, sorbitol and glucose. The strain can grow at a wide range of temperature (40-70ºC) and pH of 6-10.

The last objective was to check the antibiotic susceptibility patterns and any possible mechanisms. We checked the antibiotic resistance profile of 10 antibiotics such as Penicillin, Methicillin, Amoxicillin, Ampicillin, Erythromycin, Chloramphenicol, Gentamycin, Clindamycin, Norfloxacin, and Ciprofloxacin and interestingly found that all the isolates were susceptible against all the antibiotics used. The search was extended and minimum inhibitory concentrations was also checked. The results showed that very less minimum inhibitory concentration values for almost all the antibiotics checked. In case of Gentamycin, Vancomycin, Erythromycin, and Chloramphenicol the MIC values were generally around 0.5 µg ml^{-1}, 2 µg ml^{-1}, 2 µg ml^{-1}, and 8 µg ml^{-1} respectively. The least MIC of 0.25 µg ml^{-1} was shown in case of Penicillin G and oxacillin. The MIC was defined as the lowest concentration producing no visible growth. No internationally acknowledged criteria for susceptibility testing or for breakpoints for susceptible or resistant isolates are

accessible for thermophilic bacteria. However, the two thermophilic Campylobacter species were investigated and their breakpoints has been established (Guévremont et al, 2005). Briefly, the breakpoint values of the MIC for resistance are given in results section and thus as per Guévremont et al, 2005, the MIC values of various antibiotics against our isolates is thus very less.

Various universal resistance beta-lactam genes such as pbpA, ampC and mecA were targeted using specific primers by PCR technique. The results showed the absence of any resistant genes in our isolates. These results were also supported by whole genome sequencing of two isolates AYN2 and LYN3. The whole genome sequencing also shows the absence of any resistance genes. The genes if any present were putative with other functions also. The search for ARGs were also carried out by metagenomic analysis. It was found that the resistance genes detected in metagenomic analysis were showing >95% similarity with mesophilic bacterial genes. Thus this also suggests the absence of resistance genes in thermophilic bacteria.

Heavy metal tolerance was also checked against five heavy metals and it was shown that our isolates were resistant than that of the other *Geobacillus* species and *E Coli.* Whole genome sequencing also supported these results by showing the presence of heavy metal resistance genes. Metagenomic analysis also showed the presence of heavy metal resistance genes, however, their percentage similarity was higher with that of mesophilic bacterial genes. Hence, here the results support the assumption that the antibiotic resistance and heavy metal resistance detected through metagenomics may be due to the contamination of soil micro flora surrounding the top soil layers of hot springs. As, the MRGs were found in culturable thermophilic bacteria in contrast to their antibiotic

counterparts in this study, so it might be concluded that there may be no co-occurrence or co-selection of these genes in such isolated habitats. However, this is a broad assumption which needs to get counter check in other such habitats.

9. CONCLUSIONS

The culture dependent analysis and Phospholipid fatty acid analysis were correlative as both the methods revealed the abundance of Gram positive thermophilic bacteria as compared to Gram negative bacteria. PLFA suggested the abundance of Gram positive bacteria in all the four Hot Springs, however, the Yumthang Hot Spring possess the higher percentage of Gram positive and Gram negative bacteria than that of other Hot Spring. Whereas Actinomycetes were found in case of Yumthang Hot Spring only. The culture dependent approach revealed the abundance of genus *Geobacillus* followed by *Bacillus, Anoxybacillus* and uncultured bacteria. The lower percentage identity (<95%) and higher enzymatic potential of the isolates encourage the further investigation of these isolates as there may be enough chances of getting novel species, which may thus be exploited in industrial and biotechnological purposes. The correlation of physicochemical characteristics with PLFA data suggested that various physicochemical parameters such as temperature, alkalinity, Na, Mg, Cl, Ca and K content shapes the microbial community composition and diversity. The piper diagram also suggested that the water of Reshi and Yumthang hot springs are different such as Na-HCO^{3-} type and Na-Cl type respectively. Whereas, those of Polok and Borong are Ca- HCO^{3-} type. These can thus be predicted as marine or deep fresh ground waters and shallow fresh ground waters respectively. The present study has limitations as the conventional culture-dependent methods and thus PLFA fail to give comprehensive microbial community structure of an environment due to the large uncultivable status of the microbial world. Thus there was further scope to investigate these environmental niches using more advanced techniques such as metagenomics.

Metagenomic analysis revealed a wide and diverse bacterial population in all the four hot springs of Sikkim. These hot springs of Indo-Tibetan plateau are home to many possibly unknown and novel microbes as indicated by the abundance of 16.35% and 15.28% uncultured bacteria in Borong and Polok respectively. However, the most abundant phyla present were *Proteobacteria*, Actinobacteria, *Bacteroidetes and Firmicutes*. The correlation of physicochemical characteristics with most dominant phyla suggested that various physicochemical parameters such as temperature, pH, alkalinity, Ca^{+2}, Mg^{+2}, Cl^{+2}, and sulfur content shapes the microbial community composition and diversity. To the best of our knowledge this is the first study which revealed the microbial diversity of Polok and Borong and Reshi Hot Springs of Sikkim. The results of this study significantly expand the understanding of the microbial community structure of these hot springs and provided a basis for comparative analysis with other geothermal systems.

Antibiotic resistance is threatening the mankind but the discovery of novel parvome or development of new class of antibiotics has been a great concern in modern century of antibiotic era. Interestingly we found that all the isolates were susceptible against all the antibiotics used. These results were supported by minimum inhibitory concentrations, targeting antibiotic resistance genes, whole genome sequencing and metagenomic analysis. The significant heavy metal resistance in our isolates as compared to other *Geobacillus* species and *E.Coli* rejects the possibility of co-occurrence in these habitats we studied. The origin and evolution of the resistomes are in huge ambiguity and hence requires more attention in studying the ARGs pattern for possible LGT or HGT in future among microbial community which might wreck mayhem worldwide. However, our study may support the school of thought that anthropogenic activities cause the increase in antibiotic resistance.

Metagenomics is playing a crucial role in deciphering the unculturable microbial diversity and also hunt for ARGs and MRGs by the help of functional tools through Gene Ontology (GO) in various environments. Competitiveness among the microbes can drive resistance to antibiotics owing to genetic and physiological linkages between the two resistances. Undoubtedly, pristine landscapes and their microflora can date back the antibiotic resistance genes as antediluvian theory but the abiotic factors and anthropogenic activities, wind dispersal and fecal droppings can be the possible cofactors for distributing the resistomes. Expression of the resistomes depends on the brink of their survival strategies against human endeavor. Thus, thermophiles from hot springs of Sikkim although do harbor both antibiotic and metallo resistomes, yet these genes might be considered as putative. However, this kind of study in other less antibiotic exposed habitats such as thermal vents, high temperature oil fields etc. may shed further light on this subject.

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ℑ Appendix-1

Various Media used for isolation of Thermophilic Bacteria

Thermus Agar (TA)	g L^{-1}	**Modified Luria Bertani Agar**	g L^{-1}
Peptone	8	Tryptone	5
Yeast Extract	4	Yeast Extract	5
NaCl	2	NaCl	3
Agar	25	NaOH	1
		MgSO4	1
Nutrient Agar	g L^{-1}	CaCl2	1
Peptone	5	FeSO4	1
NaCl	5		
Yeast Extract	1.5	**YTP-2 Medium**	g L^{-1}
Beef Extract	1.5	Tryptone	2
Agar	25	Yeast Extract	2
		Sodium Pyruvate	2
Luria Bertani Agar	g L^{-1}	KCl	1
Casien Enzyme Hydrolysate	10	KNO_3	2
Yeast Extract	5	Na_2HPO4	2
NaCl	5	MgSO4	1
Agar	25	CaCl2	0.03
		Classified Tomato Juice	2ml
TR Medium	g L^{-1}	Agar	25
Tryptone	4		
Yeast Extract	2	**BP Medium**	g L^{-1}
NaCl	1	Peptone	4
$CaCl_2$	0.4mM	Beef Extract	4
$MgCl_2$	0.4mM	K_2HPO4	3
Agar	25	KH_2PO4	1
		Agar	25
GYT Medium	g L^{-1}		
Glucose	15	**Actinomycete Isolation Agar**	g L^{-1}
Yeast Extract	10	Sodium Caseinate	2
Tryptone	8	L-Asparagine	0.1
$CaCO_3$	5	Sodium Propionate	4
NaCl	2	Dipotassium Phosphate	0.5
Agar	25	Magnesium Sulphate	0.1
		Ferrous Sulphate	0.001
		Agar	25

R-2A Agar	$g\ L^{-1}$	**Starch Agar**	$g\ L^{-1}$
Casein Acid Hydrolysate	0.5	Peptone	5
Yeast Extract	0.5	Yeast Extract	1.5
Proteose Peptone	0.5	Beef Extract	1.5
Dextrose	0.5	Starch Soluble	2
Starch Soluble	0.5	NaCl	5
Dipotassium Phosphate	0.3	Agar	25
Magnesium Sulphate	0.024		
Sodium Pyruvate	0.3	**Gelatin Agar**	$g\ L^{-1}$
Agar	25	Gelatin	30
		Casien Enzyme Hydrolysate	10
Skim Milk Agar	$g\ L^{-1}$	NaCl	5
Casien Enzyme Hydrolysate	5	Agar	25
Yeast Extract	2.5		
Dextrose	1	**Mueller-Hinton Agar**	$g\ L^{-1}$
Skim milk powder	28	Meat, infusion from	300
Agar	25	Casein Acid Hydrolysate	17.5
		Starch	1.5
Plate Count Agar	$g\ L^{-1}$	Agar	25
Casien Enzyme Hydrolysate	5		
Yeast Extract	2.5		
Dextrose	1	**Urea Agar**	$g\ L^{-1}$
Agar	25	Urea	20
		Peptone	1
Carbohydrate Fermentation Broth	$g\ L^{-1}$	Dextrose	1
Sugar	5	Monopotassium phosphate	2
Peptone	10	Phenol Red	0.01
NaCl	5	NaCl	5
Phenol Red	0.018	Agar	25
1X TE Buffer (pH 8)	100ml	**50X TAE buffer***	$g\ L^{-1}$
Tris (1M)	1ml	Tris base	242
EDTA (0.5M)	0.2ml	Glacial Acetic Acid	57.1 ml
Distilled Water	98.8ml	EDTA (0.5M)	100 ml

*Mix Tris with stir bar to dissolve in about 600ml of ddH_2O and add EDTA and Acetic Acid bring final volume to 1L with ddH_2O and store at room temperature.

List of Journal Articles

Najar IN, Sherpa MT, Das S *et al.* Microbial ecology of two hot springs of Sikkim: Predominate population and geochemistry**. *Sci Total Environ* 2018;** 637–638**:730–45.**

Najar IN, Sherpa MT, Das S *et al.* *Geobacillus yumthangensis* sp. nov., a thermophilic bacterium isolated from a north-east Indian hot spring**. *Int J Syst Evol Microbiol* 2018:1–5.**

Najar IN, Sherpa MT, Das S, Thakur N. Draft genome sequence of *Geobacillus yumthangensis* AYN2 sp. nov., a denitrifying and sulfur reducing thermophilic bacterium isolated from the hot springs of Sikkim**. *Gene Rep* 2018; 10:162–166.**

List of Seminars

Presented on a topic entitled **"*Geobacillus* diversity in Hot springs of Sikkim and *Geobacillus yumthangensis* sp. nov., A novel thermophilic bacterium isolated from North-east Indian Hot spring"** in **PHMBI-2018: National Seminar on Perspectives of Human Health, Microbial Biotechnology and Innovation,** 27th-29th March 2018, Organized by Department of Microbiology and Department of Human Physiology with Community Health, Vidyasagar University, Midnapore, West Bengal.

Delivered Oral Presentation entitled **"Heavy metal tolerance and antibiotic susceptibility of various strains of *Geobacillus sp*. isolated from Yumthang and Yume Samdung Hot Springs of Sikkim"** in **Microbial World 2017:** National Seminar on **Applied Microbiology** on September 4, 2017 Organized by Department of Microbiology University of North Bengal.

Delivered a talk on **"Culture Dependent and Culture Independent-Analysis of Microbial Diversity and Geochemistry of Two Hot Springs of Sikkim, India**'' in **National conference** on **Recent trends in biological research and future prospects** organized by department of Zoology, Sikkim University on 28th-29th May, 2018.

Awards

Received **first prize** and **best presentation award** in oral presentation, entitled **"*Geobacillus* diversity in Hot springs of Sikkim and *Geobacillus yumthangensis* sp. nov., A novel thermophilic bacterium isolated from North-east Indian Hot spring**" in PHMBI-2018 : National Seminar on **Perspectives of Human Health, Microbial Biotechnology and Innovation**, on 27th-29th March 2018, Organized by Department of Microbiology and Department of Human Physiology with Community Health, Vidyasagar University, Midnapore, West Bengal.

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Draft genome sequence of *Geobacillus yumthangensis* AYN2 sp. nov., a denitrifying and sulfur reducing thermophilic bacterium isolated from the hot springs of Sikkim

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ABSTRACT

We are reporting the draft genome sequence of the *Geobacillus yumthangensis* AYN2 sp. nov. with the genome size of (~3.4 Mb) corresponding to 3712 predicted genes with (G + C) content of 42.28%. This bacterium possesses motility and chemotaxis genes, sulfur and denitrifying reductase gene clusters. This thermophilic bacterium was isolated from the Yumthang Hot Spring located in the North district of Sikkim, India.

1. Introduction

The search for new microflora constituting thermophilic and hyperthermophilic bacteria and archaea from subterranean ecosystems such as hot spring or deep thermal vents have attracted the attention of researchers due the high thermostability and various other contributions of these microflora in industrial and biotechnological sectors (Nazina et al., 2000). The biocatalytic potential of thermophiles and their enzymes such as protease, lipase and polymerase degrading enzymes such as cellulases, chitinases and amylases have been reviewed by various researchers (Sharma et al., 2017). Also at high temperature there is a significant improvement in the solubility of many reaction components. Other factors such as high metabolic rates, physically (i.e., thermally stable) and chemically stable enzymes and cells, facilitated end product recovery and the least risk of contamination (which may cause undesired complications) are very important characteristics of thermophilic bacteria (Lamed and Zeikus, 1980).

Subsequent analysis and research done by several authors, led to the discovery of new genus, phylogenetically distinct, physiologically and morphologically consistent taxon, for which they have submitted the validly-described genus name of *Geobacillus* (Nazina et al., 2001). With high levels of 16S rRNA sequence similarity ranging from 98.5 to 99.2%, the *Geobacillus* species comprise a phenotypically and phylogenetically cogent group of thermophilic bacilli (*Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans*).

The genus *Geobacillus* comprises as many as 10 validated species i.e. *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius*, *G. thermo-denitrificans*, *G. subterraneus*, *G. uzenensis*, *G. caldoxylosilyticus* and *G. toebii*. These species are usually Gram-positive, rod shaped thermophilic bacteria (Nazina et al., 2001). *Geobacillus* species are ubiquitous and can be found from hot geothermal locations to cold regions on earth and these bacteria possess a great industrial and biotechnological potential due to their thermostable nature (McMullan et al., 2004). A less genomic study has been done between mesophilic bacillus and thermophilic bacillus related species (Takami et al., 2004).

The study of thermophily in the prokaryotic cells which often survive by modifying their metabolic pathway or other mechanism the thermostability of their proteins mainly rely on the genomic information of the considered genome (Takami et al., 2017). Comparative genomics plays an essential role in mining the contender genes concomitant with thermophily (Tatusov et al., 2001). Comparing genomes based on genomic information between closely related organisms including both mesophiles and thermophiles is also an effective approach for understanding thermoadaptation with respect to evolution. Although the sequences from an appropriate set of organisms such as genus *Geobacillus* are needed, but have not yet been obtained (Takami et al., 2017). Also the study of the genomes of *Geobacillus* species led to the discovery, cloning and exploitation of natural products (Studholme, 2014). Several genomic sequences potentially related to thermostable homologues of useful enzymes have been reported. Also many genome sequences have been used to clone and express the genes of interest and

Abbreviations: Mbp, Mega Base Pairs; Bp, base pairs; G., *Geobacillus*; sp., species; ATCC, American Type Culture Collection; MTCC, Microbial Type Culture Collection and Gene Bank; DNA, deoxyribonucleic acid; tRNA, transfer ribonucleic acid; rRNA, ribosomal ribonucleic acid

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Table 1
Genome characteristics and resources of *Geobacillus yumthangensis* AYN2 nov. sp.

NCBI BIOPROJECT ID	PRJNA407404
NCBI BIOSAMPLE ID	SAMN07653191
NCBI GENOME ACCESSION NUMBER	NWUZ00000000
SEQUENCING PLATFORM	ILLUMINA HISEQ 4000
SEQUENCING MODULE	PAIRED END
TOTAL NUMBER OF READS	6,856,386,000 (~6856 Mbp)
READ LENGTH	101 bp
AVERAGE COVERAGE	99.94%
ESTIMATED GENOME SIZE	3,409,966 (~3.4 Mb)
GC CONTENT	42.11%
PROTEIN CODING GENES	3631
tRNA CODING GENES	71
rRNA Coding genes	5
PLASMID SEQUENCES	NONE

to characterize the enzyme for biotechnological potential. For example the genome of *Geobacillus kaustophillus* HTA426 was recently extracted for members of the glycoside hydrolase family 1 (Suzuki and Okazaki, 2013). Also for the first time, the nitrous oxide reductase gene from a Gram-positive and a novel thermophilic long chain alkane monooxygenase from NG80-2 genome sequence was discovered (Feng et al., 2007). Thus complete genomic studies may lead to new insights and will provide much information related to various aspects of a bacterial cell such as difference in metabolism of such bacteria and functionality and thermostability of various proteins and enzymes at molecular level. Here we report the draft genome sequence of a novel *Geobacillus* species - .*Geobacillus yumthangensis* AYN2.

2. Isolation of the bacterium

The *Geobacillus yumthangensis* AYN2 sp. nov. was isolated from the Yumthang Hot spring of North District of Sikkim, India. The bacterium was grown in Thermus medium (ATCC medium: 697 Thermus medium) [Peptone 8 g L^{-1}, Yeast Extract 4 g $L^{-1}$and NaCl 2 g L^{-1}] at 60 °C for 24 h.

3. Genomic DNA isolation, sequencing and data assembly

The genomic DNA of *Geobacillus yumthangensis* AYN2 sp. nov. was extracted from the bacteria, which was grown in Thermus medium at 60 °C using Qiagen QIAamp DNA Mini Kit (50) as per the guide lines of manufacturer. The whole genome sequencing was performed by using Illumina Hiseq 4000 sequencing technology with a paired end sequencing module (Table 1). It produced a total of 68,563,860 bp (68.56 Million) paired end reads with a maximum read length of 101 bp. For high quality data and genome assembly, the data was filtered by employing Next Generation Sequencing Quality Control (NGSQC) Toolkit and SQIT (Patel and Jain, 2012). The total QC passed high quality reads were 52,028,822 (52.03 Million). The overall quality of the data was good with more than 75.88% high quality reads. The primary genome assembly was carried out by Velvet (V 1.2.10) (Zerbino and Birney, 2008). The primary genome assembly statistics revealed the K-mer length of 71 with total number of 454 contigs. The average contig length was 7487.7 bp (~0.07 Mb) with N50 contig size of 24,353 (~0.02 Mb).

Based on paired-end directional information, the genome was assembled into 264 scaffolds, with N_{50} length 27,853 bp (0.03 Mb) and average scaffold length 12,863.51 bp (~0.01 Mb) using SSPACE v3.0 scaffolder (Boetzer et al., 2011). The obtained draft genome was assembled resulting in a total genome size of ~3.3 Mb with (G + C) content of 42.28%. The final genome draft consists of 124 scaffolds with average scaffold length of 27,499.73 bp (~0.02 Mb) and N50 contig size of 2,988,775 bp (~3.0 Mb), constituting 3,409,966 bp (~3.4 Mb) of the genome with (G + C) content of 42.11%.

Bowtie2 (v2.2.2) (Langmead and Salzberg, 2012) was used for de novo genome validation and quality control. By using this tool the reads mapped to the assembly concordantly and discordantly were 19,386,432 and 1,141,476 respectively. The overall alignment was 98.79%. The scaffolds covered were 264 with the average depth (scaffold level in x) of 1494.37 × and average coverage (scaffold level in %) of 99.94%.

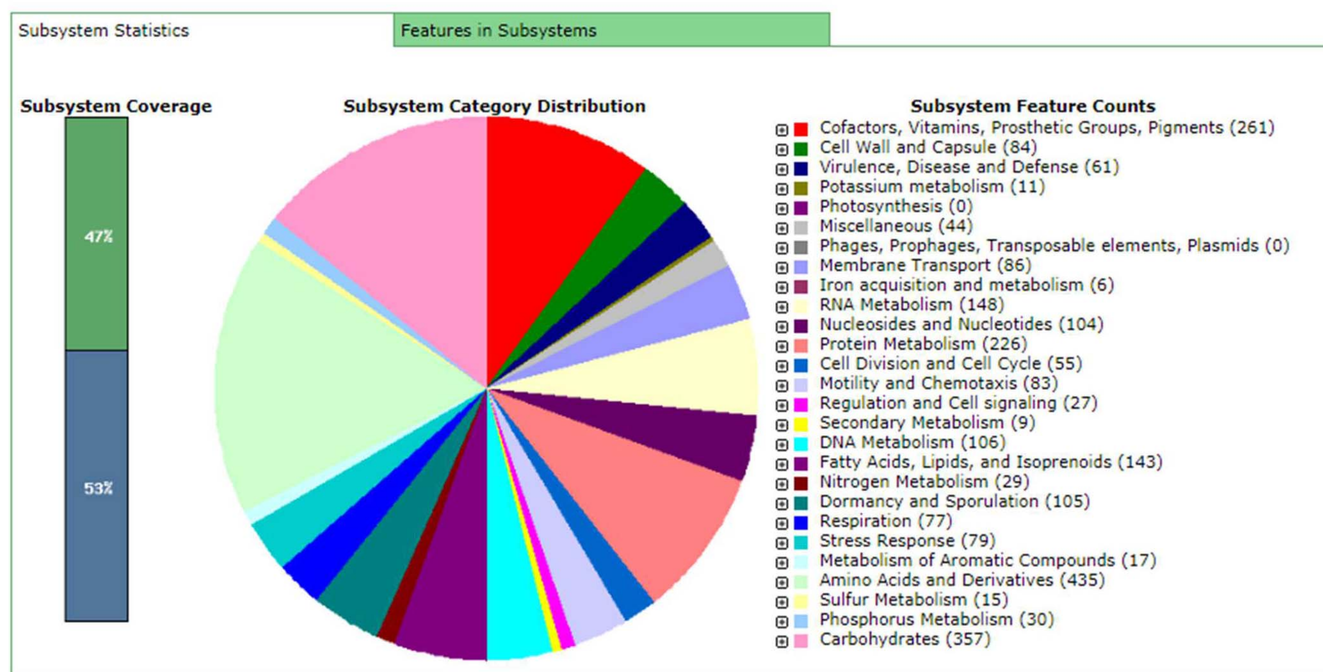

Fig. 1. Subsystem category distribution of major protein coding genes of *Geobacillus yumthangensis* AYN2 nov. sp. as annotated by the RAST annotation server. The bar chart shows the subsystem coverage in percentage (blue bar corresponds to percentage of proteins included). The pie chart shows percentage distribution of the 27 most abundant subsystem categories. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Genes involved in sulfur and nitrogen metabolism protein predicted by RAST in *Geobacillus yumthangenesis* AYN2 sp., nov.

Category	Subcategory	Subsystem	Role	SS active
Sulfur Metabolism	Inorganic sulfur assimilation	Inorganic sulfur assimilation	Sulfate adenylyltransferase, dissimilatory-type (EC 2.7.7.4)	No
			Sulfate permease, Pit-type	No
	Organic sulfur assimilation	Alkanesulfonates utilization	FMN reductase (EC 1.5.1.29)	Yes
		L-cystine uptake and metabolism	Cystathionine beta-lyase (EC 4.4.1.8)	Yes
			Cystathionine gamma-synthase (EC 2.5.1.48)	Yes
			L-cystine uptake protein TcyP	Yes
	No subcategory	Galactosylceramide and sulfatide metabolism	Alpha-galactosidase (EC 3.2.1.22)	Yes
		Thioredoxin-disulfide reductase	Alkyl hydroperoxide reductase protein C (EC 1.6.4.-)	Yes
			Alkyl hydroperoxide reductase protein F (EC 1.6.4.-)	Yes
			Thiol peroxidase, Bcp-type (EC 1.11.1.15)	Yes
			Thiol peroxidase, Tpx-type (EC 1.11.1.15)	Yes
			Thioredoxin reductase (EC 1.8.1.9)	Yes
Nitrogen metabolism	Denitrification	Denitrifying reductase gene clusters	Nitric oxide reductase activation protein NorD	Yes
			Nitric oxide reductase activation protein NorQ	Yes
			Nitrous oxide reductase maturation protein NosD	Yes
			Nitrous oxide reductase maturation transmembrane protein NosY	Yes
			Respiratory nitrate reductase alpha chain (EC 1.7.99.4)	Yes
			Respiratory nitrate reductase beta chain (EC 1.7.99.4)	Yes
			Respiratory nitrate reductase delta chain (EC 1.7.99.4)	Yes
			Respiratory nitrate reductase gamma chain (EC 1.7.99.4)	Yes
			Nitrous oxide reductase maturation protein, outer-membrane lipoprotein NosL	Yes
			Nitrate/nitrite transporter NarK	No
			Nitrous oxide reductase maturation protein NosF (ATPase)	No
			Nitrous-Oxide Reductase (EC 1.7.99.6)	No

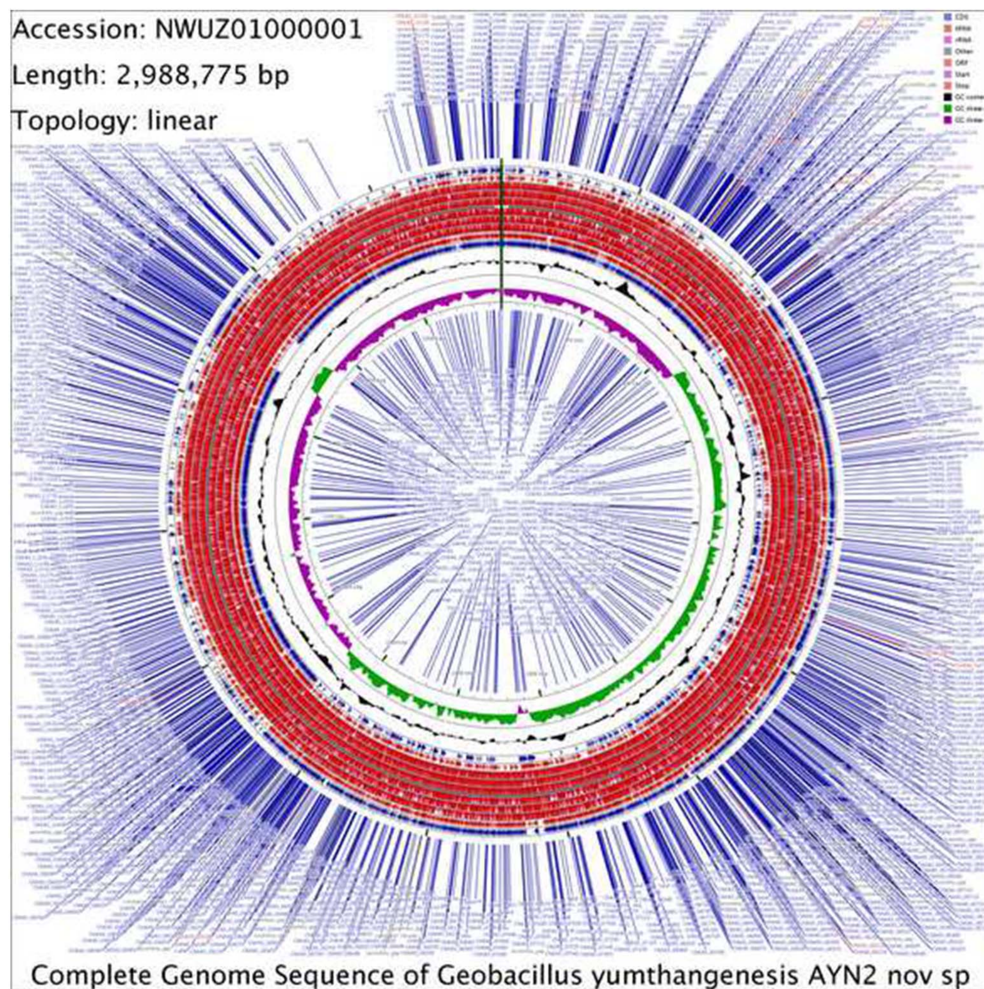

Fig. 2. Circular representation of complete genome of *Geobacillus yumthangenesis* AYN2 nov. sp. Labeling from the outside to inside circle: CDS on the forward strand (colored blue) with blast hits by reading frame, ORFs on the forward and reverse strands (colored by red), RNA genes (tRNAs maroon, rRNAs purple, other RNAs grey), (G + C) content (peaks out/inside the circle indicate values higher or lower than average (G + C) content, respectively, colored black),GC skew (calculated as (G-C)/(G + C), green/purple peaks out/inside the circle indicates values higher or lower than 1, respectively). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

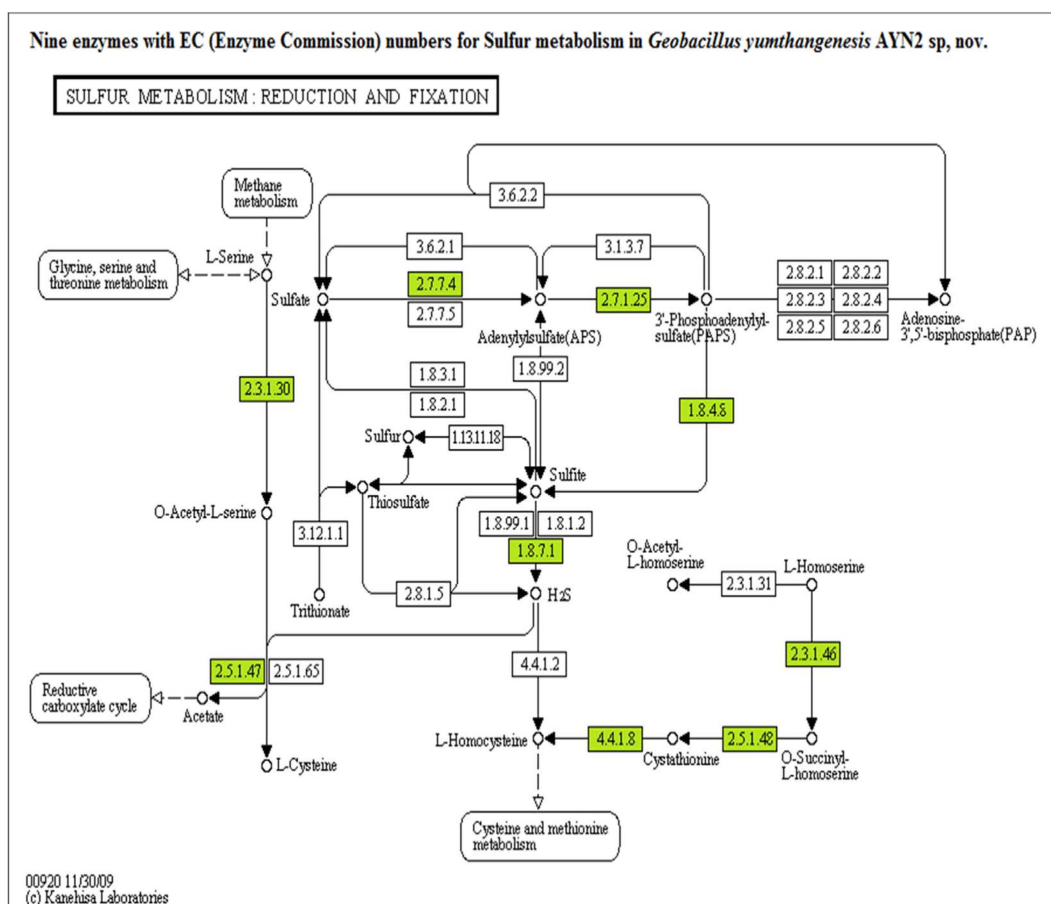

Fig. 3. Sulfur metabolism with 9 EC (Enzyme Commission) numbers colored green in *Geobacillus yumthangenesis* AYN2 sp., nov. EC-2.7.7.4 = sulfate adenylytransferase, EC-2.7.1.25 = adenylylsulfate kinase, EC-1.8.4.8 = phosphoadenylyl-sulfate reductase (thioredoxin), EC-1.8.7.1 = ferredoxin-sulfate reductase, EC-2.3.1.46 = homoserine O-Succinyltransferase, EC-2.5.1.48 = O acetylhomoserine sulfhydrylase, EC-4.4.1.8 = cystathionine beta lyase, EC-2.5.1.47 = cysteine synthase, EC-2.3.1.30 = serine acetyltransferase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The genomic elements of assembled genome were done by using ARAGORN v1.2.36 (Laslett and Canback, 2004) and RNAmmer 1.2 Server (Lagesen et al., 2007). All the three types of rRNAs and possible tRNAs were identified, indicating a high degree of completeness in the genome assembly. Using ARAGORN tRNA identification method, the number of tRNA genes identified were 71. All the three RNAs (5S rRNA, 23S rRNA and 16S rRNA) were identified. Non-core genomic elements were also screened and using PlasmidFinder (V 1.3) (Carattoli et al., 2014), no plasmid sequences were found. The draft genome was annotated and its functional characterization was done. The number of genes predicted was 3712, with 2609 characterized proteins and 1090 hypothetical/putative proteins. The number of rRNA genes and tRNA genes were 5 and 71 respectively (Table 1). The circular representation of complete genome of *Geobacillus yumthangensis* AYN2 nov. sp. is given in Fig. 2. The taxonomy identification method was performed using EzTaxon (Kim et al., 2012) and MEGA6, from which it was found that the novel species *Geobacillus yumthangensis* AYN2 is the putative species (as per sequence homology) and *Geobacillus toebii* NBRC 107807 is the closest homolog of the assembled genome (based on 16S rRNA match). *Geobacillus thermoglucosidasius* DSM 2542, complete genome was used as a reference genome (Ref Seq accession ID: NZ_CP012712.1).

The annotation tool used was Rapid Annotations using Subsystems Technology RAST (V 2.0) (Aziz et al., 2008). Using RAST, 962 proteins were annotated. Fig. 1 presents an overview of the count of each subsystem feature and the subsystem coverage. RAST functional annotation predicted (357) genes were linked to carbohydrate metabolism. The absence of any photosynthetic apparatus and gene clusters indicated that *Geobacillus yumthangensis AYN2* may be adapted to a heterotrophic metabolism. Eighty three complete sequences of the gene encoding methyl accepting chemotaxis protein (MCP) and motility were detected in this genome. Genes encoding MCPs were mainly found in genomes of motile microbes (Zhulin, 2001). Gene clusters of sulfur metabolism which includes thioredoxin-disulfide reductase, L-cysteine uptake and metabolism, alkanesulfonates utilization, galactosylceramide and sulfatide metabolism, and inorganic sulfur assimilation clusters, was also discovered in present genome. Out of total 30, the 9 (30%) distinct ECs were found and most of them were active as shown in Fig. 3, Table 2. Similarly denitrifying reductase gene clusters were detected in this genome. Denitrification is the process of reducing nitrate to dinitrogen gas. The reaction sequence of this process is Nitrate → Nitrite → Nitric Oxide → Nitrous Oxide → Dinitrogen. Denitrification is a fundamental process in the nitrogen cycle by which many prokaryotes and a few fungi are able to use nitrogen oxides instead of oxygen as terminal electron acceptors (Simon et al., 2013). Four gene clusters control each step of denitrification: the *nar* gene cluster (nitrate reductase), the *nir* gene cluster (nitrite reductase), the *nor* gene cluster (nitric oxide reductase), and the *nos* gene cluster (nitrous oxide reductase) (Alvarez et al., 2011). Among facultative strains, the use of nitrogen oxides, especially nitrate, is a widespread electron acceptors. In fact, the genomes of several phylogenetically ancestral extreme thermophiles and hyperthermophiles include genes encoding enzymes for denitrification, which is one of the few steps of the nitrogen cycle that remain active above 65 °C. The probable ancestral nature of thermophiles and of the denitrification process itself is an important argument in favor of the study of denitrification in thermophilic models (Cava and Hidalgo, 2009; Alvarez et al., 2011). Also our data

employing ICPMS (Inductive Coupled Mass Spectroscopy) suggests the normal range of nitrate concentrations (10 mg/L) in the hot spring water, which supports that the present thermophilic isolate might be also carrying out denitrification and using nitrate as an alternative electron acceptor. The genes corresponding to these four clusters are present in the *Geobacillus yumthangensis* AYN2 nov. sp. genome. Nine functional genes were present in the present genome as shown in Table.2. In the summary the genome information of the thermophilic *Geobacillus yumthangensis* AYN2 nov. sp. provides insights into investigating the metabolic and regulatory mechanisms and other phenotypic characteristics of interest particularly present in the Hot Springs.

3.1. Nucleotide sequence accession and culture accession numbers

The *Geobacillus yumthangensis* AYN2 nov. sp. genome sequence has been deposited at NCBI GenBank under accession number NWUZ00000000 (Table 1). The bacterial culture has been submitted to Microbial Type Culture Collection (MTCC) and its accession number is MTCC12749.

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Geobacillus yumthangensis sp. nov., a thermophilic bacterium isolated from a north-east Indian hot spring

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Abstract

A thermophilic, spore-forming, rod-shaped bacterium isolated from the Yumthang hot spring in North Sikkim, India was subjected to taxonomic studies. The thermophilic bacterial isolate was designated as strain AYN2^T. Cells were Gram-stain-positive, aerobic, motile, rod-shaped, catalase-positive and methyl red-negative. Strain AYN2^T was able to grow in the pH range from 6 to 10 (optimum, pH 7.5–8.0), at 40–70 °C (60 °C) and in NaCl concentrations of 0–4 % (1 %). The major cellular fatty acids were iso-$C_{15:0}$ (12.8 %), iso-$C_{16:0}$ (13.9 %) and iso-$C_{17:0}$ (13.8 %). No matches were found in the RTSBA6 Sherlock libraries. The G+C content of the genomic DNA was 42.11 mol%. Based on phylogenetic analysis of the 16S rRNA gene sequences, strain AYN^T showed highest sequence similarity to the type strain of *Geobacillus toebii* (96 %). However, the phenotypic properties of strain AYN2^T were clearly distinct from those of *G. toebii* and related species. On the basis of polyphasic analysis, strain AYN2^T represents a novel species in the genus *Geobacillus*, for which the name *Geobacillus yumthangensis* sp. nov. is proposed. The type strain is AYN2^T(MTCC=12749=KCTC=33950= JCM 32596).

In 1991, based on 16S rRNA sequences, Ash *et al.* analysed many species of *Bacillus* and further investigations found that they fell into five distinct groups [1]. Subsequently, using phylogenetic, physiological and morphological characteristics led to the description of *Geobacillus* [2]. With high levels of 16S rRNA sequence similarity of 98.5–99.2 %, the *Geobacillus* species include a cogent group of thermophilic bacilli (*Bacillus sterothermophilus*, *Bacillus thermoleovorans*, *Bacillus thermocatenulatus*, *Bacillus kaustophillus*, *Bacillus thermodenitrificans* and *Bacillus thermoglucosidasius*). *Geobacillus* species are endospore-forming, Gram-stain-positive, aerobic or facultative anaerobic rods [3]. They normally grow within the range of temperatures from 35°C to 80 °C, depending on the strain [4]. The *Geobacillus* species can be found in various harsh environments that include high-temperature oilfields [5], marine vents [6], corroded pipeline in an extremely deep well [4], African [7] and Russian [8] hot springs, and the Mariana Trench [9]. In addition, they can also be found in hay compost [10] and garden soil [11]. Their thermostable characteristics make members of *Geobacillus* attractive to the biotechnology industry as sources of thermostable enzymes [12], as platforms for biofuel production [13] and as attractive constituents of bioremediation strategies [14]. Here we describe a novel species of the genus *Geobacillus,* which can grow in wide range of pH.

Water samples were taken from the source of the Yumthang hot spring, North Sikkim, India (27° 47′ 34.50″ N 88° 42′ 30.96″ E). The *in situ* temperature and pH values were measured using a Multiparameter Water Quality Checker U-50 Series (Horiba). The temperature of the source was between 42–45 °C and the pH values ranged from pH 7.5 to 8. Strain AYN2^T was isolated on solid thermus agar containing 8 g l^{-1} peptone, 4 g l^{-1} yeast extract and 2 g l^{-1} NaCl with an incubation of 24 h at 60 °C. Strain AYN2^T was also able to grow on actinomycetes agar with the composition of 2 g l^{-1} sodium caseinate, 0.1 g l^{-1} L-asparagine, 4 g l^{-1} sodium propionate, 0.5 g l^{-1} dipotassium phosphate, 0.1 g l^{-1} magnesium sulphate, 0.001 g l^{-1} ferrous sulphate and 25 g l^{-1} agar. The colonies were round and creamy-white coloured. The Gram-stain reaction and spore staining were performed using a Gram-staining kit (Difco) and a spore-staining kit, respectively, following the recommended protocols. Strain AYN2^T was found to be a spore-forming Gram-stain-

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Keywords: *Geobacillus yumthangensis* sp. nov.; thermophiles; hot spring; polyphasic taxonomy; Sikkim.
Abbreviations: FESEM, field emission scanning electron microscope; SEM, scanning electron microscope; KCTC, Korean Collection for Type Cultures; MTCC, Microbial Type Culture Collection; JCM, Japan Collection of Microorganisms; FAME, Fatty Acid Methyl Ester; PCR, Polymerase Chain Reaction.
The GenBank accession numbers for the 16S rRNA gene and the whole genome are MG603320 and NWUZ00000000, respectively.
Two supplementary figures and two supplementary tables are available with the online version of this article.

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positive bacteria. Scanning electron microscope (SEM) analysis, i.e. field emission scanning electron microscopy (FESEM), was performed as per Hagen *et al.* [15, 16] using a Sigma FESEM (Zeiss). The SEM analysis showed that cells were 2.5–5 µm long and 0.4–0.6 µm wide (Fig. S1, available in the online version of this article).

The effect of temperature, pH and NaCl on growth of bacteria was determined as per Aaniz *et al.* [17]. Strain AYN2^T could grow between 40 and 70 °C with the optimal temperature of 60 °C and in the pH ranges from 6 to 10 with the optimum pH of 7.5–8.0 at the optimum growth temperature. The NaCl concentration for the growth of strain AYN2^T was found to be in the range between 0 and 4%, with the optimum NaCl concentration of 1 % at the optimum pH and temperature, as shown in Fig. S2(a), (b) and (c). By comparing these results with the closely related species of *Geobacillus*, especially *Geobacillus toebii* (the closest one), it was found that there is considerable distinction between them. For example, if we compare the pH ranges, strain AYN2^T grows over a wide range of pH, i.e. from pH 6–10 as compared to other *Geobacillus* species which thus adds to its novelty (Table 1). Also, many biochemical characteristics such as: production of acids from various sugars such as ribose, inositol, glycerol and cellobiose; hydrolysis of casein, gelatin and starch; utilization of acetate, formate and lactate; and fermentation of glucose are quite different from those of *G. toebii* and other related species as shown in Table 1. All of the phenotypic and biochemical tests were performed manually or by using the Biolog system.

The fatty acid analysis of 16 h grown culture of strain AYN2^T was performed at 50 °C. The fatty acid extraction and analysis were performed by following the instructions of the Microbial Identification System (MIDI) as reported previously [18]. The RTSBA6 method was used and the

Table 1. Phenotypic characteristics that differentiate *Geobacillus yumthangensis* sp. nov. strain AYN2^T from its phylogenetic neighbours

Strains: 1, AYN2^T (manual); 2, AYN2^T (Biolog); 3, *Geobacillus toebii* [10]; 4, *Geobacillus thermoglucosidasius* [32]; 5, *Geobacillus uzenensis* [2]; 6, *Geobacillus subterraneus* [2]; 7, *Geobacillus stearothermophilus* [33]; 8, *Geobacillus thermocatenulatus* [34]; 9, *Geobacillus thermoleovorans* [35]; 10, *Geobacillus kaustophilus* [36]; 11, *Geobacillus thermodenitrifcans* [37]. +, Positive; −, negative; D, 11±89 % of strains positive; ND, not determined.

	1	2	3	4	5	6	7	8	9	10	11
Cell width (µm)	0.4–0.6	ND	0.5–0.9	<3	0.9–1.3	0.8–1.5	0.6–1	0.5–1.2	0.9	1.5	0.5–1.0
Cell length (µm)	2.5–5	ND	2–3.5	<0.9	4.7–8	4.7–8	2–3.5	3.0–7.0	6.0–8	3.5	1.5–2.5
Motility	+	ND	+	ND	+	+	+	+	+	−	ND
Production of acid from:											
Adonitol	ND	ND	−	+	−	−	ND	−	ND	ND	ND
L-Arabinose	−	ND	−	−	+	−	D	−	−	D	+
Cellobiose	+	+	−	+	+	+	−	+	+	+	+
Galactose	−	+	−	D	+	+	−	−	+	+	+
Ribose	+	ND	−	−	+	+	ND	ND	ND	+	+
Glycerol	ND	+	−	−	+	+	+	+	+	D	+
Inositol	−	+	+	+	−	−	−	−	−	−	ND
Lactose	−	+	−	−	−	−	−	−	−	−	+
Rhamnose	−	+	−	−	−	−	−	+	−	−	−
Sorbitol	−	+	−	−	−	−	−	+	ND	−	ND
D-Xylose	−	ND	−	+	−	−	D	+	−	D	+
Hydrolysis of:											
Gelatin	−	+	−	+	+	−	D	−	−	ND	ND
Casein	−	−	+	+	−	−	D	+	ND	+	−
Starch	+	+	−	+	+	+	+	+	−	D	+
Aesculin	−	ND	−	−	+	+	ND	+	ND	ND	ND
Utilization of:											
Formate	ND	+	−	D	−	+	−	ND	ND	ND	ND
Acetate	ND	+	−	−	+	+	−	ND	ND	ND	ND
Lactate	ND	+	−	−	+	+	−	ND	ND	ND	ND
Citrate	−	−	−	+	−	−	D	D	+	ND	ND
Fermentation of glucose	+	+	−	−	−	−	D	−	+	−	ND
Methyl red test	−	ND	−	−	−	+	D	D	ND	ND	ND
Denitrification	ND	ND	+	ND	−	+	−	−	+	ND	+
NaCl concentration for growth (%, w/v)	0–5	0–5	0–5	0–5	0–4	0–5	0–5	0–1.5	0–4	ND	0–3
pH range for growth	6.0–10	>6.0	6.0–9.0	6.0–8.0	6.2–7.8	6.0–7.8	6.0–8.0	6.5–8.5	6.2–7.8	6.2–7.5	6.0–8.0
Temperature range for growth(°C)	40–70	ND	45–70	37–68	45–65	45–70	37–65	42–69	35–78	40–75	45–70

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Table 2. Fatty acid composition of strain AYN2^T and type strains of *Geobacillus* with validly published names

Strains: AYN2^T; *Geobacillus toebii* [10]; *Geobacillus thermodenitrificans* [2]; *Geobacillus thermoleovorans* [2]; *Geobacillus thermocatenulatus* [2]; *Geobacillus stearothermophilus* [38]; *Geobacillus thermoglucosidasius* [38]. The values in bold signifies the major fatty acids present in individual bacterial strains.

	iso-C14 : 0	C14 : 0	iso-$C_{15:0}$	a-$C_{15:0}$	$C_{15:0}$	iso-$C_{16:0}$	$C_{16:0}$	Iso-$C_{17:0}$	a-$C_{17:0}$	C-$_{17:0}$	Iso-$C_{18:0}$	$C_{18:1}$	$C_{18:0}$	References
G. yumthangensis AYN2^T	–	3.3	**12.8**	–	–	**13.9**	7.0	**13.7**	3.2	4.7	2.3	3.2	2.6	This paper
G. toebii	–	–	**34.0**	–	–	**17.0**	–	**34.0**	–	–	–	–	–	[10]
G. thermodenitrificans	0.4	1.8	**33.6**	1.8	2.3	9.5	**11.0**	**26.6**	7.3	2.9	0.2	1.3	1.3	[2]
G. thermoleovorans	1.0	1.4	**22.6**	1.3	2.1	**21.0**	**11.2**	**18.5**	4.6	1.3	0.9	1.2	3.4	[2]
G. thermocatenulatus	1.3	0.6	**25.5**	0.6	1.3	**31.8**	8.3	**21.0**	3.1	2.3	1.3	0.7	2.2	[2]
G. stearothermophilus	0.1	1.5	**39.8**	6.4	0.5	6.2	9.2	**17.1**	**13.3**	–	–	–	–	[38]
G. thermoglucosidasius	–	0.6	**22.0**	1.6	–	**10.4**	**11.6**	**30.3**	**16.6**	0.8	–	–	–	[38]

results were analysed by using Sherlock version 6.2. The predominant fatty acids were iso-$C_{15:0}$ (12.8 %), iso-$C_{16:0}$ (13.9 %) and iso-$C_{17:0}$ (13.7 %). The results of a comparison between the various fatty acids of strain AYN2^T and its closest relatives are shown in Table 2. Although the major fatty acids are similar, the total amount or the percentage of total fatty acids vary considerably. Thus these fatty acid profiles supports the inclusion of strain AYN2^T in the genus *Geobacillus*. When analysing the results, no matches were found in the RTSBA6 Sherlock libraries, thus suggesting that strain AYN2^T is novel.

The 16S rRNA sequence was amplified by using universal primers (27F and 1492R) as described by Hugenholtz [19] and the product was purified by using the QIAquick PCR purification kit (Qiagen). The purified 16S rRNA gene was further sequenced by using the BigDye Terminator cycle sequencing kit (version 3.1, Applied Biosystems) in an automated DNA Sequencer (ABS/Genetic 3500 Analyzer). The sequences were assembled by CodonCode Aligner (version 7.1) and the assembled sequences were identified by performing BLAST searches. Strain AYN2^T shared only 96 % identity with the *G. toebii* strain R-35642, which confirms the novelty of strain AYN2^T. The 16S rRNA gene sequence of strain AYN2^T was aligned with representative 16S rRNA gene sequences of related taxa using CLUSTAL_W software [20]. A phylogenetic tree was reconstructed by using the neighbour-joining method [21] and the software package MEGA 7 to demonstrate the relationship of strain AYN2^T to other members of the family *Geobacillus* (Fig. 1). Also, three more conserved genes, i.e. *rpoB, DnaK* and *dnaJ* genes, were investigated using primers *rpoB*1698F (5′-AACATCGG TTTGATCAAC-3′; corresponding to *E.coli* position 1643) and *rpoB*2041R (5′-CGTTGCATGTTGGTACCCAT-3′; corresponding to *E. coli* position 2041) [22]; *dnaK*F (5′-C TCCGTGGACCTTCTCTTGG-3′) and *dnaK*R (5′-ATGA TCTGCTTGTGGGCCTC-3′) [23]; and *dnaJ*F (5′-CAGA TCGAGGTSACCTTCGAC-3′) and *dnaJ*R (5′-CGTCRYCA TMGAGATCGGCAC-3′) [24]. After performing BLAST searches for the *rpoB* gene, the results showed 88 % identity with *G. thermoglucosidasius*. The heat shock chaperone genes, i.e. *dnaK* and *dnaJ*, showed 89 and 90 % identity with *G. thermoglucosidasius* and *Bacillus thermoglucosidasius* respectively. Thus, these lower values shown for the *rpoB*, *Dnak* and *DnaJ* genes support AYN2^T being a novel species.

The draft genome sequence of the species *Geobacillus yumthangensis* AYN2^T has been published recently [25]. The whole genome accession numbers are given in Table S1. RAST (version 2.0) [26] was employed, which annotated 962 proteins. The genome of AYN2^T was 3.4 Mbp and contained 3721 predicted genes [25]. The number of rRNA genes and tRNA genes were 5 and 71, respectively. The absence of any photosynthetic apparatus and gene clusters indicated that strain AYN2^T may be adapted to a heterotrophic metabolism. The G+C content of AYN2^{T}was also checked using Valvet (version 1.1.10) and SSPACE (version 3.0) in our previous study [25]. The G+C content was found to be 42.11 mol%, which is quite different from the other species of genus *Geobacillus* (Table S4). Whole genome sequencing results [25] showed that the novel organism does not possess any plasmid, as compared to the other closest *Geobacillus* species such as *G. thermoglucosidasius* [27], *G. sp,* WCH70 [28], *G. stearothermophilus* [29], etc. The original methods of determining average nucleotide identity and average nucleotide identity of orthologous genes were calculated using the Orthologous Average Nucleotide Identity Tool (version 0.93) [30]. The original average nucleotide identity and the average nucleotide identity of orthologous genes between strain AYN2^T and the closest species, *G. toebii*, were 97.6 and 97.8 %, respectively. Digital DNA–DNA hybridization values were determined using the Genome-to-Genome Distance Calculator (version 2.1) [31]. The digital DNA–DNA hybridization values were 69.10 %. Although the average nucleotide identity and digital DNA–DNA hybridization values were a little higher or comparable to the cut-off values of ~96 and <70 %, respectively. However, the cell morphology, biochemical characteristics, sugar fermentation, 16S rRNA, *rpoB*, *dnaJ* and

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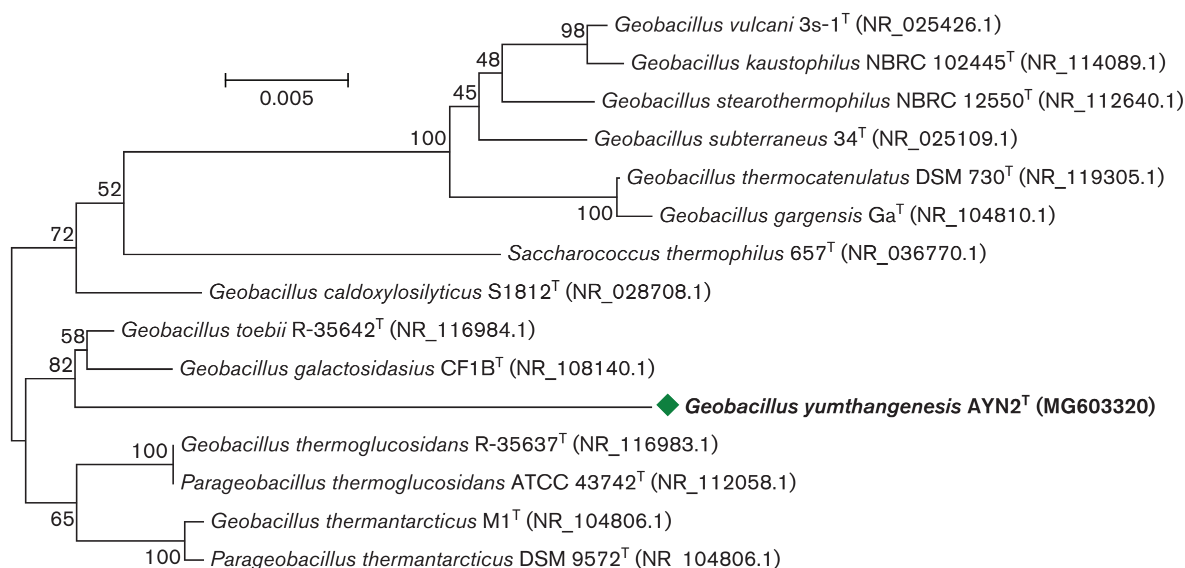

Fig. 1. Phylogenetic tree showing the position of strain AYN2^T among *Geobacillus* species and related taxa. The evolutionary history was inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches The evolutionary distances were computed using the maximum-composite-likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA7.

dnaK gene similarity, G+C content, absence of plasmids, and fatty acid methyl ester analysis suggested that strain AYN2^T represents a novel species.

DESCRIPTION OF *GEOBACILLUS YUMTHANGENSIS* SP. NOV.

Geobacillus yumthangensis (yum.thang.en.sis.N.L.masc.adj. *yumthangensis* of Yumthang referring to Yumthang Hot Spring from where the type strain was isolated).

Cells are Gram-stain-positive, aerobic, rod-shaped, 2.5–5 µm long and 0.4–0.6 µm wide. They form endospores located at the sub-terminal to terminal positions. Growth is observed between 40 and 70 °C with the optimum temperature of 60 °C. The pH for growth ranges from pH 6 to 10 with the optimum pH of 7.5–8 at the optimum growth temperature. The NaCl concentration for the growth of AYN2^T is in the range between 0 and 5%, with the optimum NaCl concentration of 1 % at the optimum pH and temperature. No growth is observed at 80 °C and at pH below 6 and above 10. No growth is observed at concentrations of 5 % NaCl and above. Cells are catalase-positive and methyl red-negative. Able to utilize lactate, formate and acetate. Acids are produced from cellobiose, lactose, galactose, sorbitol and glucose, but not from arabinose, ribose or xylose. The major cellular fatty acids are iso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{17:0}$. The G+C content is 42.11 mol%. The isolate was isolated from Yumthang hot spring, North Sikkim, India. The type strain is strain AYN2^T (MTCC=12749=KCTC=33950=JCM=32596) (Table S3).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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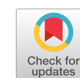
Microbial ecology of two hot springs of Sikkim: Predominate population and geochemistry



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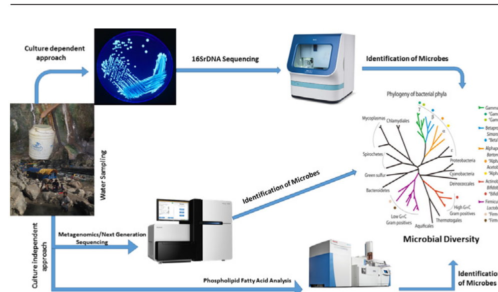
HIGHLIGHTS

- 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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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 abovementioned 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.

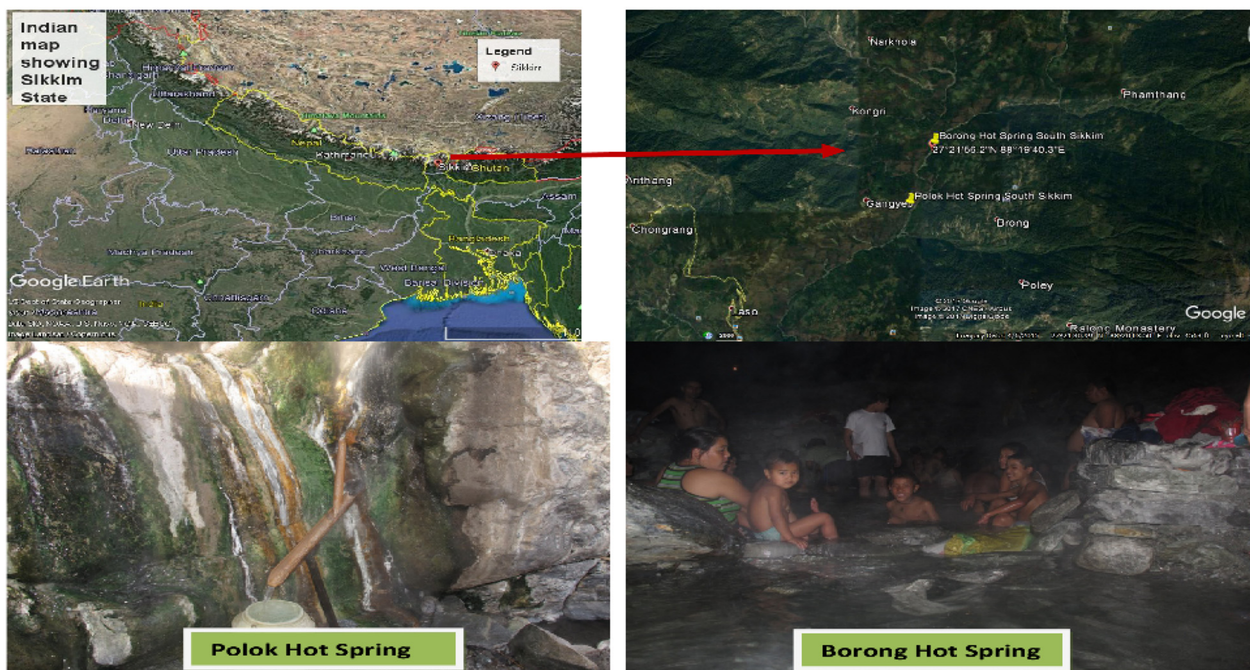

Fig. 1. Google based map of two hot springs Borong and Polok of South Sikkim India. The geographical position of coordinates and elevation range of Polok and Borong hot spring were measured with the help of GPSMAP 78S (Garmin, India).

2.2.2. Morphological, physiological and biochemical characterizations of the isolates

Colony morphology (color, form, margins, elevation, and density) was checked. The general cell morphology was observed under light microscope. The shape (short rods, long rods, filaments, commas, spirals), and arrangement (simple, pair, chains, clusters) were recorded systemically. Gram-staining and spore staining was done by standard methods (Arayan et al., 2008; Arya et al., 2015). The physiological characteristics including the effect of temperature, pH, and NaCl concentrations on growth were measured. The optimal physiological temperature for growth was determined by incubating the isolates in a gradient of temperature from 20 to 80 °C in Thermus Agar medium. The pH tolerance and effect of NaCl concentration was tested in a range of 4.0–10.0 and 0.5–5% in TA medium respectively (Arya et al., 2015). The biochemical characterization of the isolates was done by qualitative analysis of various enzymes such as catalase, amylase, protease, lipase, urease, oxidase, and nitrate reductase. The carbohydrate fermentation test was performed using dextrose, maltose, fructose, mannitol, mannose, raffinose, ribose, sucrose, xylose, and arabinose (Arayan et al., 2008; Arya et al., 2015)

2.2.3. Molecular identification and phylogeny

For culture-dependent techniques, the DNA was extracted using Qiagen QIAamp DNA Mini Kit (Qiagen, USA) as per the manufacturer's instructions. The extracted DNA was stored at −80 °C for further analysis. The 16S rRNA gene amplifications were done by using two universal primers 27F(5′-AGAGTTTGATCMTGGCTCAG-3′) and 1406R(5′-GACGGGCGGTGTGTRCA-3′). The polymerase chain reaction was performed in 25 μL volume using 12.5 μL GoTaq Green Master Mix 2× (Promega), 20 pM of forward and reverse primers and 2 μL of the template. The PCR cycle was designed as, 5 min at 94 °C; 35 cycles of 1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C; and a final extension step of 10 min at 72 °C. The PCR products were analyzed by electrophoresis at 100 V for 1 h in a 0.8% Agarose gel (Sigma Aldrich, USA). The PCR product was purified using a QIAquick PCR purification kit (Qiagen, USA) for cycle sequencing. The purified 16S rDNA was then sequenced using a BigDye™Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) as manufacturer's instructions using Automated DNA Sequencer (ABS/Genetic 3500 Analyzer). The sequence obtained was assembled with Codon Code Aligner (ver. 5.2). Assembled sequences were compared with nr/nt database of NCBI using BLAST sequence homology search for taxonomic identification. A phylogenetic tree was constructed to identify the evolutionary lineage of the isolates. The tree was constructed using neighbor-joining method (Saitou and Nei, 1987) with jukes-cantor evolutionary distance measurement (Erickson, 2010) in MEGA 7.0 software.

2.3. Culture independent techniques

2.3.1. Phospholipid Fatty Acid Analysis (PLFA)

For PLFA analysis, the phospholipids were extracted according to the standard protocol (Fan et al., 2017) (Quideau et al., 2016) and were analyzed using Sherlock-MIDI identification system. The calibrated standards were used by the microbial identification system (MIDI) for annotation of generated phospholipid peaks. The Equivalent carbon length (ECL) values in comparison to the expected ECL value in the PLFA peak are mentioned in Supplementary Table S2. The minimum limit of detection (LOD) for the MIDI-PLFA method per 2uL injection, is 1 ng of fatty acid.

2.3.2. Metagenomic DNA extraction

Environmental DNA was extracted using DNeasy PowerWater Kit (MO BIO Laboratories, Carlsbad, CA, USA) in accordance with the manufacturer's instruction. Quality of the DNA was checked on 0.8% agarose gel and DNA was quantified using Qubit Fluorometer (Thermofisher Scientific, USA), with a detection limit of 10–100 ng/μL.

Table 1
Physical Parameters of Polok and Borong hot springs.

Hot spring	Temperature (in °C)	pH	Conductivity mS/cm	D.O. (mg/L)	D.O. (%)	TDS (g/L)
Polok	76.3	7.52	0.756	5.56	92	0.483
Borong	52.3	5.32	0.205	6.56	98.3	0.133

Table 2
Elemental analysis of Polok and Borong hot springs.

SNO.	1	2	3	4	5	6	7
Test parameters	Color	Aluminium	Ammonia	Boron	Calcium	Chloride	Copper
Unit	Hazen	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}
Polok	<1	<0.03	<0.5	<0.5	14	70	<0.05
Borong	<1	<0.03	<0.5	<0.5	12	17	<0.05

SNO.	9	10	11	12	13	14	15
Test parameters	Free residual chlorine	Iron	Magnesium	Manganese	Nitrate	Phenolic compounds	Selenium
Unit	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}
Polok	0.22	0.08	7	<0.1	10	0.07	<0.01
Borong	0.2	0.06	4	<0.1	8	0.18	<0.01

SNO.	17	18	19	20	21	22	23
Test parameters	Sulfide	Total alkalinity	Zinc	Cadmium	Cyanide	Lead	Mercury
Unit	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}
Polok	0.4	327	<0.05	<0.003	<0.05	<0.01	<0.001
Borong	0.4	75	<0.05	<0.003	<0.05	<0.01	<0.001

SNO.	25	26	27	28	29	30	31
Test parameters	Nickel	Total arsenic	Total chromium	COD	BOD	Colloidal sulfur	Total phosphate
Unit	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}
Polok	<0.02	<0.01	<0.05	30	11	8.7	<0.05
Borong	<0.02	<0.01	<0.05	20	8.4	10.2	<0.05

2.3.3. 16S metagenomic sequencing library preparation and sequencing

Amplifications of the V3 and V4 regions of bacterial 16S rRNA gene were done using two primers (16SV3F = 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; 16SV3R = 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC3′) (Klindworth et al., 2013). The amplicon libraries were prepared using Nextera XT Index Kit (Illumina inc.), accordance with 16S metagenomic sequencing library preparation protocol (Faircloth et al, 2014). The amplicon library was purified with AMPure XP beads. The amplified library was checked by Bioanalyzer 2100 (Agilent Technologies) using High Sensitivity (HS) DNA chips and concentration was quantified by Qubit fluorometer. Based on the data obtained from the Qubit fluorometer and the bioanalyzer, 500uL of the 10pM library was loaded into MiSeq cartridge for cluster generation and sequencing. Paired-end sequencing method was used. After the sequencing, high-quality metagenome reads were trimmed to remove the barcode and adaptor sequences.

2.3.4. Metagenomic data analysis

The adapter trimmed sequence were subjected to pre-processing for De-replication, Singleton removal, OTU Clustering, Chimera filtering with SolexaQA. Sequences with Phred score lower than 20 and

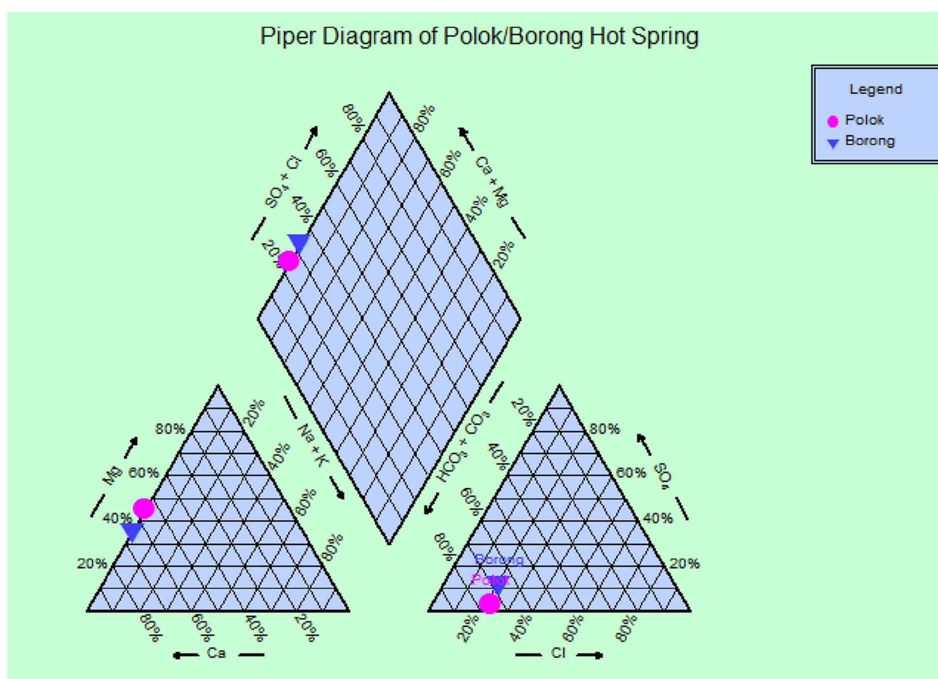

Fig. 2. Piper diagram of Polok and Borong hot spring.

Table 3.a
Morphological and biochemical characterization of various isolates; (+) indicates positive (−) indicates negative results.

Isolates	Gram stain	Spore	Form	Biochemical tests				Carbohydrate fermentation								
				Catalase	Amylase	oxidase	Nitrate reductase	Maltose	Mannitol	Dextrose	Mannose	Ribose	Sucrose	Xylose	Fructose	Cellobiose
TP3	+	+	Rods	+	–	–	–	+	+	+	–	+	–	–	+	–
TP2	+	+	Rods	+	–	–	–	+	+	+	–	–	–	–	+	–
BPP2	+	+	Rods	+	–	–	+	+	–	–	+	–	–	–	+	+
TP5,10PHP1	+	+	Rods	+	–	–	+	+	+	+	–	+	–	–	+	–
10PHP2	+	+	Rods	+	–	+	+	+	–	+	–	–	–	–	+	–
TP9	+	–	Rods	+	+	–	–	+	+	+	–	–	+	+	+	–
TP11	+	+	Rods	+	–	–	+	+	+	+	+	+	–	+	+	–
BPP1	+	+	Rods	+	–	–	–	+	–	–	+	–	–	–	–	–
TB10	+	–	Rods	+	–	–	–	+	+	+	+	+	–	+	+	–
TB7	+	–	Rods	+	–	–	–	+	+	+	+	+	–	–	+	–
TB3	+	+	Rods	+	–	–	+	+	+	+	–	+	–	–	+	–
BPB1	+	+	Rods	–	–	–	+	+	–	–	–	–	–	–	–	+
10PHB1	+	+	Rods	+	–	–	+	+	+	+	–	–	–	–	+	–
YTPB1	+	+	Rods	+	–	+	+	+	–	+	–	+	–	–	–	–
TRB1	+	+	Rods	+	–	–	+	+	–	+	–	+	–	+	+	–
TB9	+	+	Rods	+	–	–	–	+	+	+	+	+	–	–	+	–
TB1	+	–	Rods	+	–	–	+	+	+	+	+	+	–	–	+	–

ambiguous bases having primer mismatch and low read length <100 bp were removed. Annotation and normalization of operational taxonomic unit (OUT) was done using UPARSE OUT clustering and QIIME at 97% similarity (Edgar, 2013). For normalization, inbuilt script as well as METAGEN assist was used. The resulting representative OUT was aligned and given taxonomic classing using Greengenes database (http://greengenes.lbl.gov/). The output of this workflow is a classification of reads at several taxonomic levels: kingdom, phylum, class, order, family, genus, and species. Sequences without homologous pair were classified as unknown.

2.3.5. Statistical analysis

The principal component analysis (PCA) was used to correlate between bacterial diversity and physicochemical parameters of the samples using PAST. The Analysis of variance was used to check the significance of various data with the help of Graph Pad Prism and XLSTAT. The piper diagram was made with the help of Rock Ware AQUA version 1.5. The Shannon diversity indices, chao1 were calculated with the help of EstimateS and PAST software (Chao et al., 2006). The heat map was used to analyze the comparative bacterial diversity among each of the two hot springs and between previously reported microbial diversity of different hot springs from Central India, North-east India, and Tibet with the help of R software using Bray Curtis Dissimilarity matrix (package: ggplot, function: heatmap.2).

2.4. Data availability and accession number

Raw metagenomics reads were submitted to Sequence Read Archive (SRA), NCBI under accession numbers SAMN08038921, SRA: SRS2697425 for Polok Hot Spring with sample name as POLV4 and SAMN08038632, SRA: SRS2697438 for Borong Hot Spring with sample name as BORV4. The BioSample records will be accessible with the following links for Polok and Borong hot springs respectively http://www.ncbi.nlm.nih.gov/biosample/8038921 and http://www.ncbi.nlm.nih.gov/biosample/8038632.

3. Results

3.1. Site description

The geographical location of Polok and Borong hot spring was determined with the help of GPSMAP 78S (Garmin, USA) as per the guidelines are given by the manufacturer. The coordinates of the sites of

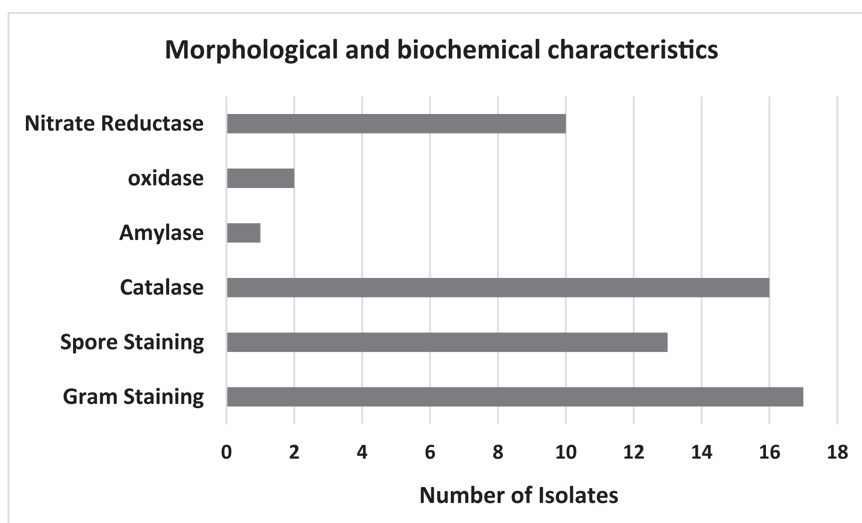

Fig. 3. Morphological and biochemical characteristics of isolates isolated from both Polok and Borong hot springs.

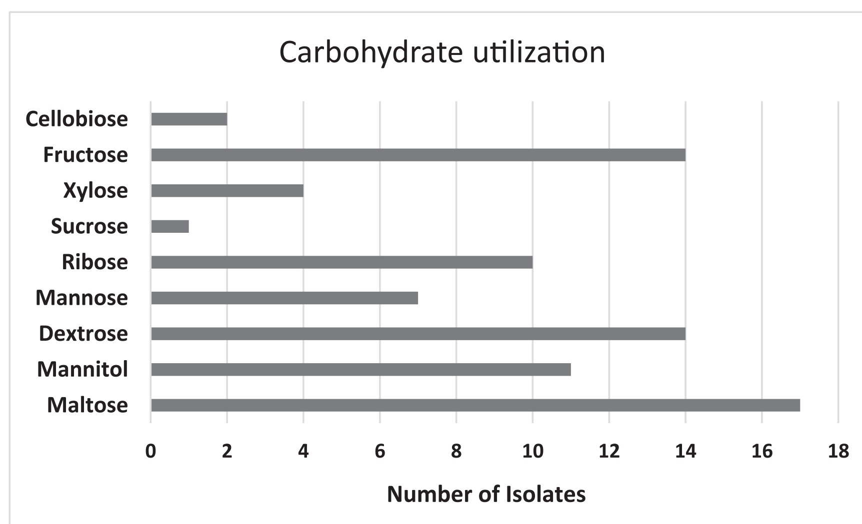

Fig. 4. Carbohydrate utilization of isolates isolated from both Polok and Borong hot springs.

Polokhot spring is 27°21′00.29″N longitude and 88°19′21.99″E latitude. The Borong is located at 27°21′93.57″N longitude and 88°19′67.01″E latitude. The elevation range of the locations was 3108 m and 3404 m for Polok and Borong hot springs respectively.

3.2. Physicochemical analysis of two hot springs

The temperature and pH of the sites were found considerably different. The Polok hot spring was little warmer than the Borong with a temperature of 75–77 °C, Borong was recorded with 50–52 **°**C. The pH of Polok hot spring was in between 7.5 and 8.5 defining the normal to the alkaline condition of the water while the water of Borong was acidic in nature with a pH of 5.1–5.6. The conductivity, dissolved oxygen and TDS were measured as 0.75 mS/cm, 4.5–5.5 mg/L, and 0.4 g/L respectively for Polok hot spring while the Borong hot spring had 0.20 mS/cm, 5.5–6.2 mg/L, and 0.13 g/L respectively (Table 1). The elemental analysis with ICP-MS showed more or less similar pattern of elemental composition in both the springs (Table 2). However, the Polok hot spring was found to be rich in chloride, calcium, magnesium along with COD and BOD (Table 2). Ionic concentration of elements in the hot springs was plotted as piper diagram (Fig. 2) for classification on the basis of chemical composition (Piper, 1944). Piper diagram is a combination of triangle plots representing anionic and cationic element on a common baseline. The apexes of the cation plot were magnesium, calcium, sodium, and potassium cations, while the apexes of the anion plot were chloride, sulfate, carbonate and hydrogen carbonate anions. The two ternary plots are then anticipated onto a diamond which can be used to describe different water types. Piper divided water into four basic types conferring to their location near the four corners of the diamond. Water that plots at the top of the diamond is high in $Ca^{2+} + Mg^{2+}$ and $Cl^{-} + SO4^{2-}$, which results in an area of permanent hardness. The water that plots near the left corner is rich in $Ca^{2+} + Mg^{2+}$ and HCO^{3-} and is in an area of temporary hardness. Water plotted at the lower corner of the diamond is mainly composed of alkali carbonates ($Na^{+} + K^{+}$ and $HCO^{3-} + CO3^{2-}$). Water present near the right-hand side of the diamond may be reflected as saline ($Na^{+} + K^{+}$ and $Cl^{-} + SO4^{2-}$). 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 (Fig. 2).

3.3. Bacterial isolation and physicochemical characterization

A total of 100 thermophilic bacteria were isolated from two springs. On the basis of morphological and biochemical differences, 25 isolates from each hot spring were selected for further analysis. The molecular identification based on 16S rRNA further identified 17 distinct bacteria, which were taken as a representative of autochthonous thermophiles from the springs. The cell morphology of the bacteria suggested that most of the isolates were Gram-positive rods, however, some of the isolates showed Gram variable reaction. Most of the isolates were spore-forming except few such as TP9, TB10, TB7, and TB1 (Table 3.a). Of the 17 isolates, 16 were catalase positive, whereas amylase, oxidase, and nitrate reductase activity were less common among the isolates (Fig. 3). Carbohydrate fermentation test showed that most of the isolates were

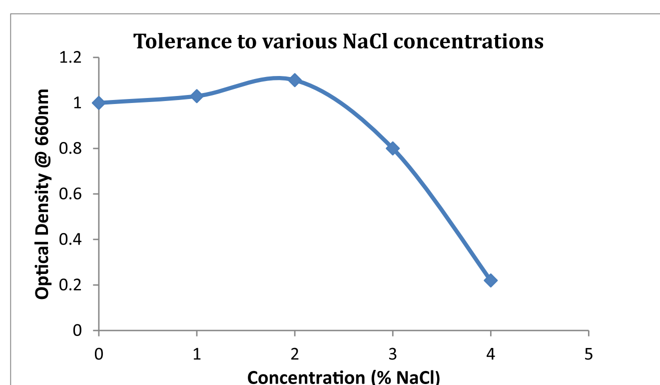

Fig. 5. Tolerance to different NaCl concentrations.

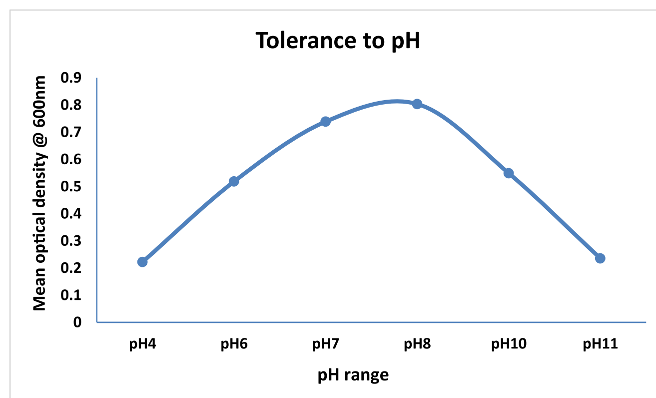

Fig. 6. Tolerance of isolates to different pH.

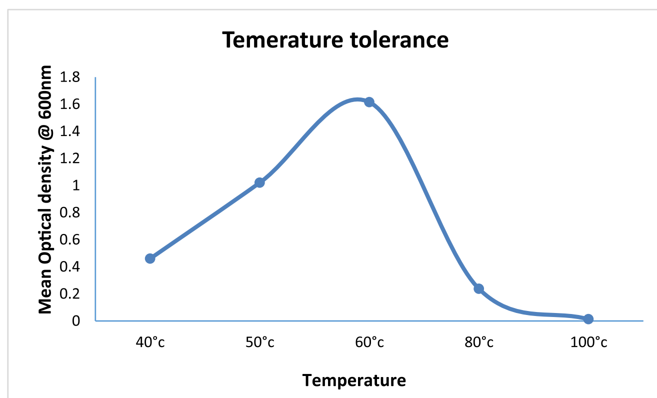

Fig. 7. Tolerance of isolates to various temperatures.

active utilizers of simple sugars like dextrose, maltose, ribose, fructose, mannitol, while they were unable to utilize complex sugars like sucrose, arabinose, raffinose, cellobiose, and dulcitol (Fig. 4). Physiological analysis showed the isolates could tolerate a wide range of temperature, pH and NaCl concentration as mentioned in Figs. 5–7. Growth was observed up to 5% of NaCl concentration (Fig. 5) while interestingly the isolates were able to grow both at acidic and alkali condition of pH ranging from 4 to 11. However, optimum pH for most of the isolate was pH 8.0 though few isolates showed growth up to pH 11.0 (10PHP1, 10PHP2, and 10PHB1) (Fig. 6). The isolates could actively grow in the temperature gradient of 30 °C −80 °C. However, most of the isolates showed the optimum temperature of 60 °C (Fig. 7).

3.4. Molecular identification and phylogeny

Molecular identification showed the singular dominance of phylum *Firmicutes*. Major genus found in the study is *Geobacillus* with a few representatives of genus *Anoxybacillus*. Identified isolates of *Geobacillus* were as *G. lituanicus* TP11, *G. toebii* TP3/TP5, *Parageobacillus toebii* 10PHP2, *G. Kaustophillus* YTPB1, *G.* sp., BPP2, and *G sp.*, TB7. The representative isolates of genus *Anoxybacillus* were *Anoxybacillus gonensis* TP9 and *Anoxybacillus Caldiproteolyticus* TRB1. The alignment and similarity search of 16S rRNA sequence with nr/nt database of NCBI have shown that many of the isolates have a distinct percentage of identity from <95%. These results suggested the novelty of these isolated bacteria. The identified species, the percentage of identity and their NCBI Accession numbers are given in Table 3.b. The phylogenetic tree was made by Neighbor-joining method using the jukes-cantor model as shown in Fig. 8.

Table 3.b
Identification of bacteria isolates based on 16SrRNA, there percentage identity and accession numbers.

Isolates	Identification based on 16SrRNA	% identity	Accession No.
TP3	*Geobacillus toebii* TP3	94	MG603308
TP2	*Geobacillus* sp. TP2	91	MG603309
BPP2	*Geobacillus* sp. BPP2	91	MG603313
TP5,10PHP1	*Geobacillus toebii* TP5	90/88	MG603315
10PHP2	*Parageobacillus toebii* 10PHP2	95	MG731573
TP9	*Anoxybacillus gonensis* TP9	100	KX894322
TP11	*Geobacillus lituanicus* TP11	99	MG603317
BPP1	Geobacillus *toebii* BPP1	95	MG731574
TB10	*Geobacillus* sp. TB10	91	MG603310
TB7	*Geobacillus* sp.TB7	91	MG603311
TB3	*Geobacillus toebii* TB3	90	MG603312
BPB1	*Geobacillus* sp.BPB1	90	MG603314
10PHB1	*Geobacillus toebii* 10PHB1	90	MG603316
YTPB1	*Geobacillus kaustophillus* YTPB1	99	MG603318
TRB1	*Anoxybacillus caldiproteolyticus* TRB1	98	MG603319
TB9	*Geobacillus* sp.TB9	93	MG731576
TB1	*Geobacillus* sp.TB1	95	MG731575

3.5. PLFA studies

The major fatty acids significantly varied among the Polok and Borong hot springs. It was found that the straight chain fatty acids were abundant in case of Borong (11.28) as compared to Polok (3.34), whereas branched-chain fatty acids were similar in both the springs (B – 26.79; P – 27.07). However, the polyunsaturated fatty acids (PUFA) were relatively higher in case of Polok as shown in Table 4. The PLFA results showed that the two hot springs, i.e., Polok and Borong were considerably different with respect to their biomass content. The biomass content of Borong was higher (1044.939 nmoles/g) than that of Polok hot spring (838.859 nmoles/g) (Table 5). Fatty acid marker analysis with Sherlock PLFA tool defined the community structure of two springs with the abundance of Gram-positive bacteria, Gram-negative bacteria, anaerobic bacteria, fungi, and eukaryotes. The results showed that Gram-positive bacteria were relatively higher in Borong hot spring (30.03%) than the Polok (27.94%), while Gram-negative bacteria were higher in Polok (7.17%) than the Borong (4.85%). The percentage of Fungi and Eukaryotes were similar in both the hot Springs as shown in Table 5 and Fig. 9. The abundance of various fatty acid types was also investigated and it was found that there was no much distinction between the fatty acids present in both the hot springs (Supplementary S2). The fatty acid found in the springs were 15:3 ω3c, 15:0 anteiso, 16:00, 17:1 iso ω9c, 17:0 anteiso, 18:2 ω6c, 18:1 ω9c, 19:3 ω3c and 21:3 ω3c. However, the fatty acid 12:0 was abundant in Borong hot spring which was not recorded in Polok. The abundance of various fatty acids was represented by plot matrix as shown in Fig. 10. The principle component analysis (PCA) of the fatty acids showed 15:3 ω3c, 15:0 anteiso, 17:0 anteiso, 19:3 ω3c and 21:3 ω3c are positively correlated between the two springs while 12:0 fatty acid was correlated to the Borong (Fig. 11).

3.6. Metagenomic analysis

3.6.1. Diversity index and rarefaction curve

The diversity indices such as Shannon H, Fisher Alpha, and Chao1 were estimated using PAST and EstimateS software packages. The results have shown that the Polok is more diverse than Borong hot spring. The Shannon index was 3.54 and 2.78 for Polok and Borong respectively (Fig. 12). The Chao1 index was also higher in case of Polok hot spring (Table 6).

Rarefaction allows the calculation of species richness in a sample. The curve is a plot of a total number of species annotated as a function of the number of sequences sample (Das et al., 2017). The steep slope at the beginning on the left side signifies most common species have been identified and the plateau at the right side signifies further intensified sampling could lead to the identification of few rarest species (Fig. 13)

3.6.2. Metagenomic studies

The 16 s rRNA gene library of Borong was comprised of 3, 72,480 reads with average sequence length of 301 bp. The average GC content was 53%. While, 3, 98,782 reads were obtained from 16 s rRNA gene library of Polok hot spring with an average sequence length of 301 bp. The mean GC content was estimated to be 54%. A total of 409 OTUs were clustered using UClust. Approximately, 104 OTUs were obtained from Borong hot spring and 360 OTUs were obtained from Polok hot spring. Annotation with reference library showed, both the hot spring possesses lesser amount of archaeal communities (<1% but they are significantly distinct). Borong hot spring consisted of *Crenarchaeota* (0.96%), whereas Polok had *Euryarchaeota* (0.56%). The order and genus under *Crenarchaeota* were identified as *Desulfurococcales* and *Desulfurococcus* respectively and in case of *Euryarchaeota*, the order and genus were identified as *Methanomicrobiales* and *Methanospirillum*. The bacterial community showed significant variation between the springs. The phylum wise diversity showed the dominance of *Proteobacteria* (62.50%), *Bacteroidetes* (15.38%), *Acidobacteria* (3.85%),

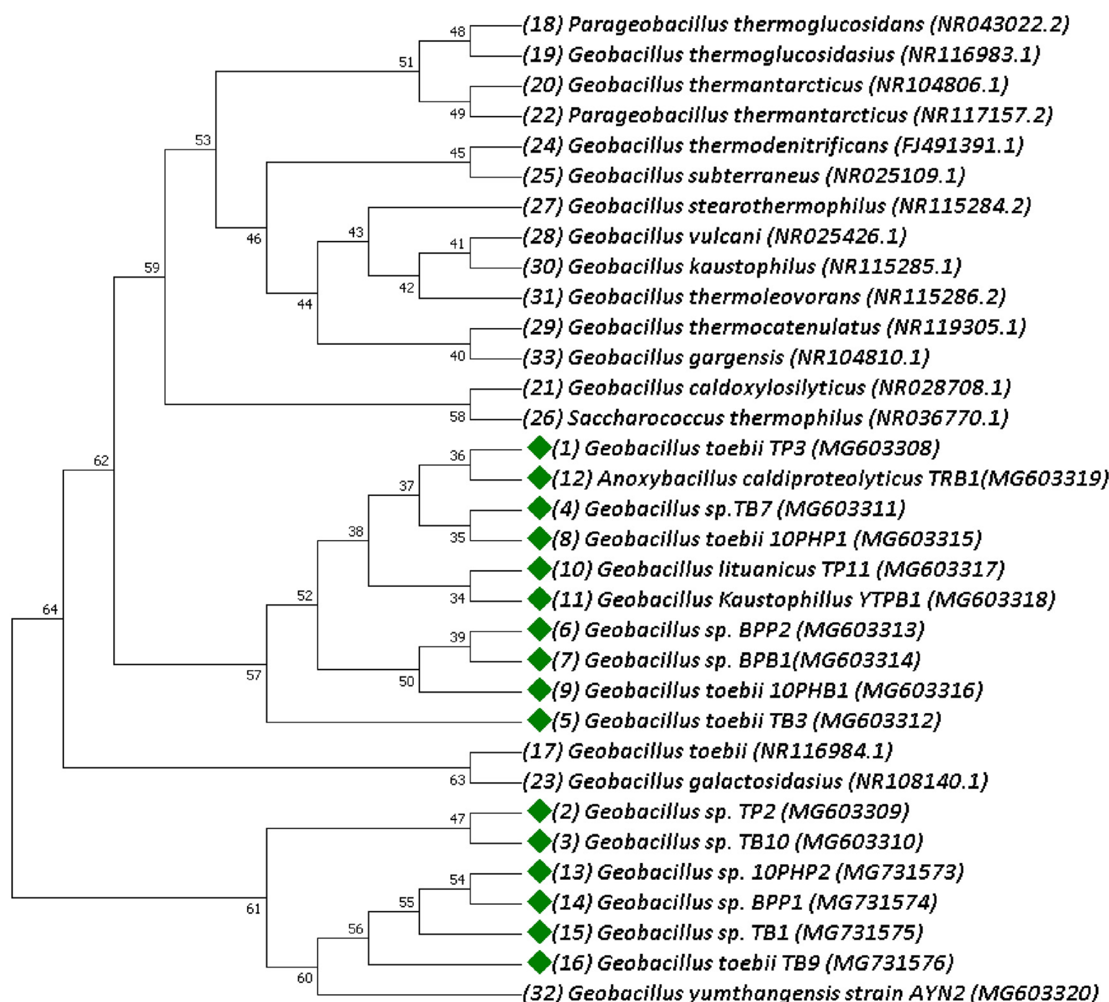

Fig. 8. Phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.39 is shown. The evolutionary distances were computed using the Jukes-Cantor method [2] and are in the units of the number of base substitutions per site. The analysis involved 33 nucleotide sequences. All positions containing gaps and missing data were eliminated. There. Evolutionary analyses were conducted in MEGA7.

Nitrospirae (3.85%) and *Firmicutes* (2.88%) in Borong whereas Polok was dominated by *Proteobacteria* (47.22%), *Bacteroidetes* (3.61%), *Firmicutes* (3.06%), *Parcubacteria*(3.06%) and Spirochaetes (2.50%) (Figs. 14, 16). The major genus present in Borong hot spring was *Acinetobacter* (7.69%), *Flavobacterium* (3.85%), *Vogesella* (3.85%), *Ignavibacterium* (2.88%), *Sediminibacterium* (2.88%), *Thermodesulfovibrio* (2.88%) and *Acidovorax* (1.92%). While the major genera in Polok was *Flavobacterium* (3.33%), *Parcubacteria genera Incertae sedis* (3.06%), *Sediminibacterium* (2.78%), *Pseudomonas* (1.67%), *Treponema* (1.68%) and *Opitutus* (1.39%) Figs. 15, 17.

3.6.3. Correlation of bacterial diversity and geochemistry of Polok and Borong hot spring

Principle component analysis was accomplished to analyze the correlation between bacterial diversity at phylum level and geochemistry of the two hot springs. The top five phyla from both the hot springs and geochemical parameters such as temperature, pH, alkalinity, Ca^{+2}, Mg^{+2}, Cl^{+2}, and sulfur were studied as shown in Fig. 18. The PCA revealed that the community composition was differentially correlated to various concerned geochemical parameters. The *Proteobacteria* were highly correlated to temperature, whereas, *Bacteroidetes* showed a high correlation to chlorine and alkalinity. Contrary, Ca^{+2}, Mg^{+2}, sulfur, and pH were positively correlated with *Parcubacteria*, *Firmicutes*, and *Spirochaetes*. However, *Parcubacteria*, *Firmicutes*, and *Spirochaetes* were negatively correlated with temperature, chloride and alkalinity.

3.6.4. Comparison of microbial diversity

Microbial community structure of hot springs reported earlier from different regions of northeast India (Panda et al., 2015) were compared with our studies using heatmap plot with Bray Curtis dissimilarity method. Phylum level diversity comparison showed that the community structure of Borong and Polok were correlative with the Yumthang hot spring of Sikkim (Panda et al., 2017) as earlier reported. Whereas, Jarkem hot spring of Meghalaya showed comparatively different microbial community structure. The major phylum of hot springs Polok and Borong are *Proteobacteria* and *Bacteroidetes* while major phylum of Jarkem is *Firmicute* sand *Chloroflexi*. This difference indicates possible role of geographical location in shaping the microbial community (Fig. 19.)

Table 4
Major Fatty acids types present in both the hot springs.

Hot spring sample info	Straight 001: straight	Branched 002: branched	Cyclo 004: cyclo	MUFA 005: MUFA	PUFA	DMA	18:1 w9c	18:2 w6, 9c	10-methyl	Hydroxy
Borong	11.28	26.79	–	3.04	55.32	0.54	1.25	1.79	–	–
Polok	3.34	27.07	–	4.75	60.46	0.94	1.44	1.98	–	–

Table 5
PLFA analysis of two hot springs. Fatty acid types converted to microbial groups by Sherlock software.

Hot spring	Gram positive	Gram negative	Anaerobe	Actinomycetes	AM fungi	Fungi	Methanobacter	Eukaryote	Total biomass (nmoles/g)
Borong	30.03	4.85	0.61	–	–	2.03	–	62.41	1044.9
Polok	27.94	7.17	0.43	–	–	2.05	–	62.41	838.8

4. Discussion

The systems with a continuous circulation of heat and fluid, where fluid enters the reservoir from the recharge zones and leaves through discharge constitute geothermal fields. The unique spots within these fields are mainly hot springs, which are present all over the world. Although the hot springs are not merely the ponds where hot water oozes out, these thermal springs have been in use for religious and/or medicinal tenacities before 2000 BCE in India and for hundreds of years in China, Egypt, Japan, Turkey and in many European and Middle-Eastern countries as shown by archaeological marks (Olivier and Jonker, 2013). Many thermal springs urbanized into thriving centers of religion, culture, and health, such as those at Bath in England, Vichy in France and Baden-Baden in Germany (Olivier et al., 2011).

The main importance of these hot springs is the microbiota present in them. The microorganisms present in such hot springs can thrive under extreme temperatures. These microorganisms are known as thermophiles, which can grow into the temperatures above 45 °C (Stetter, 1999). The ideal growth temperature of the thermophiles may vary from 45 °C to above 100 °C (Andrade Jr et al., 1999). Some extremophiles have been known for >40 years, but the detection and isolation of new thermophilic microorganisms have increased for the last decades or ever since thermophilic bacteria were first discovered in the 1960's (Rampelotto, 2013). Many bacteria and archaea have been isolated from hot springs. *Aquificales* are the dominant group of thermophilic bacteria. Also, *Thermotoga*, *Thermus* (*T. thermophilus*), *Bacillus*, *Clostridium*, *Synechococcus*, *Chloroflexus* etc., have also been identified from many hot springs. In environments with a temperature above 90 °C, archaeal communities are dominating (Reysenbach and Shock, 2002). Archaea such as *Methanopyrus*, *Pyrodictium*, *Thermoproteus*, *Methanothermus*, *Archaeoglobus*, *Thermofilum*, *Thermococcus*, *Sulfolobus* etc. have been isolated from many such springs (Rampelotto, 2013; Stetter, 1999). It is important to study the thermophiles of hot springs as their chemical constituents and various metabolites are stable especially at high temperature. Thermophilic enzymes of these microorganisms got a special attention from the scientists from all over the world. These enzymes not only withstand high temperature but are also stable at different pH and resistant to different

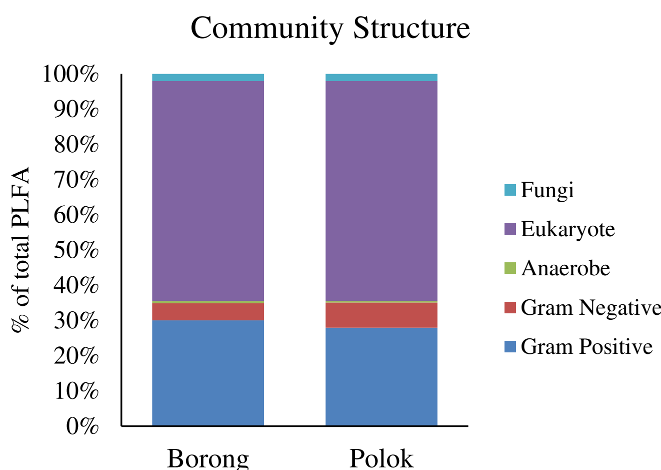

Fig. 9. Community Structure of two hot springs based on PLFA analysis.

kinds of chemical reagent. These thermophilic enzymes have vast applications in industries and also in genetic engineering. Thus it is of great importance to investigate the microflora of these hot springs by culture-dependent and culture-independent techniques to find new promising enzymes or protein compounds.

The biodiversity of several hot environments such as hot springs, oil reservoirs, composter were studied using shotgun metagenomics sequencing.

(Kotlar et al., 2011; Martins et al., 2013; Mehetre et al., 2016). These new molecular methodologies have enhanced the microbial ecology studies by aiding in the analysis of comprehensive microbial community structure of an environment. Geothermal craters in earth crust are naturally diverse in microbial community structure. However, due to the uncultivated status of the major taxa in hot springs, culture-dependent analysis led to the identification of few distinct genera from the Polok and Borong hot springs. The major or the singular dominant phyla identified was *Firmicutes* and the major genus identified was *Geobacillus*. Homology with reference strains from nucleotide database of NCBI showed identity percentage as low as 93%, which predict the possibilities of finding novel species from the springs. The various microorganisms identified were *G. lituanicus* TP11, *G. toebii* TP3/TP5, *Parageobacillus toebii*10PHP2, *G. Kaustophillus* YTPB1, *G.* sp., BPP2, *G* sp., TB7, *Anoxybacillus gonensis* TP9 and *Anoxybacillus Caldiproteolyticus* TRB1. The genus *Geobacillus* is earlier reported from the Himalayan geothermal provinces of Himachal Pradesh (Mehta et al., 2012; Sharma et al., 2013) and Uttarakhand regions (Dheeran et al., 2010). But, to the best of our knowledge, it is the first report of finding the *Geobacillus* species from the hot springs of the northeastern region of India. Apart from Himachal, there was no such known report of *Geobacillus* from other geothermal provinces in India such as Sohana geothermal province (Sareen and Mishra, 2008), west coastal geothermal province (Mehetre et al., 2016), Mahanadi geothermal province (Badhai et al., 2015). The biochemical characterization indicated the isolated bacteria could use a wide range of carbon and nitrogen sources for energy. Most of the strains are able to utilize simple sugars like dextrose, maltose, fructose, mannose etc. The physiological characterization showed the thermophilic nature of the isolates. The isolates could grow in wide range of temperature (30–80 °C) with optimum of 60 °C. The pH-dependent growth analysis showed acidic to extreme alkali nature of the isolates which could actively survive and grow in pH 3–12. Correlative results could be found from the study of Pandey et al. (2015). Isolated bacteria from Soldhar and Ringigad hot springs of Uttarakhand, India exhibited tolerance to a wide range of temperature (20–80 °C), from mesophilic(+20° to +45 °C) to thermophilic (+46° to +75 °C); and few in hyperthermophilic range (+76 °C). The isolates also had a wide range of pH (4–14) and moderate salt tolerance (Pandey et al., 2015). The possible reasons could be hypothesized that this wide range of temperature and pH tolerance shown by these bacteria may be due to their presence in fluctuating surrounding environments at different geothermal heights. The hot waters present in reservoirs deep down to earth flows out through high pressures. The geochemistry of water as in the deep-down geothermal point's get changes in the surface with changing physicochemical parameters. The bacteria present may come in contact with varying water contents with varying physicochemical composition at varying heights. Thus these fluctuating environments with respect to distinct physicochemical contents help these bacteria to become tolerant and adaptive to the changing environment.

The conventional culture-dependent methods fail to give comprehensive microbial community structure of an environment due to the

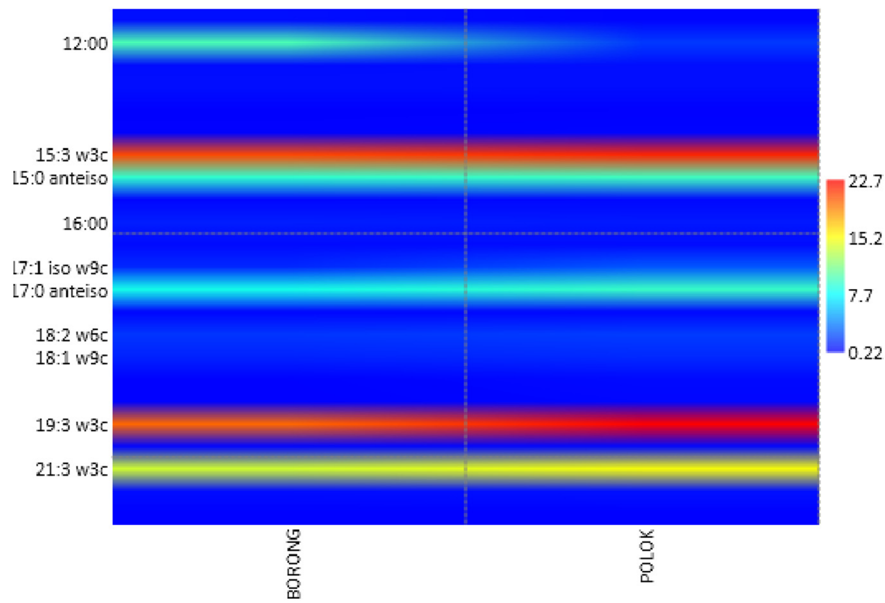

Fig. 10. Abundance of various fatty acids in Borong and Polok hot springs.

large unculturable status of the microbial world. The development of wide range of culture-independent methods has widened the scientific knowledge about previously unknown microorganisms and their involvement in different biological processes. Culture-independent techniques such as PLFA analysis which was a pioneer in the field and first used in 1979 to estimate the microbial biomass from marine sediments (White et al., 1979). This technique is important to determine viable microbial biomass, microbial community composition and metabolic activity in an environment (Rzonca and Schulze-Makuch, 2003). Phospholipid fatty acids (PLFA) are an essential structural component of microbial cell membrane, which makes it an important chemotaxonomic marker. PLFA analysis is widely used to estimate the total biomass and to observe the changes in community composition of the microbiota in soil and aqueous environment. The PLFA results have shown the abundance of Gram-positive bacteria in both the hot springs. Borong had 30% of Gram-positive bacterial community while Polok had 27%. Gram-negative bacteria were 4% and 7% respectively in Borong and Polok. The Anaerobes, Fungi, and Eukaryotes were equally abundant in both the hot springs. The various fatty acids abundant in both the hot springs were 15:3 ω3c, 15:0 anteiso, 16:00, 17:1 iso ω9c, 17:0 anteiso, 18:2 ω 6c, 18:1 ω 9c, 19:3 ω 3c and 21:3 ω 3c. The abundant fatty acids showed the dominance of biomarkers for Gram-positive bacteria such as 15:0 anteiso, 16:00, 17:0 anteiso, 18:0 (Willers et al., 2015). The total biomass of Borong hot spring (1044.9 nmoles/g) was higher

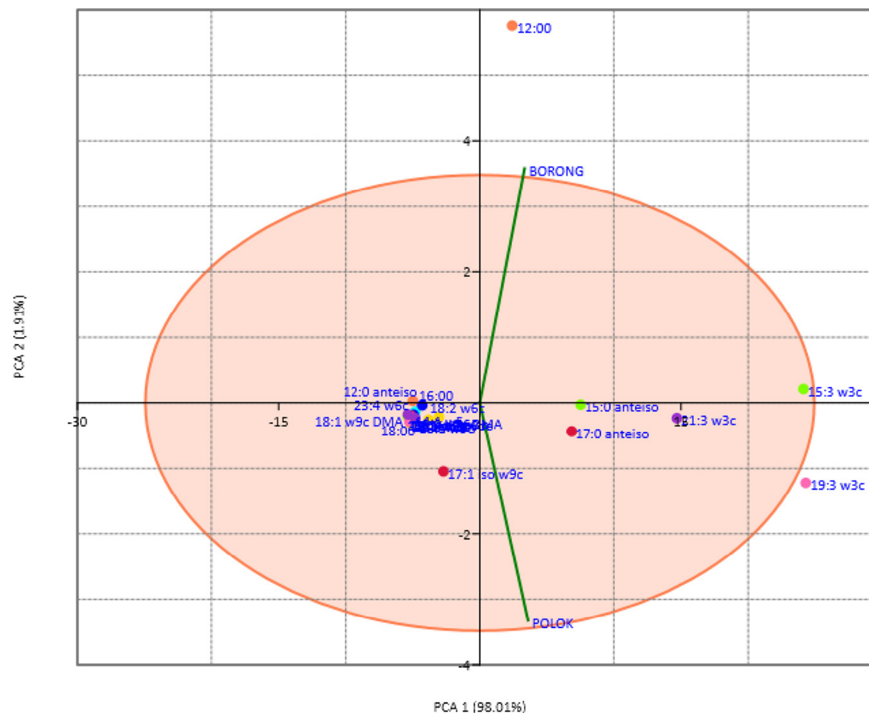

Fig. 11. Principle component analysis of various fatty acids with respect to Polok and Borong hot springs.

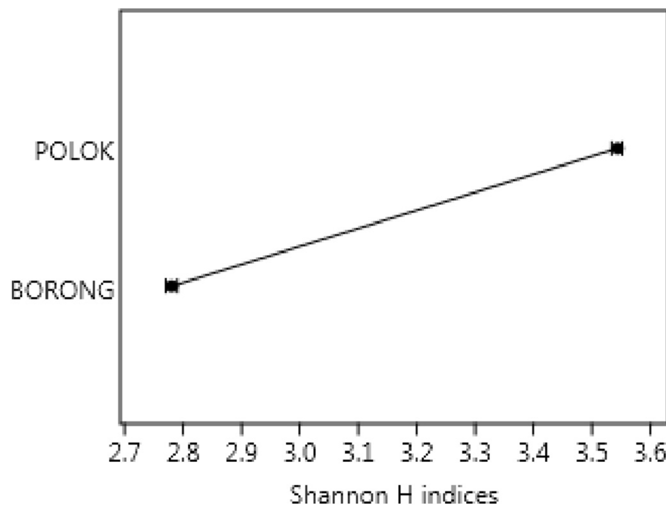

Fig. 12. Representation of the abundance of bacterial diversity in both the hot springs with Polok having higher Shannon H index than Borong hot spring.

than that of Polok hot spring (838.8 nmoles/g). The similar studies have been performed by (Rzonca and Schulze-Makuch, 2003), however, they have found an abundance of Gram-negative bacteria than that of Gram-positive bacteria.

The metagenomic studies revealed the major dominance of *Proteobacteria* and *Bacteroidetes* in both the hot springs. The *Proteobacteria* constituted 62.50% and 47.22% of the total community in Borong and Polok hot springs respectively. The second abundant phylum in both the hot springs was *Bacteroidetes* which constituted 22.78% and 15.38% of the metagenome library of Polok and Borong hot springs respectively. In many geothermal environments, the phylum *Proteobacteria* and *Bacteroidetes* have been found as the dominant phylum (Amin et al., 2017; Lau et al., 2009; López-López et al., 2015). An earlier report on the microbial diversity of the Yumthang hot spring of north Sikkim similarly recorded the abundance of *Proteobacteria* (83.68%) and *Bacteroidetes* (10.93%) as the dominant phyla (Panda et al., 2016).

As the main characteristic feature of any hot spring is temperature, thus, the abundance of a particular phylum in the hot springs has been correlated and interpreted by many researchers as a function of temperature. Subudhi et al. (2017) have shown the predominant shifting of thermophilic cyanobacteria as a function of temperature and also, have shown the abundant growth of different strains at different temperatures (Subudhi et al., 2017). Similarly, Sahoo et al., 2017, have correlated and linked the predominant nature of Proteobacteria in the hot springs of Odisa, India, as a function of temperature (Sahoo et al., 2017). In our study, the phylum level diversity of two hot springs is similar to a great extent, but the relative abundance is considerably different. Borong hot spring is having higher abundance of Proteobacteria than that of Polok hot spring. These hot springs are located in the same area, only few miles apart and at a similar altitude and similar chemical constituents. Thus having similar geographical and geological features, this migt be the reason for having similar bacterial diversity in the two hot springs. However there is a considerable difference in their temperature. The phylum Proteobacteria are known to dominate in moderate temperatures (Wang et al., 2013). Since, Borong possess the lower temperature than Polok hot spring and therefore, the function temperature may be the reason for proteobacterial predominance in Borong hot spring. The other characteristic feature of phylum Proteobacteria is that they are known to tolerate higher concentration

Table 6
Diversity indices of hot spring microbial communities.

Hot springs	Total number of reads	G + C content	Average sequence length	Total number of OTUs	Shannon H index	Fisher alpha	Chao1
Borong	372,480	53	301	104	2.78	11.46	104
Polok	398,782	54	301	360	3.54	44.5	360

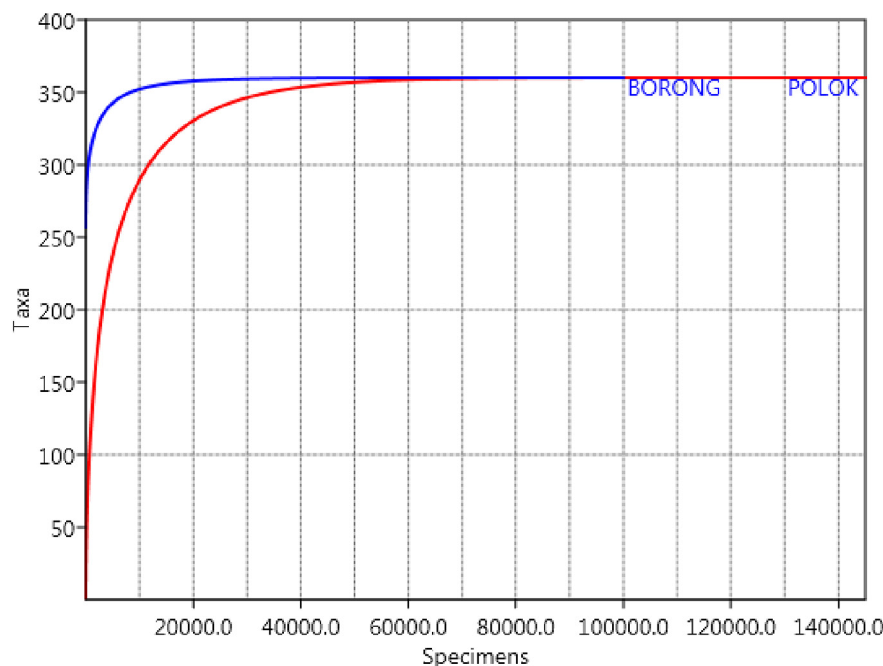

Fig. 13. Rarefaction curve, red curve shows species richness in Polok Hot Spring whereas blue line represents Borong hot spring. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

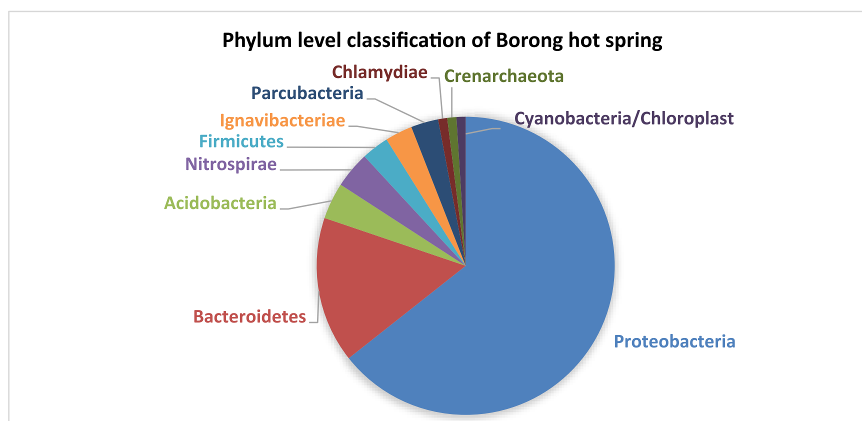

Fig. 14. Phylum level classification of Borong hot spring.

of sulfur and utilize sulfur as an electron donor during their physiological process (Bolhuis et al., 2014). Therefore, it is not surprising that Borong hot spring has higher abundance of proteobacteria, as Borong hot spring possess high sulfur content then Polok hot spring which was confirmed by ICPMS results. This correlation of temperature and sulfur concentration to phylum Proteobacteria has been supported by our PCA results which is in accordance with Sahoo et al., 2017 (Sahoo et al., 2017).

Subdivision of *Proteobacteria* to class level hierarchy showed the presence of *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* and *Deltaproteobacteria* in both the hot springs with the dominance of *Gammaproteobacteria*. The *Epsilonproteobacteria* was least while zetaproteobacteria was not recorded. Following the *Proteobacteria* and *Bacteroidetes*, the other dominant phyla were quite distinct with respect to each spring. *Verrucomicrobia* (3.61%) and *Firmicutes* (3.06%) are the third and fourth dominant bacterial phyla in Polok followed by *Parcubacteria* (3.06%) and *Spirochaetes* (2.50%). However, Borong hot spring was dominated by *Acidobacteria* (3.85%) and *Nitrospirae* (3.85%).

The major genus present in Borong hot spring was *Acinetobacter* (7.69%), *Flavobacterium* (3.85%), *Vogesella* (3.85%), *Ignavibacterium* (2.88%), *Sediminibacterium* (2.88%), *Thermodesulfovibrio* (2.88%) and *Acidovorax* (1.92%). While the major genera in Polok was *Flavobacterium* (3.33%), *Parcubacteria genera Incertaesedis* (3.06%), *Sediminibacterium* (2.78%), *Pseudomonas* (1.67%), *Treponema* (1.68%) and *Opitutus* (1.39%). Most of the genus represented was Gram-negative in nature. The genus *Acinetobacter* possess Gram-negative, non-fermentative bacteria which are ubiquitous organisms in soil, water, and sewage. These are known for the biopolymers and biosurfactant production (Towner, 2006). The genus *Flavobacterium* contains Gram-negative aerobic rods that are motile by gliding and are widely distributed in soil and freshwater habitats (Bernardet et al., 1996). The genus *vogesella* contains singular or paired short chained bacteria. The colonies are a deep royal blue with a metallic copper-colored sheen, due to the production of indigoidine (Krieg, 2015). The genus *Ignavibacterium* possess Gram-negative bacteria which are strictly anaerobic, moderately thermophilic, neutrophilic and obligately heterotrophic in nature (Iino et al., 2010). The genus *Sediminibacterium* belongs to phylum *Bacteroidetes* which includes Gram-negative, strictly aerobic, rods, motile by gliding and having isoC15: 1 G and isoC15: 0 as the major cellular fatty acids (Kang et al., 2014). The genus *Thermo desulfovibrio* was described as a group of obligately anaerobic, curved rod-shaped, thermophilic bacteria that reduce sulfate and other sulfur compounds (Maki, 2015). At species level, the diversity of bacteria is quite distinct in both the hot springs. However, the uncultured bacteria dominated both the hot springs. The top five species of Polok hot spring were *Sediminibacterium goheungense*, *Opitutusterrae*, *Treponema caldarium*, and *Ignavibacterium album*. Top five species of Borong hot spring were *Ignavibacterium album*, *Thermodesulfovibrio yellowstonii*, *Flavobacterium cheonhonense*, *Rheinheimera aquatic*, and *Sediminibacterium goheungense*.

Comparison with the reported microbial diversity of hot springs from different provinces of India showed a significant pattern of differentiation and correlation along the community structure. This difference in microfloral diversity may be due to the geographical and geochemical distinction among the hot springs. The heatmap constructed with Bray Curtis Dissimilarity distance showed two major clades or groups with two sub-clades each and one out-group. Four of the Tibetan hot springs (Rongma, Gulu, Jiwa1 and Jiwa2) (Huang et al., 2011) and two hot springs from Barkeshwar, West Bengal, India (Bengal et al., 2017), Junagarh, Gujrat, India (TulsiShyam) (Ghelani et al., 2015), one from Shillong, Meghalaya, India (Jakrem) (Panda et al., 2015) and one from Odisha (Atri) (Badhai et al., 2015), formed a single group, where the diversity of Atri and Jakrem was similar with the dominance of *Chloroflexi*. The other hot springs formed the second sub group

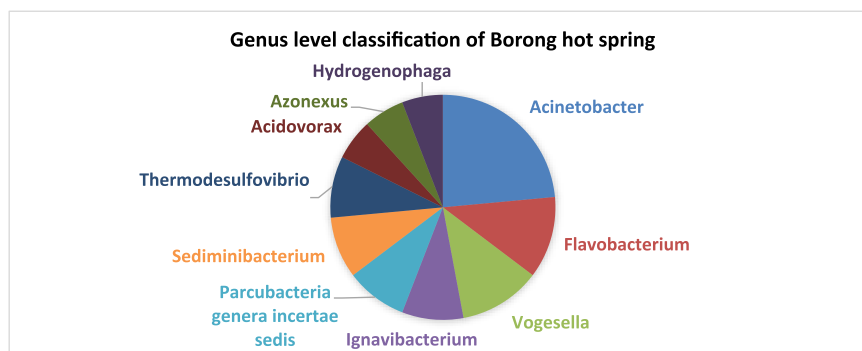

Fig. 15. Genus level classification of Borong hot spring.

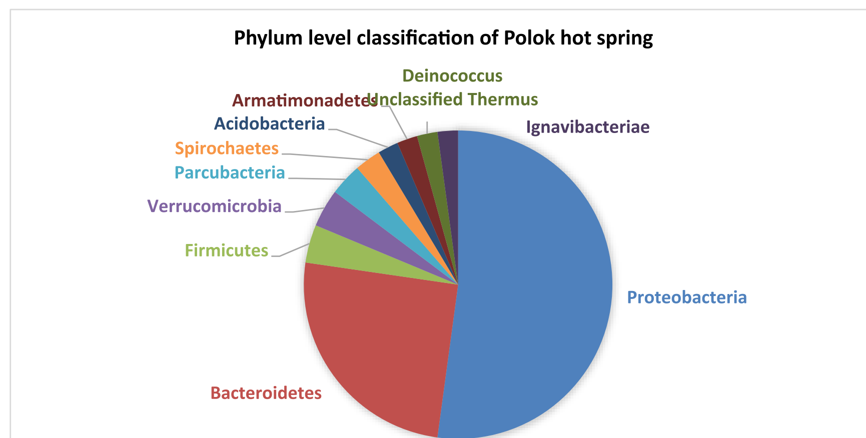

Fig. 16. Phylum level classification of Polok hot spring.

where the dominant group is *Firmicute*s. The Rongma of Tibet formed a single branch in the second subgroup of first clade as it the only spring in first group with dominance of *Acidobactera* and *Bacteroidete*s. The second clade was made by seven hot springs including three hot springs of Sikkim (Polok, Borong and Yumthang) and one from Shimla, Himachal (Tattapani) (Mohanrao et al., 2016), two hot springs from Odisha, India (Athamallik and Tarabalo) (Badhai et al., 2015), and one from Tibet (Gulu 2) (Huang et al., 2011) respectively. The hot springs from the second clade were dominated by *Proteobacteria*. Polok and Borong in the second clade showed correlative diversity with the dominance of *Proteobacteria* and *Bacteroidetes*. Anhoni hot spring from Madhya Pradesh, India (Saxena et al., 2017) formed the out-group, where the relative abundance of the phyla was least in comparison to the other springs.

In the present study, the culture-dependent taxonomic profiling and PLFA studies showed a positive correlation. Both the methods showed the dominance of Gram-positive bacteria over Gram-negative in the hot springs. Some of the additional studies with environmental samples from our lab (unpublished data) also supports the similar findings, where PLFA and culture dependent data are correlative. The similar results were found by Pandey et al., 2015. They have also found only Gram positive bacteria while investigating the diversity of two hot springs of Uttrakhand (Pandey et al., 2015). However, the NGS data was relatively contradictory to both the PLFA and culture-dependent approach. The dominant bacterial phylotypes in the metagenome library were Gram-negative (*Proteobacteria*). The earlier findings of dominant Gram-negative bacteria with a metagenomic approach from the *Yumthang* hot springs of Sikkim correlates with our results (Panda et al., 2017) In order to limit the contradictions, various media were used to culture the enriched samples along with different pH and temperature. However, the results were synonymous. Thus a probable reason for these findings can be hypothesized that the ubiquitous nature of phylum *Firmicutes* (the dominant phyla in culture-dependent study) and their ability to adopt a wide range temperature, pH, and salinity makes them stable and culturable. But, the large dominance of unculturable Gram-negative bacteria (*Proteobacteria*) in the environment masked the dominance of Gram-positive phyla (*Firmicutes*) in the metagenomic study. Also the present study including the two different culture independent techniques i.e., PLFA and metagenomics which are based on the analysis of two different molecules such as fatty acids and DNA respectively. It is also evident that the DNA is more stable than phospholipid fatty acids as the later gets degraded immediately after the decease of bacteria. Whereas in case of metagenomics study, the DNA of both the live and dead bacterial cell are present and investigated. Thus this makes it important to study the microbial diversity of any ecological niche with the help of diverse techniques.

Geochemical parameters have often been the driving factor in shaping the microfloral community in such environment holds an important aspect of such studies. Several researchers in order to determine the evolution of thermophilic microbial communities have compared the metagenomic data with geochemistry of hot springs (Rzonca and Schulze-Makuch, 2003). The piper diagram for physicochemical parameters revealed, both the hot springs are Ca-HCO^{3-} type and can be predicted as shallow fresh ground waters. However, in veracity, sampled spring waters almost always represent mixtures from deep thermal fluids diluted by more shallow ground waters, therefore they must be considered as deep geothermal waters derivatives (Goff and Grigsby, 1982). The correlation between various physicochemical parameters

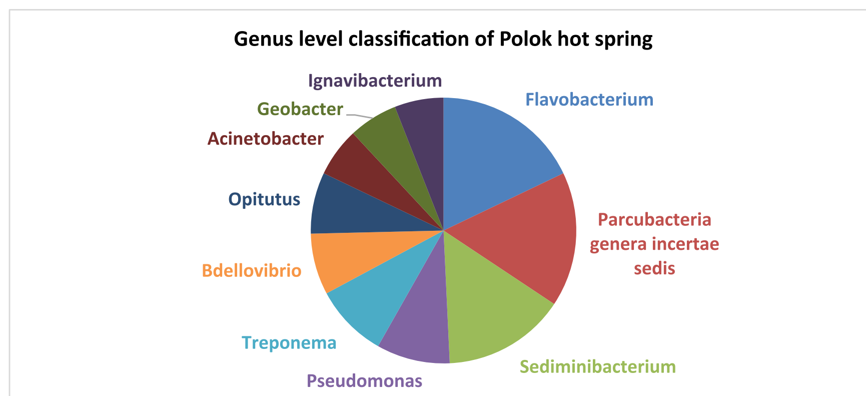

Fig. 17. Genus level classification of Polok hot spring.

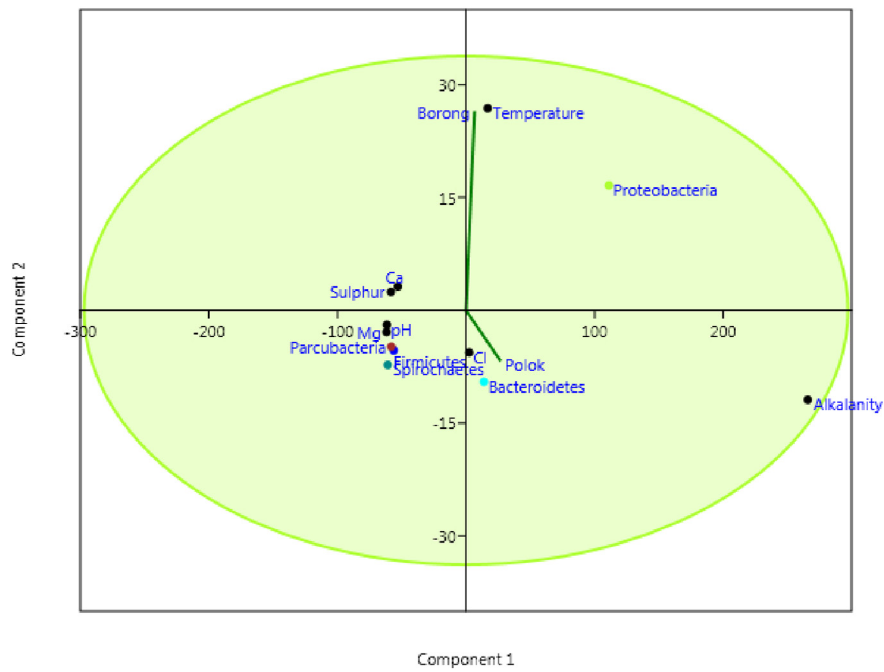

Fig. 18. Principle component analysis (PCA), to analyze the correlation between bacterial diversity at phylum level and geochemistry of the Polok and Borong hot springs.

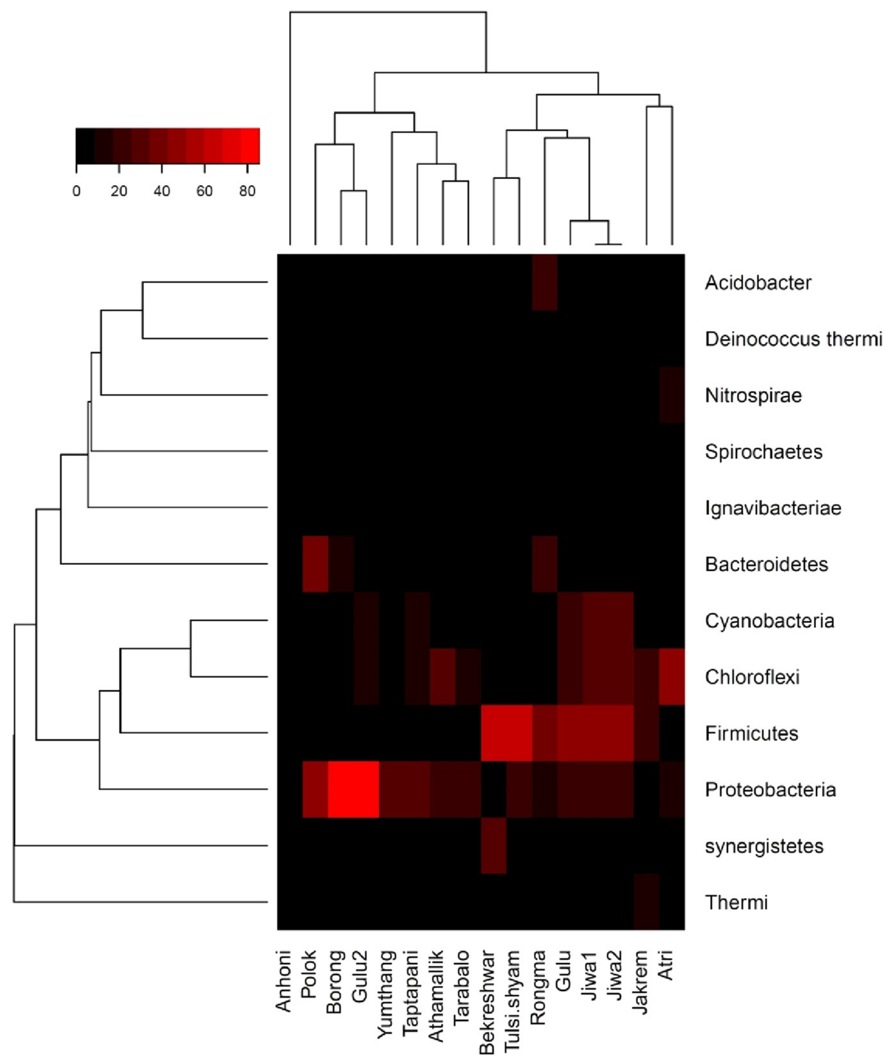

Fig. 19. Heat map (Comparative analysis of top phylums among various hot springs of central India, North-east India and Tibet).

and bacterial diversity from both the hot springs showed chlorine, alkalinity was positively correlated with *Bacteroidetes* and temperature is closely correlated with the abundance of *Proteobacteria*. These results are supported by earlier studies (Rzonca and Schulze-Makuch, 2003; Panda et al., 2016). They have found the significant correlation between community composition and various physicochemical parameters such as temperature, dissolved SiO_2, elemental sulfur, total sulfide, and calcium.

5. Conclusion

Metagenomic analysis revealed a wide and diverse bacterial population in both the Polok and Borong hot springs of Sikkim. These hot springs of Indo-Tibetan plateau are home to many possibly unknown and novel microbes as indicated by the abundance of 16.35% and 15.28% uncultured bacteria in Borong and Polok respectively. However, the most abundant phyla present were *Proteobacteria* and *Bacteroidetes*. The PLFA analysis showed the abundance of Gram-positive bacteria and eukaryotes in both the hot springs. Also the culture dependent 16S rRNA analysis showed the abundance of genus *Geobacillus* followed by *Anoxybacillus*. The correlation of physicochemical characteristics with most dominant phyla suggested that various physicochemical parameters such as temperature, pH, alkalinity, Ca^{+2}, Mg^{+2}, Cl^{+2}, and sulfur content shapes the microbial community composition and diversity. The piper diagram suggested that the water of both the hot springs are $Ca\text{-}HCO^{3-}$ type and can be predicted as shallow fresh ground waters. To the best of our knowledge this is the first study which revealed the microbial diversity of Polok and Borong hot springs of Sikkim. The results of this study significantly expand the understanding of the microbial community structure of Polok and Borong hot springs and provided a basis for comparative analysis with other geothermal systems.

Conflict of interest

Authors have no conflict of Interests.

Author's contribution

NT designed the study, INN did the experimental works, MTS and SKD helped in the sample collection and field study, INN and SD did the analysis and prepared the manuscript, NT reviewed and edited the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.05.037.

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SUMMARY

The microbial communities residing in extreme environmental niches are very important in terms of industrial and biotechnological perspectives. Among all the microbes living in such extreme conditions, thermophiles (temperature loving microbes) got widespread devotion from various researchers in recent decades. The prime advantage is the thermostable nature of these microbes and their enzymes thus this characteristic made them the choice of various industrial and biotechnological scientists (Coker 2016).. The dwelling places of these thermophiles are scattered all over the world in the form of Hot Springs, hydrothermal vents, thermal composts, marine trenches etc. (Anderson, Sogin and Baross 2014; Brumm, Land and Mead 2016; Mohammad *et al.* 2017). Among other Asian countries, India is also blessed with a lot of Hot Springs. There are almost 400 Hot springs in India (Singh *et al.* 2016) and most of them are pristine. Sikkim, one of the biodiverse states of India holds many Hot Springs in its vicinity and most of them are immaculate with respect to the microbial diversity. Various neo methodologies and approaches such as next generation sequencing (NGS) technologies have been developed to study such a microbial world. Thus there is an immediate need to explore the microbial diversity of these Hot Springs, which may lead to the understanding and accomplishing novel microbes and their enzymes for industrial and biotechnological benefits. Thus the present study was aimed to investigate the microbial diversity of these Hot Springs by culture dependent and culture independent techniques such as next generation sequencing (NGS) and PLFA approaches.

The first aim was to check the physicochemical properties of four hot springs such as Yumthang, Polok, Borong and Reshi hot springs. It was found that besides being distantly located all the four hot springs possess similar pattern of elements. The concentration of various elements was also

similar, however, Reshi hot spring was rich in several elements such as chloride, calcium and magnesium. The Reshi hot spring was followed by Polok hot spring in elemental rich ness. The sulfur concentration was found to be higher in case of Yumthang. The temperature of Polok hot spring was found to be highest followed by Borong hot spring. The pH analysis shows that all the hot springs are alkaline in nature, however, Borong hot spring is slightly acidic in nature. Statistically piper analysis shows that the deep ancient ground water nature in case of Yumthang and Reshi whereas shallow fresh ground water in case of Polok and Borong Hot spring.

The second aim was the isolation and characterization of thermophilic bacteria from these four hot springs. 218 isolates were isolated and on the basis of morphological and biochemical characterization only 152 isolates were taken to further analysis. All the isolates were Gram positive, aerobic, rod-shaped and most of them were able to form endospores. Carbohydrate fermentation analysis shows that the isolates were able to utilize simple sugars such as dextrose, maltose, ribose, fructose and mannitol, however, they were unable to utilize cellobiose, dulcitol, melezitose etc. Our results have found 47 isolates as amylase enzyme producers and 7 protease enzyme produces. Based on Morphological, Biochemical characterization and carbohydrate fermentation it can be predicted that the isolates might belong to group *Bacillus.*

The third aim was to identify the bacteria on the basis of 16S rRNA sequencing. The dominance of phylum *Firmicutes* was observed. Major genus found in the study was *Geobacillus* with a few representatives of genus *Anoxybacillus* and *Bacillus.* Also, some uncultured bacteria were reported such as Uncultured 17R2 and Uncultured TRR2. The alignment and similarity search of 16S rRNA sequence with nr/nt database of NCBI have shown that many of the isolates have a distinct percentage of identity from <95%. These results suggested the novelty of these isolated bacteria. The identification of few bacterial isolates was also done by FAME (Fattyacid methyl ester)

analysis. The three isolates AYN2, LYN3 and CTRL6 were identified. The fatty acids present in them were distinct and with variable concentrations. These results represent the unique nature of these isolates which has been also confirmed by their SI (Sim Index) values. By analyzing the results using RTSBA6 Sherlock libraries, In case of strain AYN2 no matches were found with (SimIndex-0.00). The strain LYN3 was showing similarity with *Geobacillus stearothermophilus* possessing Sim Index of 0.201. And lastly the strain CTRL6 was showing similarity with *Paenibacillus macerans* having Sim Index of (SI-0.385). The small diversity found in culturable techniques led us to extend the work and to check the bacterial diversity by culture independent techniques such as Next Generation Sequencing (NGS) and Phospholipid Fatty Acid (PLFA) analysis.

The PLFA analysis shows the abundance of Gram positive bacteria followed by Gram negative, which was thus correlating with the culture dependent diversity analysis. PUFA and branched chain fatty acids were abundant fatty acids in all the hot springs. However, apart from these major fatty acids Yumthang hot spring was possessing straight chain fatty acids also which were absent in other hot springs. Total biomass was found to be highest in Reshi followed by Borong and Reshi. Metagenomic analysis shows the prevalence of Phylum *Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria* with major species as Uncultured, *Ignavibacterium album, Sedimentibacterium, Rhodococcus ruber, Bacillus pumilus, Thiobacillus, Geobacter, Thermoanaerobacter, Thermus sp., Flavobacterium aquaticum* and so on.

A novel bacterium was claimed after polyphasic characterization such as Biochemical Identification, Biolog, FAME analysis, Whole Genome Analysis etc, and these results have found that there is a considerable distinction between our isolate AYN2 and to that of other closely related Geobacillus species thus can be confirmed as a novel species. The strain was named as

Geobacillus Yumthangensis AYN2 based on the hot spring where from the strain was isolated. The Gram-positive, endospore forming aerobic rods, with 2.5-5 µm long and 0.4-0.6 µm wide. The cells are able to utilize cellobiose, lactose, ribose, sorbitol and glucose. The strain can grow at a wide range of temperature (40-70ºC) and pH of 6-10.

The last objective was to check the antibiotic susceptibility patterns and any possible mechanisms. We checked the antibiotic resistance profile of 10 antibiotics such as Penicillin, Methicillin, Amoxicillin, Ampicillin, Erythromycin, Chloramphenicol, Gentamycin, Clindamycin, Norfloxacin, and Ciprofloxacin and interestingly found that all the isolates were susceptible against all the antibiotics used. The search was extended and minimum inhibitory concentrations was also checked. The results showed that very less minimum inhibitory concentration values for almost all the antibiotics checked. In case of Gentamycin, Vancomycin, Erythromycin, and Chloramphenicol the MIC values were generally around 0.5 µg/ml, 2 µg/ml, 2 µg/ml, and 8 µg/ml respectively. The least MIC of 0.25 µg/ml was shown in case of Penicillin G and Oxacillin. The MIC was defined as the lowest concentration producing no visible growth. No internationally acknowledged criteria for susceptibility testing or for breakpoints for susceptible or resistant isolates are accessible for thermophilic bacteria. However, the two thermophilic Campylobacter species were investigated and their breakpoints has been established (Guévremont et al, 2005). Briefly, the breakpoint values of the MIC for resistance are given in results section and thus as per Guévremont et al, 2005, the MIC values of various antibiotics against our isolates is thus very less.

Various universal resistance beta-lactam genes such as pbpA, ampC and mecA were targeted using specific primers by PCR technique. The results showed the absence of any resistant genes in our isolates. These results were also supported by whole genome sequencing of two isolates AYN2 and LYN3. The whole genome sequencing also shows the absence of any resistance genes. The

genes if any present were putative with other functions also. The search for ARGs were also carried out by metagenomic analysis. It was found that the resistance genes detected in metagenomic analysis were showing >95% similarity with mesophilic bacterial genes. Thus this also suggests the absence of resistance genes in thermophilic bacteria.

Heavy metal tolerance was also checked against five heavy metals and it was shown that our isolates were resistant than that of the other *Geobacillus* species and *E Coli*. Whole genome sequencing also supported these results by showing the presence of heavy metal resistance genes. Metagenomic analysis also showed the presence of heavy metal resistance genes, however, their percentage similarity was higher with that of mesophilic bacterial genes. Hence, here the results support the assumption that the antibiotic resistance and heavy metal resistance detected through metagenomics may be due to the contamination of soil micro flora surrounding the top soil layers of hot springs. As, the MRGs were found in culturable thermophilic bacteria in contrast to their antibiotic counterparts in this study, so it might be concluded that there may be no co-occurrence or co-selection of these genes in such isolated habitats. However, this is a broad assumption which needs to get counter check in other such habitats.

Department of Human Physiology with Community Health
&
Department of Microbiology

VIDYSAGAR UNIVERSITY

Midnapore, West Bengal

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in three day National Level Seminar on 'Perspectives of Human Health, Microbial Biotechnology & Innovation' from 27 - 29 March, 2018.

Prof. Ranjan Chakrabarti	Prof. Chandradipa Ghosh	Dr. Sandip kr. Sinha	Dr. Keshab C. Mondal
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Organizing Secretary, PHMBI-18