MICROBIAL ECOLOGY, DIVERSITY, AND BIOCHEMICAL POTENTIAL WITHIN BASALTIC INDIAN HOT SPRING

Thesis submitted to AcSIR for the award of the degree of

DOCTOR OF PHILOSOPHY

In Biological Sciences

BY

GAJANAN T. MEHETRE

(10BB13J26033)

Dr. MAHESH S. DHARNE
(Research Supervisor)

Dr. SYED G. DASTAGER
(Research Co-supervisor)

NCIM RESOURCE CENTRE

DIVISION OF BIOCHEMICAL SCIENCES

CSIR-NATIONAL CHEMICAL LABORATORY

PUNE 411008, Maharashtra, INDIA

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Dedicated to

My beloved parents

सीएसआईआर - राष्ट्रीय रासायनिक प्रयोगशाला
(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद)
डॉ. होमी भाभा मार्ग, पुणे - 411 008, भारत
CSIR - NATIONAL CHEMICAL LABORATORY
(Council of Scientific & Industrial Research)
Dr. Homi Bhabha Road, Pune - 411 008, India

CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis entitled, **"MICROBIAL ECOLOGY, DIVERSITY, AND BIOCHEMICAL POTENTIAL WITHIN BASALTIC INDIAN HOT SPRING"** submitted by Mr. **Gajanan T. Mehetre** to the Academy of Scientific and Innovative Research (AcSIR), New Delhi, India, is in fulfilment of the requirements for the award of the **Doctor of Philosophy** and embodies original research work under my supervision and guidance. I further certify that this work has not been submitted to any other University or institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table, used in the thesis from other sources, have been cited and acknowledged.

(Student)	(Supervisor)	(Co-supervisor)
Gajanan T. Mehetre	Dr. Mahesh S. Dharne	Dr. Syed G. Dastager
(10BB13J 26033)		

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DECLARATION

I hereby declare that the work of the thesis entitled, **"MICROBIAL ECOLOGY, DIVERSITY, AND BIOCHEMICAL POTENTIAL WITHIN BASALTIC INDIAN HOT SPRING"**, submitted in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy (Biological Sciences) to AcSIR, has been carried out by me at NCIM resource centre, CSIR-National Chemical Laboratory, Pune 411008 (MS, India), under the supervision of Dr. Mahesh S. Dharne and Dr. Syed G. Dastager. This is the original work and no part of this thesis work has been submitted for any other degree or diploma to any other University or institutions. The assistance and help received during the course of this investigation have been fully acknowledged.

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Gajanan T. Mehetre

(Research scholar)

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LIST OF ABBREVIATIONS

ANI: Average Nucleotide Identity

APHA: American Public Health Association

ARDRA: Amplified Ribosomal DNA Restriction Analysis

AST: Antimicrobial susceptibility testing

ATR: Attenuated total reflection

BHA: Bushnell Haas Agar

BLAST: Basic Local Alignment Search Tool

bp: Base pairs

BRIG: BLAST Ring Image Generator

BS: Biosurfactants

CDS: protein coding sequence

CLSI: Clinical and Laboratory Standard Institute

CTAB: Cetyl Trimethyl Ammonium Bromide

DGGE: Denaturing gradient gel electrophoresis

DNA: Deoxyribonucleic acid

dNTP: Deoxy ribonucleotide triphosphate

DPC: 1, 5- diphenylcarbazide

EI: Emulsification index

FISH: Fluorescent in situ hybridization

FLASH: Fast Length Adjustment of SHort reads

FTIR: Fourier-transform infrared spectroscopy

GC-MS: Gas chromatography-Mass Spectrometry

GO: Gene Ontology

h: Hour

Hcl: Hydrochloric acid

HRMS: High Resolution Mass Spectrometry

Kb: Kilobase(s)

KD: Kilo Dalton

KEGG: Kyoto encyclopedia of genes and genomes

KO: KEGG Orthology

LB: Luria Bertani

LCMS: Liquid chromatography mass spectrometry

LP: Lipopeptides

M: Molar

MEGA: Molecular Evolutionary Genetic Analysis (MEGA)

MEGAN: MEtaGenome ANalyzer

MG-RAST: metagenomics Rapid Annotation using Subsystem Technology

min: Minutes

mM: Mili mol

MSM; Mineral salt medium

MW; Molecular weight

NCBI: National Center for Biotechnology Information

ng: Nano gram

NGS: next-generation sequencing

OD: Optical density

ORF: Open reading frame

OTUs: Operational taxonomic units (OTUs)

PAHs: Polycyclic aromatic hydrocarbons

PATRIC: Pathosystems Resource Integration Center

PCR: Polymerase chain reaction

PERL: Practical Extraction and Reporting Language

pM: Pico Mole

PyNAST: Python Nearest Alignment Space Termination

QIIME: Qualitative Insight Into Microbial Ecology

RAST: Rapid Annotation using Subsystem Technology

RISA: Ribosomal RNA (rRNA) intergenic spacer analysis

RNA: Ribonucleic acid

Rnase: Ribonuclease A

Rpm: Rotation per minute

rRNA: Ribosomal ribonucleic acid

RT: Retention time

S: Svedberg unit

sec: second

SEM: Scanning Electron Microscopy

sp: Species

SRA: Sequence Read Archive

SSCP: Single-strand conformation polymorphism

ST: Surface tension

Taq: Thermus aquaticus (polymerase)

TE; Tris-HCl EDTA

TLC: Thin layer chromatography

T-RFLP:Terminal restriction fragment length polymorphism

UHPLC: Ultra high-performance liquid chromatography (UHPLC)

UPGMA: Unweighted Pair Group Method with Arithmetic Mean

USA: United States of America

UV: Ultra violet

v/v: Volume per volume

w/v: Weight per volume

WIMP: What's in my pot

µg/L: Micrograms per liter

µg/mL: Micrograms per milliliter

µg/µL: Micrograms per microliter

µg: Micro gram

µL: Microliter

µM: Micromole

%: Per cent

°C: Degree Celsius

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Abstract

This thesis is focused on the microbiological studies of the Indian hot spring, which was previously unexplored. Unkeshwar hot spring situated in a central region of India was found to largely underexplore in terms of microbiological analysis. We performed comprehensive microbiological studies over this hot spring for understanding the microbial communities and impacts of several environmental factors, seasonal variations on its composition and functions and on the intrinsic properties of hot springs. In the comprehensive analysis of microbial community composition and functions performed using high-throughput sequencing revealed that Unkeshwar hot spring is predominated by *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Deinococcus-Thermus*, *Actinobacteria*, *Cyanobacteria* and few of the candidate division phyla. A comparative analysis of microbial communities between Unkeshwar hot spring and its associated sites such as a borehole, bath tank etc has been carried out. It showed that the environmental conditions and anthropogenic activity have a greater impact on intrinsic properties of hot springs their microbial assemblages and function of the microbial communities. Further, a comparative account among few of the selected hot springs of India including Unkeshwar based on in silico analysis showed that the microbiota associated with each of the hot spring is distinct and majorly found to be influenced by temperature and other geochemical conditions of that hot spring. Hot springs microbial communities are important in term of biotechnological applications. Therefore, we also performed few functional analysis studies of microbial communities of this hot spring. The result revealed that the hot springs members of the microbial community might be having some functions in biogeochemical cycling of carbon, nitrogen, and sulphur. In addition, they also have high metabolic capacities for degradation of various xenobiotic compounds to be harnessed for various biotechnological and environmental applications.

The second aspect of the study was to analyze microbial composition using several cultivation methods. This is important, as microbes associated with hot spring often regarded as valuable resources for various applications due to their unique macromolecular properties. However, due to limitations of cultivation methods, a majority of such microorganisms remain uncultivated and unexplored. Here, we have done an attempt to isolates bacteria which were found to be difficult to culture and requires special culture conditions. The results revealed that this hot spring has microbial diversity which could be cultured using specialized cultivation techniques. Here, numbers of bacterial strains were isolated, which found to belong to 54 different bacterial genera. Among these genera, few were such as *Ramlibacter*, *Phretobacter*, *Methyloversatilis*, *Rhodobacter*, *Caldimonas*, *Methaloterigenea*, *Actinomodura*, *Barrentisimonas*, and *Dietzia* found to be rare bacterial species, isolated for the first time form hot spring environments. Most of the isolated bacteria were also reflected in the metagenome studies previously done over the few other hot springs, indicating that, isolation of them requires the developments of specialized culture techniques. Furthermore, the imputed functional analysis (in-silico) was also performed to gain insights into the metabolic potential of the isolated strains. The results obtained were found to be almost similar as revealed in the metagenome based analyses, which preferably indicated that these are the most important function of these microbial communities. Based on the metabolic functional analyses, we have performed bioprospecting for the important enzymes productions, their heavy metal tolerance properties. These studies have revealed that most of the isolated strains were found to producing some of the industrial importance enzymes, whereas other strain had shown higher tolerance to few of the heavy metals. Furthermore, the heavy metal tolerance associated with few of the selected strain was co-investigated along with the antibiotic resistance. This study was performed based on the few of noticeable observation carried regarding the anthropogenic influences over this hot springs. We found

that a coexistence of heavy metal and antibiotic resistance among few strains such as *Achromobacter*, *Ferrovibrio*, *Pseudomonas*, *Staphylococcus*, *Chronobacter*, *Hydrogenophaga* and *Micrococcus* was found. This could be because of an indirect selection process that occurs through the coupled physiologically (cross-resistance) and genetically (co-resistance). Such observations show that hot springs environments influenced by anthropogenic pressure need more attention in this research area.

In the third part of the study, a functional aspect of the hot spring microbes was studied. In the imputed functional analyses we found that most of the metabolic pathways observed in the microbial community were associated with the xenobiotic compounds, particularly for the degradation of aromatic hydrocarbons such as Polycyclic aromatic hydrocarbons (PAHs) are a group of organic priority pollutants. In general, bioremediation processes are influenced by various environmental conditions, such as temperature, nutrients and the nature of the toxic compounds. Therefore, implementations of the naturally occurring microorganisms, which are well adapted to stressful conditions, may provide a solution to problems. Therefore, here we have used few of the thermo-tolerant microorganisms isolated under PAH enriched conditions from this hot spring for biodegradation of PAH compounds. Based on various isolation and screening techniques, a few of the selected strains belonging to Bacillus group were used for degradation of PAHs. Various strategies were also designed to find out the influence of different factors such as mixed compounds, degradation in crude oil, temperatures and nature and number of degrading strains, total microbial community and amendment with biochar to maximize the degradation rates. The results revealed that degradation depends on the nature and chemical type of compounds. Use of mixed culture at higher temperature found more effective for degradation of low molecular weight (LMW) PAH compounds. In contrast, amendment with biochar and degradation by the whole community; enhanced degradation of High molecular weight (HMW) was observed.

Moreover, PAHs degrading strains were also found to producing biosurfactants, which may also be involved in the enhancing the degradation of PAH compounds.

Overall, this study has shown an improved census of microbial community structure and functions of Unkeshwar hot spring. The knowledge of which could provide a foundation for future studies for exploration of microbial diversities from this as well as other hot springs present in India. The study also potentially highlighted the uses and advantages of cultivation-based methods for comprehensive explorations of untapped and unique bacterial diversities and their utilities for various biotechnological and environmental applications. Lastly, it also inferred from the study that, hot springs microbes could have the metabolic properties for the degradation of toxic aromatic hydrocarbon compounds. A systematic development of bioremediation strategy using these microbes, therefore, could provide a solution to the PAHs and crude oil contaminated sites.

In summary, Unkeshwar hot springs microbial communities host important microbes for the potential use in various biotechnological as well as environmental applications, which therefore necessities more studies on this hot springs for various bioprospecting activities. A more important observation was also made about the influence of human activities on these hot springs. Unkeshwar hot spring and most of the other hot springs present in India were found to be surrounded by temples, which therefore attract more devotees to take bath in the belief to cure them of major skin ailment and perform spiritual activities, which therefore potentially had an impact on the natural microbial communities of the hot springs. Therefore, the study also suggests the protection of these natural ecosystems using the long-term environmental impact assessments.

CHAPTER 1

Introduction and Review of literature

1.1: Introduction

Microbial diversity is a unique asset of a planet. Microorganisms evolved in such a way that they can adapt to every possible habitat like heat, cold, salt, acidity, anoxic niches present on the Earth, are termed as "extremophiles". However, progress in achieving the understanding of microbes and their environments was hampered by the general necessity to culture them in the laboratory (Pace et al. 2009). Over the last few decades, improvements in molecular biology techniques and bioinformatics have permitted an unprecedented access to the uncultured microbial fraction of any given environment (Escalante et al. 2014). These methods are generally referred as "culture-independent", as they do not require growing cultures under the laboratory conditions. The ongoing surveys of microbial diversity within different habitats have enabled us to understand complexities that exist in various ecosystems ranging from simple to complex groups of microorganisms. Many novel extremophilic microbial phyla have been isolated, identified, cataloged and further exploited for various applications. Among such extremophiles, thermophiles are known to thrive and dominate the high-temperature environments (55°C - 80°C) are of considerable importance due to their unique macromolecular properties. Hot springs (also known as geothermal springs) represent modern analog of the primitive ancient environment and are the most favorable habitat of thermophiles. Thermophilic microorganisms are getting much recognition in the field of microbial technology because of production of the broad range of metabolites, enzymes, which exhibits unique properties for several biological applications such as antibacterial, antiviral or antitumor (Shivlata and Tulasi 2015). Thermophiles are also used as model organisms for studying extraterrestrial life and evolution of life on earth and for understanding the microbial cell organization (Hall et al. 2008).

Hot springs microbiology is an important aspect of extremophilic research for understanding the ecosystem functioning. Microorganisms present in these systems are

involved in diverse metabolic activities, biogeochemical and nutrient cycling. Therefore, studies of thermophiles also help in understanding the physiological adaptations and survival mechanisms used for adaptation to the high-temperature conditions. The fundamental knowledge about hot spring ecosystem functioning, adaptive survival strategies of thermophiles will help for better understanding of some underlying questions about the origin of life and evolutionary biology. Furthermore, a stability of macromolecules of thermophiles is an important aspect of the biotechnology industry. The enzymes from thermophilic microorganisms (famously known as "thermozymes") are thermo-stable and therefore are extremely valuable as biocatalysts for industrial processes, which are generally performed at the high temperatures.

Microbial communities of the hot springs are majorly featured by the physiochemical, geological and biological factors. Different hot springs have different geochemistry due to the geographic locations; therefore characterizing microbial communities of hot springs is of considerable interest for any microbiologist. Moreover, microbial ecological functions found to respond differently to the geochemical conditions of hot springs. A study of geographically closer hot springs with similar temperatures and a comparable pH, found to have different microbial compositions (Blank et al. 2002). Apparently, understanding the microbial ecology and exploration of thermophiles of hot springs located at different geographic locations is crucial for getting a more comprehensive view of microbial populations and their underlying functions. With the advancements in DNA sequencing tools, investigations into the hot springs microbial diversities are gaining more attention. Various studies were found to focuses on the understanding of the fundamental role of hot spring microbial communities in biogeochemical and nutrient cycling of the matter (Inskeep et al. 2010). In addition, thermophlies were found to be centre of attention for various biotechnological applications studies.

1.2: Review of literature

1.2.1: Terrestrial hot spring ecosystems

Hot springs are formed by the emergence of hot water from the earth's crust due to disturbance or imbalance created by tectonic movement or volcanic eruption. When groundwater percolates deeply into the crust and gets heated by the heat of hot rocks or from the magma. Most of the high-temperature hot springs were found to present in the volcanic geothermal system. In the active volcanic zones such as Yellowstone National Park (YNP), water is heated by the magma (Boyd et al. 1961). In the non-volcanic regions, hot springs are formed due to the heat of the geothermal gradient.The important factor of any hot springs is its temperature, which is constant and found to be stable over the time. Numbers of hot springs are distributed throughout the world with low to high temperatures. Different types of hot springs are present with a starting temperature of 20°C to the more than 100°C, depending on the location of a hot spring in the geothermal area (Madigan et al. 2003). Some of the globally well known hot springs with their temperature ranges are shown in table 1.1

Table 1.1: Well known global level hot springs

Sr. No	Name of the hot spring	Temperature	Reference
1	Yellowstone National Park (USA)	75 - 93°C	Hugenholtz et al. 1998
2	Kamchatka (Russia)	68 - 87°C	Kublanov et al. 2009
3	Kamojang (Indonesia)	90 - 92°C	Aditiawati et al. 2009
4	Tunisian (Tunisia)	43 - 72°C	Sayeh et al. 2010
6	Hveragerdi (Iceland)	50 - 75°C	Sonne-Hansen et al. 1999
7	North Iceland (New Zealand)	45 - 55°C	Wahlund et al. 1991
8	Nakabusa (Japan)	72 - 80°C	Nakagawa et al. 2003
9	Tengchong (China)	50 - 75°C	Pagaling et al. 2012

1.2.1.1: Physico-chemical aspects of hot springs

Hot springs have a wide range of geochemical characters, creating extreme environment analogous to the ancient environment in which life may have originated (Cavicchioli et al. 2002). Temperature is one of the most important factors of the hot springs, which control the composition and diversity of the microbial populations. Therefore, understanding the influence of temperature variation on the structure and function of microbial communities and interaction among different microbial communities is an important aspect in microbiological studies. Along with temperature, some other parameters such as acidity, oxidation/reduction, solute concentrations, gas compositions, mineralogy, and nutrient are also the unique characteristics of the hot springs (Whitaker et al. 2003; Keller et al. 2004). Various types of geochemical factors were also known to have a great impact on the microbial community and assemblages of the hot springs. In addition, local environmental heterogeneity, anthropogenic disturbances may also be responsible for the assemblages and distribution of microbial flora of these systems. Several other climatic factors such as light intensity, oxygen, and sulfide concentration also affect the distribution of several primary producers of hot springs ecosystems (van der Meer et al. 2005).

1.2.2.2: Microbiological aspects of hot springs

Due to presence of wide range of geochemical factors, hot springs are most favourable geothermal environments hosting diverse range of thermophilic microorganisms (Huber and Stetter 1998; Lau et al. 2009). Earlier studies from these environments were majorly hampered due to the limitations associated with microbial cultivation. The discovery of the isolation of the remarkable thermophilic microorganisms from the boiling springs of Yellowstone National Park had created an interest in the explorations of thermophlies (Brock TD 1997). Since then, thermopiles were isolated from the different types of geothermal springs of the world. Many novel microbial species with important applications in

biotechnology, medicine, and bioremediation were identified (Bergquist et al. 1987; Cardoso et al. 2011).

Microbes inhabiting hot springs are majorly featured by physicochemical characteristics such as pH, redox potential, temperature and concentration of trace elements (Siering et al. 2006; Mathur et al. 2007). Among these factors, water temperature controls microbial distribution within hot springs. Cole et al. (2013) showed an inverse relationship between hot spring temperatures and the degree of microbial diversity. Hot springs microorganisms thrive under multiple environmental stresses and to survive under such stresses, microbial communities use mutualistic or commensalistic symbiotic relationships (Chan et al. 2015). Along with physicochemical characteristics of the hot spring water, other factor, such as dissolved gases, minerals, and trace elements also influences the survival of these microbes in such conditions. The physical, chemical and biological characteristics of every hot springs have been shown to affect the structures of its microbial community. In this view, studies focusing on microbiological investigations of the hot springs have gained much interest of scientific communities (Sharma et al. 2015).

1.2.2: Approaches and methods for microbial ecology studies

1.2.2.1: Traditional approaches: Culture dependent methods

During 19th and early 20th century revival of microbiology takes place from the studies of Pasteur's and Koch's work, which is often reffered as the golden age of microbiology (Blevins et al. 2010). This has shifted research philosophy from ecosystem-oriented view to the identification, characterization of individual microorganism and their etiological agents responsible for some microbial phenomena (Youssef et al. 2014). However, the work of Sergei Winogradsky (1890) in the field of microbial ecology enable microbiologist to reconceive microbes in their natural habitats. Behaviour of a microorganism in the natural environment and in an isolated form varies greatly, due to the differences in nutrient and

resource availability and the interactions with the co-existing microbial taxa of the habitats (Dworkin 2012). Further research by various eminent microbiologists such as Beijerinck, van Neal, and Kluyver led to foundation of microbial ecology as an independent field in the 1960s. As a result, culturing microorganism using different enrichment techniques become the first route to study the uncultivable microorganisms (Strohl et al. 1978). However, due to the temperature issues associated with the hot springs microbes, culturing them was more prompting. Earliar studies on thermophiles were performed over the Yellowstone National Park (YNP) hot springs using cultivations (Davis 1897). However, the isolation of hyperthermophilic organisms from hot springs has initiated numbers of studies in the hot spring microbiology (Brock 1978). Since then various culturing techniques were developed for the isolation of thermophiles and hyperthermophiles form different hot springs of the world.

1.2.2.2: Molecular approaches: Culture independent methods

Introduction of molecular phylogeny has provided a new way for the identification and characterization of microorganisms in their natural habitats (Pace et al. 1986). This approach is based on the comparison of a small-subunit (16S rRNA) sequence, which has revolutionized the world of microbial ecology. A numbers of studies were initiated to identify a specific microbial population in the natural environments based on the molecular phylogeny (Muyzer et al. 1995; Amann et al. 1995). As a result, information of the evolutionary relatedness of microorganisms was used to construct phylogenetic trees, which became a gold standard for microbial taxonomy studies (Pace et al. 2009). Initial studies of microbial taxonomy and characterization of microbial assemblages were performed over the deep-sea hydrothermal vents and copper leaching pond (Amann et al. 1995). However, in these studies, the 5S rRNA was used, which is relatively small and not suitable for fine characterization of microbial community. For better characterizations, use of 16S rRNA was

preferred as it contains sufficient sequence information of hyper-variable regions for phylogenetic analysis. The major advantage of using this molecule is that this gene lack artifact of lateral transfer between contemporaneous organisms and thus a relationship between rRNA reflect the evolutionary relationships of the organisms (Olsen et al. 1986). The hypervariable regions present in this gene provide a species-specific signature sequence that is useful for the bacterial identification process. The 16S rRNA is a highly conserved gene, its secondary structure is partitioned into four well-defined domains; 5′ terminal, central, 3′ major and 3 ′ minor domain (Olsen et. al.1986). The 5′ domain forms the bulk part of the structure and consists of 19 double helices. The central domain forms the platform. The 3′ major domain forms the head part and consists of 15 helical elements and the 3′ minor domain projects from subunit to interact with the 50S subunit and consist of 2 helical elements (Fig. 1.1).

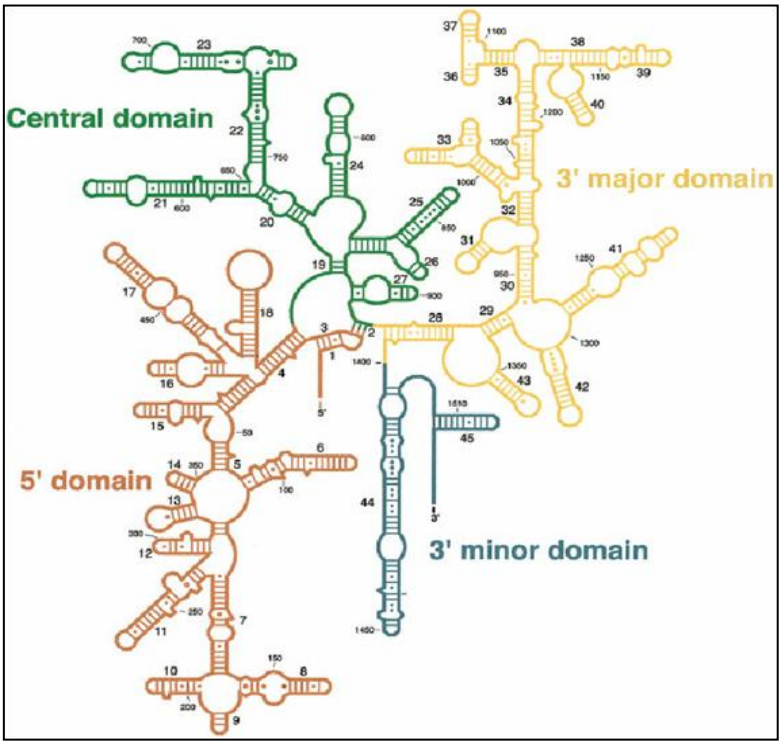

Fig. 1.1: Secondary structure 16S rRNA gene along with the four well defined domains (Source: Yang et al. 2016).

The Phylogenetic analysis using 16S rRNA gene has become the basis for the taxonomic classification of microbial diversities and identification of bacterial species (Stephen et al. 2008). Using the sequences of 16S rRNA, microbial species of communities were classified in terms of operational taxonomic units (OTUs), which forms discrete categories of microorganisms based on phylogeny. The OTUs are often used to quantify the total diversity of an ecosystem (Schloss et al. 2011). Based on such phylogenetic approaches, many new insights into the composition of uncultivated microbial populations as well as their functions were gained. These methods have also allowed the characterization of many rare and poorly understood microorganisms from various environments (Head et al. 1998). Although, the use of 16S rRNA is widely accepted for taxonomic studies of microbial communities associated with different environments. However, this approach only gives the taxonomic status of the microorganisms present in a microbial community, and does not provide information about the community functions. In general, in metagenomics, two different approaches (function based and sequence-based) are used for taxonomic as well as functional characterization of microbial communities (Lewin et al. 2013). In the first approach, the environmental DNA is directly cloned to form cloned libraries, which are then could be screened for a gene of interest as specified for a particular function, or production of metabolites (function-based screening). In the second approach, a gene of interest, or total community DNA could be amplified using PCR amplification, or it can be directly sequenced using a high throughput sequencing techniques. This latter approach is generally used for analyzing and profiling of microbial communities along with functions from various extreme environments (Fig. 1.2).

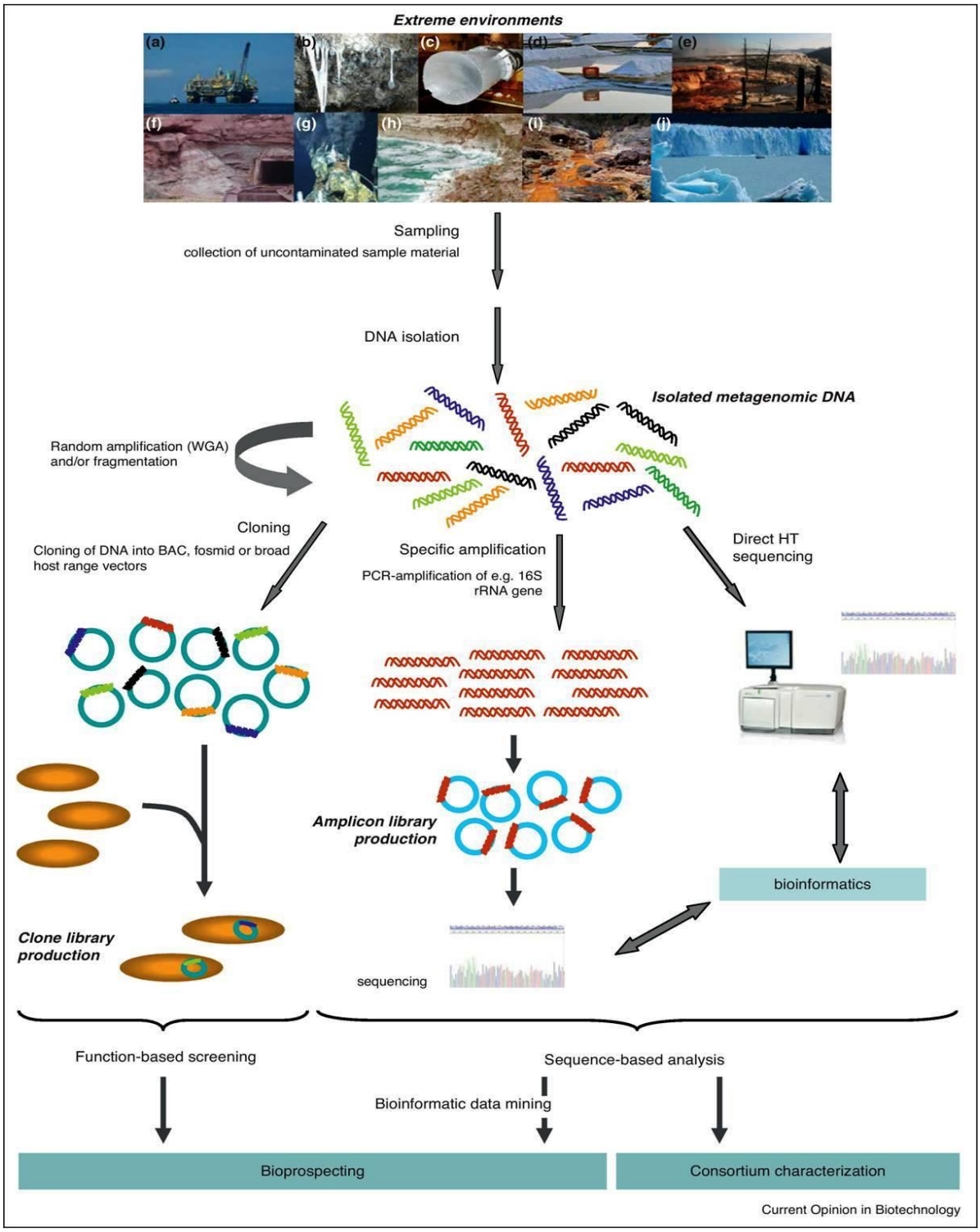

Fig. 1.2: Schematic overview of metagenomics approaches used for analysing both structural and functional aspects of the microbial communities of extreme environments (Source: Lewin et al. 2013).

1.2.2.3: Changing paradigm of microbial analysis

In the earlier period of 1970's and 80's, due to the limitations of microbial cultivations, the understanding of microbial communities as a whole has lagged behind. Culture-independent methods were the new tool, which could give more comprehensive descriptions of the microbial complexities that exists in natural extreme environments (Neelakanta and Sultana 2013). Traditional microbiological approaches have provided us the knowledge about the importance of microorganism, whereas, new approach of metagenomics expanding it further to understand the microbial capabilities as a whole in a community as well as an individual microorganism. However, in these metagenomics approaches, there is a changing trend of technologies depending on the advancement of sequencing technologies (Fig. 1.3).

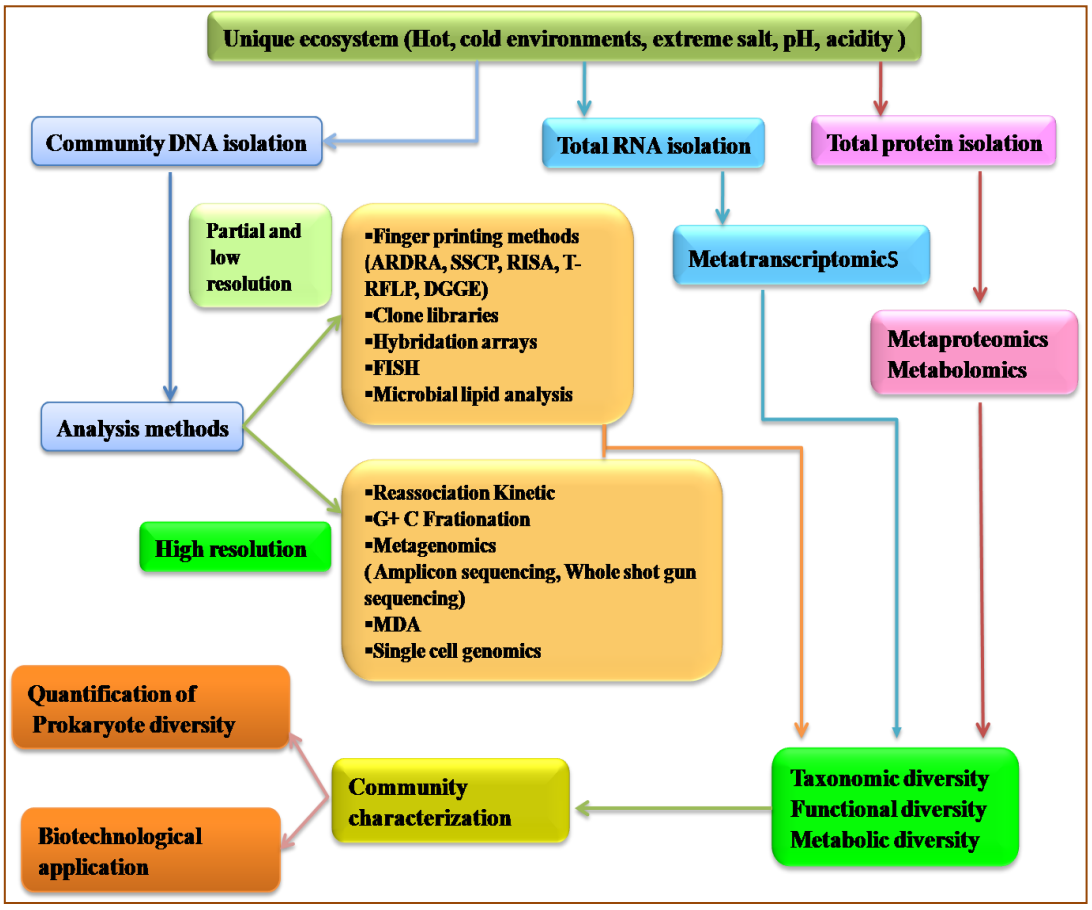

Fig. 1.3: Different levels of methods for microbial community characterizarion (Modified from Rastogi et al. 2011)

Various techniques were developed, among which the earlier techniques could give a low or partial resolution of microbial communities. Some of the deep scale analysis techniques allow assessment of the total genetic diversity of microbial communities (Torsvik et al. 1996). The fingerprinting based methods like ARDRA, SSCP, RISA, T-RFLP, and DGGE are based on PCR amplification of 16S rRNA gene and then separation of PCR amplified 16S rRNA based of use of a different parameter to obtain a specific pattern (Rastogi et al. 2011). In case of ARDRA and related methods, amplified 16S rRNA gene digested using tetra cutter restriction enzymes and the band pattern is different for the representative species (Smit et al. 1997). PCR-denaturing gradient gel electrophoresis (DGGE) analysis gives slightly higher resolution and could provide information about the changes in the whole community structure (Muyzer et al. 1993). In DGGE, separation of PCR-amplified gene segments is performed in an acrylamide gel with the increasing concentrations of denaturants. Thus, DNA separation occurs based on the denaturation properties of the double-stranded DNA, which is based on the differences in the composition of nucleotides in the DNA of each species. Such simultaneous separations result in the formation of a profile of bands, which gives community profile and additionally, these bands can be re-amplified for further sequencing analysis (Ward et al. 1998). Techniques such as clone libraries, Hybridization arrays, FISH, are the sequence and probe-based methods (Rogers et al. 2007). Although using such techniques an enormous microbial diversity form various environmental samples could be analyzed, these techniques have certain limitations. For example, 16S rRNA gene based methods were found unable to provide a high resolution at the species level (Konstantinidis et al. 2006). Secondly, there is a difficulty in assembling contigs into discrete genomes due to the computational limitation (Zhang et al. 2006). To overcome the limitations of analysis of cloned DNA library, next-generation sequencing techniques are being employed in the environmental metagenomics. These high throughput sequencing techniques have

transformed the field of microbial ecology by increasing the speed as well as the depth of the data they can generate (Zau et al. 2015). Several such platforms were developed and are widely being used; these include the Roche 454 GS FLX+, SOLiD, Ion Torrent/Ion Proton and Illumina (e.g., HiSeq, MiSeq) platforms (Loman et al. 2012; Liu et al. 2012). These are often called as next-generation sequencing (NGS) techniques and are widely used in the field of metagenomics, metatranscriptomics and in genomic analysis. Metagenomic studies are usually performed either by 16S rRNA sequencing (amplicon) or whole-genome shotgun (WGS) approaches. Among these, the 16S rRNA gene sequence-based analysis is more widely used and has driven a dramatic change in expansions of the diversity of microbial life of our planet (Shokralla et al. 2012). However, these methods have certain limitations. In such analyses, the information obtained is based on the DNA sequence present between the primers, therefore a primer mismatches may result in missing of some lineages (Zhau et al. 2010). The limitation associated with primers could be addressed and resolved by whole metagenome sequencing approach, which could provide a more view of microbial diversities and community compositions (Poretsky et al. 2014). Though several such high throughput methods are available, the differences in sequencing platforms, DNA preparation methods, and the complexities of the samples may possibly lead to different or biased observations (Quince et al. 2009; Poretsky et al. 2014). Recently, Oxford Nanopores has introduced the beta-testing sequencer, which is the first mobile DNA sequencer, and could be powered using a USB port (Eisenstein, 2012). Therefore, in the future, the advanced sequencing approaches should drive advances in the whole genome sequencing studies (Urbeta et al. 2015).

1.2.3: Microbial taxonomy and diversity

1.2.3.1: Culture dependent approaches

Till date only less than 1% of all prokaryotes could be cultured due to limitation of cultivation methods. Development of culture conditions or media that suits all the specific

growth requirements such as nutrients, pH, incubation temperatures and most important the requirement of microbial interactions is difficult (Vartoukian et al. 2010). Also, different organism has different growth patterns such as some have sporadic growth and some other have slow growth rates, which also makes difficulties in isolation of the individual organism (Madsen 2008). Classical cultivation techniques are therefore found to be inappropriate for understanding the microbial life under extreme environmental conditions. Nevertheless, from the past few decades, studies performed on hot springs lead to the isolation of many novel bacterial and archaeal species (Table 1.2), also few exploited for various biotechnological applications.

Table 1.2: Novel microbial species isolated from terrestrial hot springs

S. No	Hot spring	Novel species	Unique properties	Reference
1	Obsidian Pool YNP, USA	*Thermosphaera aggregans*	Hyperthermo philic	Huber et al. 1998
2	Costa Rica hot spring, USA	*Thermocrinis minervae*	Sulphur oxidation	Caldwell et al. 2010
3	Arkansas hot spring, USA	*Fontimonas thermophila*	-	Losey et al. 2013
4	Kamchatka, Russia	*Pyrobaculum ferrireducens*	Arsenate reduction	Slobodkina et al. 2015
		Brockia lithotrophica	Anaerobic	Perevalova et al. 2013
		Carboxydocella manganica	Mn (IV) reduction	Slobodkinaet al. 2012
		Fervidicoccus fontis	-	Perevalovaet al. 2010
		Crboxydocella thermautotrophica	CO-utilizing	Sokolova et al. 2002

5	Kunashir, Russia	*Thermogutta terrifontis*	Anaerobic respiration	Slobodkina et al. 2015
6	Xiamen, China	*Albidovulum xiamenense*	-	Yin et al. 2012
		Pleomorphobacterium xiamenense	-	Yin et al. 2013
7	Changbai, China	*Caldicellulosiruptorchangbaiensis*	Cellulolytic activity	Zheng et al. 2015
8	Colombian Andes, Colombia	*Desulfosoma caldarium*	Sulphate reduction	Baena et al. 2011
9	Jaivana, Colombia	*Caloramator quimbayensis*	Anaerobic	Rubianoet al. 2013
10	Hamam, Tunisia	*Desulfotomaculum hydrothermale*	Anaerobic	Haouari et al. 2008
11	Waiotapu, New Zealand	*Venenivibrio stagnispumantis*	Hydrogen-oxidizing	Hetzer et al. 2008
12	Furnas, Portugal	*Sulfurihydrogenibium azorense.*	Hydrogen-oxidizing	Aguiar et al. 2004
13	Oguni-cho, Japan	*Pyrobaculum oguniense*	Hyperthermophilic	Sako et al. 2001
14	Nagano, Japan	*Ignavibacterium album*	Anaerobic	Lino et al. 2010
15	Hakuba, Japan	*Thermotoga profunda*	Anaerobic	Mori et al. 2014
16	Atri, India	*Thiomonas bhubaneswarensis*	Thiosulfate oxidizing	Panda et al. 2009
17	Puttur, India	*Calidifontibacter indicus*	-	Ruckmani et al. 2011
18	Suraj kund, India	*Tepidiphilus thermophilus*	-	Poddar et al. 2014

Note (-) means not mentioned

1.2.3.2: Culture-independent approaches (Metagenomics)

With the adavancement of genomic as well as high throughput sequencing techniques, numerous microbial diversity surveys were performed to track the unseen microbial diversity of hot spring environments. Most of these studies are focused on Yellowstone hot springs (Walter et al. 1972; Madigan et al. 1984). The YNPs have wide ranges of geochemistry, therefore has resulted in the identification of the majority of novel microbial lineages and tremendous metabolic potential of microbes (Meyer-Dombard et al. 2005). Apart from the YNPs, other hot springs such as Great Basin, USA (Cole et al. 2013), Philippines (Huang et al. 2013), Canada and New Zealand (Sharp et al. 2014) were also studied, which revealed enormous diversity among thermophiles. These microbiological surveys have dramatically increased our knowledge at the diversity scale, with the identification of many novel microbial lineages at the higher taxonomic level such as phyla, divisions that were previously unrecognized (Hugenholtz et al. 1998). Some other related extreme environments such as acid mine drainage (Baker et al. 2003), hydrothermal vents under deep sea (Sogin et al. 2006), marine environments (Delong et al. 1992), soda lakes (Jones et al. 1998), subsurface hot soils (Zhou et al. 2004), Antarctica (Wynn-Williams et al. 1996) and many more ecosystems were also investigated. These and many other studies led to identification of various novel microbial lineages, and also understanding their environmental roles in biogeochemical cycling of the matter (Vartoukian et al. 2010).

1.2.4.: Biotechnological applications

1.2.4.1: Thermo-stable enzymes and industrial microbiology

Thermophilic microorganisms are of key interests in industrial processes for the production of various biotechnological products and have attracted researcher's attention (Kikani et al. 2010). The demand for various thermostable enzymes; such as proteases, amylases, cellulases, lipases, xylanases, and DNA polymerases is increasing in food, chemical and

pharmaceutical industries and environmental biotechnology due to their high thermostability and feasibility to the industrial processes (Niehaus et al. 1999; Haki et al. 2003). Thermophiles can survive at high temperatures and other harsh conditions by producing the enzymes which can function under extreme conditions with great enzymatic stability and are usually resistant against chemical denaturants (Antranikian et al. 1999). Several advantages of using thermostable enzymes are that they reduce the risk of microbial contamination, lower the viscosity, and improve transfer rates and solubility of substrates (Bruins et al. 2001). Since high temperatures often promote better enzyme penetration and cell-wall disorganization of the raw materials, thermostable enzymes are also better choices in the biorefineries (Turner et al. 2007).

1.2.4.2: Environmental applications

In recent years, thermophiles are also getting attention for their use in various environmental applications such as in bioremediation processes. Bioremediation is an approach used for the treatment of pollutant using microorganisms (Bamforth et al. 2005). The use of extremophilic microorganisms has become a promising alternative to the bioremediation of various toxic pollutants such as heavy metals, hazardous aromatic compounds because of their high stress and metabolic properties.

1.2.4.2.1: Heavy metal resistance and toxicity

Several studies have reported that, hot springs microbes could be the potential source of a biological agent for bioremediation of heavy metal contaminated sites (Valls et al. 2002). A few of the heavy metals such as chromium, cadmium, zinc, mercury were found in the industrial effluents and causes contaminations of water bodies. Arsenic also found in the underground water and causes the contamination of the drinking water sources. These metals have the hazardous effect on human and ecosystem health, therefore, removal or detoxification of these metals from the environments becomes necessary (Wu et al. 2010).

Different species of bacteria such as *Pseudomonas*, *Burkholderia*, *Sphingomonas*, *Ralstonia*, *Comamonas*, *Achromobacter*, *Alcaligenes*, *Rhodococcus* and *Acidithiobacillus* were reported to have potential to detoxify such metals (Lloyd et al. 2003; Natarajan et al. 2011). Thermophilic microorganisms were also known to detoxify several heavy metals either by solubilising the toxic forms of heavy metal through sulfur and/or iron metabolic oxidation or by reducing them using a coupled oxidation with different organic and inorganic substrates (Urbieta et al. 2015). Therefore, thermophiles could be used in the bioremediation processes for immobilizing toxic heavy metals and even in hot wastewater disposal sites. Several species of bacteria such as *Geobacillus* species, *Anoxybacillus flavithermus*, and *Thermus thermophiles* isolated from hot springs or other thermal environments were reported for the detoxification of different heavy metals (Sar et al. 2013).

1.2.4.2.2: Degradation of toxic and complex aromatic compounds

Temperature plays a significant role in controlling the bioavailability of low-solubility hydrocarbons and hence the nature and the extent of microbial metabolism (Margesin and Schinner 2001). Due to the potential advantages of using elevated temperatures for bioremediation, several researchers have recently studied thermophiles for degradation of hydrocarbons, including long-chain alkanes, aromatics and PAH compound (Perfumo et al. 2007). These organisms predominantly belong to the genera *Thermus* or *Geobacillus* (Feitkenhauer et al. 2003; Zhang et al. 2012) and have been isolated or enriched from deep subsurface hydrothermal vents, high-temperature petroleum reservoirs or from composting sites. Among thermophiles, strains of *Bacillus* and *Geobacillus* were reported to degrade long-chain alkanes (Perfumo et al. 2007), aromatic compounds such as phenol and cresol (Feitkenhauer et al. 2001). The degradation of high molecular weight PAH compounds under thermophilic condition has been studied using mixed culture approaches (Feitkenhauer et al. 2003).

1.2.4.3: Function driven approaches

Functional metagenomics is a powerful experimental approach for studying gene function of mixed microbial populations. The information obtained from the functional metagenomics studies found helpful for annotations of gene function and could serve as a complement to sequence-based metagenomics analyses (Lam et al. 2015). In terms of biotechnological application such as in health, it may become possible to identify the new drug targets via functional genomics (Kramer et al. 2004), in the screening of novel products as a potential candidate drug molecules (Zhang et al. 2005; Schmeisser et al. 2007). However, functional metagenomics relies on sequencing data which therefore requires high assistant of computational as well as bioinformatics tools (Pop et al. 2008; Teeling et al. 2012). Moreover, it also becomes necessary to develop new databases for more efficient throughput functional screenings (Quast et al. 2012). In functional metagenomics, libraries are screened based on the expression of a selected phenotype on a specific medium. A wide variety of biochemical activities have been discovered in environmental metagenomic libraries (Rondon et al. 2000). Therefore, further refinement and with improved methods will profoundly change the traditional way of bioprospecting for novel enzymes and compounds as well as for the determination of gene function.

1.2.5: Indian hot springs

India also has some low to high-temperature hot springs which are reported to be formed due to tectonic movements in the geothermal regions. R.D. Oldham has documented around three hundred hot springs in India (Oldham et al. 1888). Based on geo-tectonic setup Indian hot springs are classified into six geothermal provinces (Sharma et al. 2010). These are (a) Himalayan Province - Tertiary Orogenic belt with Tertiary magmatism, (b) Areas of Faulted blocks Aravalli belt, Naga-Lushi, West coast regions, and Son-Narmada lineament. (c) Volcanic arc - Andaman and Nicobar arc. (d) A deep sedimentary basin of Tertiary age such

as Cambay basin in Gujarat. (e) Radioactive Province Surajkund, Hazaribag, Jharkhand, (f) Cratonic province peninsular India (Bisht et al. 2011). In these geothermal provinces, few of the well known hot springs are shown in the table 1.3.

Table 1.3: Hot springs present in different geothermal province of India

Sr. No	Hot spring name	Location	Temperature	Reference
1	Vajreshwari	Mandakini Mountain, Maharashtra	45 - 50 ºC	Pillai et al. 2008
2	Unkeshwar	Deccan basalt, Maharashtra	42 - 60 ºC	Pathak et al. 2015
3	Bakreshwar	Chhotanagpur Plateau, West Bengal	66 - 69 ºC	Ghosh et al. 2003
4	Taptapani	Berhampur, Odisha	40 - 55 ºC	Sen et al. 2015
5	Atri	Bhubaneswar, Odisha	57 - 58 ºC	Mohanty et al. 2014
6	Thingbu	Tawang , Arunanchal Pradesh	45 - 50 ºC	Bisht et al. 2011
7	Mannikaran	Maniikaran, Himachal Pradesh	89 - 95 ºC	Kumar et al. 2014
8	Gauri Kund	Uttaranchal	45 - 50ºC	Jugran et al. 2014
9	Panamik	Ladakh, Jammu and Kashmir	50 - 70ºC	Craig et al. 2013

1.2.5.1: Microbiological aspects

The microbiological investigation of Indian hot springs began much later than that of global hot springs. Initial studies were limited to the culturable approaches. Using microbial cultivation methods, many novel species were isolated and identified and bioprospected for

biotechnological potentials (Kumar et al. 2004). Most of these culture-based studies were performed on Himalayan geothermal belt; such as Soldhar and Ringigad (Kumar et al. 2004; Pandey et al. 2015), Himachal Pradesh (Sahay et al. 2017). Recently, few other hot springs such as Manikaran (Bhatia et al. 2015; Kaur et al. 2018), Jakrem and Yumthang (Panda et al. 2016), Tulsi Shyam (Ghelani et al. 2015), Taptapani, Arti hot springs (Badhai et al. 2014) and few others (Poddar and Das, 2017) were also analysed using metagenomic sequencing.

1.2.5.2: Scope and formulating a research problem

Apart from very few studies of Indian hot springs, most of the other hot springs of India are still not explored for the microbiological analysis. Unkeshwar hot springs are one among the several such unexplored hot springs. Though few of the geological studies had shown that, Unkeshwar hot spring has a unique geochemistry, till date it's microbiology was not studied in a comprehensive and systematic way. In addition, none of the hot springs of India has been studied in a comprehensive manner for the microbiological analysis. In absence of such studies, a detailed understanding of the microbial communities, their function, and impacts of various environmental factors, seasonal variations on the intrinsic properties of hot springs is still lacking. Moreover, most of the Indian hot springs are believed to have medicinal properties, which attracts the local people to take bath in hot spring water in the belief to cure of their major skin ailment and to perform spiritual activities. Such activities pose the anthropogenic load on the hot spring which could have an influence on the natural microbial populations of the hot springs. Therefore to assess the impact of such activities on microbial communities also becomes important for the conservation of these natural resources for future biotechnological applications. Thus, a more comprehensive microbiological analysis and its correlation with environmental factors as well as human activities are necessary. Furthermore, a potential gap between various Indian hot springs microbiological analysis needs to evaluate for a better understanding of microbiology of Indian hot springs. In the

present study, we made an attempt to fulfil these gaps by studying the Unkeshwar hot spring with the following objectives.

1.3: Aims and objectives

Following broad objectives were defined to comprehensively understand Unkeshwar hot spring microbiology and associated metabolic potentials

- Long-term monitoring, comparison and systematic understanding of structural and functional aspects of hot spring microbial ecology, and correlation with physicochemical, and environmental factors using high throughput sequencing approach
- Comprehensive isolation and prospecting of diverse bacterial species and exploitation of their metabolic potentials for biotechnological applications
- Development of mixed bacterial consortia for degradation of selected model polycyclic aromatic hydrocarbons (PAHs)
- Strategies for enhanced biodegradation of model PAH compounds and crude oil components

1.4: References

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CHAPTER 2

Microbial Ecology using high throughput sequencing

Abstract

Hot spring microbial communities play a key role in the fundamental biogeochemical pathways and energy metabolism of the host ecosystems. In the present study, comprehensive analysis of microbial community composition and functions were analyzed using high-throughput sequencing techniques during period of 2012-2016. The results revealed that, the hot spring predominated by *Proteobacteria, Bacteroidetes Firmicutes, Deinococcus-Thermus, Actinobacteria, Cyanobacteria* and few of the candidate division phyla. For a more improved census of microbial communities, profiling of water, sediment and mat samples was also performed, which has shown the difference in composition of indigenous microbial communities between the samples of Unkeshwar hot spring. In addition, the impacts of anthropogenic activity on the ecology and functions of microbial communities were investigated by analysing the comparative study between subsurface (borehole), bath tank, discharged water and sediment samples. This analysis revealed that, the environmental conditions and anthropogenic activities have a greater impact on intrinsic properties of hot springs microbial assemblages and functions of the microbial communities. A comparative account among few selected hot springs of India including Unkeshwar showed that, these hot springs formed a distinct cluster, suggesting a different microbial community composition and structure amongst them, largely because of the differences in geochemical factors. Functional analysis revealed that, the hot springs has high metabolic capacities, which could be harnessed for various biotechnological applications. Overall the study has shown an improved census of microbial community structure in this hot spring, which could provides a foundation for exploring microbial biogeochemical cycling and microbial function in Indian hot spring ecosystems.

Keywords: Hot springs, Boreholes, Microbial Communities, Anthropogenic, Functions

Introduction

The studies of microorganism in their natural habitats and analyzing the interactions among them form the basis of microbial ecology. Investigating the nature of microorganisms residing within a specific habitat is an extremely important component of a microbial ecology. Such microbial diversity surveys aim to determine the identity, physiological preferences, metabolic capabilities, and genomic features of microbial taxa within a natural ecosystem (Youssef et al. 2015). The advent of high-throughput sequencing technologies has led to an exponential identification and characterization of a large number of microbial communities ever since from the proposal of the tree of life (Shokralla et al. 2012). Since then, the tree is growing rapidly with additions of never phyla and uncultured lineages identified through the genomic data accessed from a variety of environments including the hot spring ecosystems. Microbial communities of hot springs are unusual in the sense that they can tolerate extremes of temperature and constitute a vast reservoir of metabolic capacities which have drawn a considerable scientific interest. Various global hot springs present in different geographical regions such as Yellowstone National Park, USA (Meyer-Dombard et al. 2005; Miller et al. 2009; Kozubal et al. 2013), Great Basin, USA (Costa et al. 2009; Cole et al. 2013), Philippines (Huang et al. 2013), Canada and New Zealand (Sharp et al. 2014) and China (Yim et al. 2006; Huang et al. 2011; Wang et al. 2013), India (Poddar and Das 2017) were studied by different groups with the aspects such as taxonomic and phylogenetic, physiological, biochemical and for biotechnological applications. Such studies furthermore are likely to highlights an even higher diversity of thermophiles in near future with a better understanding of their taxonomy and ecological role as well as for use in biotechnological processes (DeCastro et al. 2016).

In general, diversity in the microbial populations of extreme environments mainly occurs as a consequence of the interactions between microorganisms and their environmental

conditions (Ward et al. 1998). Various environmental factors were known to have an impact on the overall microbial composition of an extreme environment. In the case of hot springs, the temperature is considered as the main factor that controls the overall microbial composition and diversity (Cole et al. 2013; Wang et al. 2013). Along with temperature, other physicochemical factors such as pH of the thermal water, ionic concentrations of various elements, salts present in the water are also known to shapes the microbial communities of these ecosystems (Siering et al. 2006; Mathur et al. 2007). Nevertheless, most of the abiotic factors collectively contribute to the dynamicity of hot spring microbial communities but still our current understanding about their relationship found to be limited (Chan et al. 2017; Chiriac et al. 2017). Other environmental conditions such as geographic locations of hot springs, seasonal variations also contribute and were known to have an impact on microbial populations. For instance, hot springs having circum-neutral pH were reported to have a lesser influence of seasonal variations than the acidic hot spring of temperate zone, whereas those present in tropical zones have a higher influence of seasonal variation on the microbial communities (Brigg et al. 2014; Ferris and Ward 1997; Norris et al. 2002).

Hot springs present in the form of boreholes in a geothermal region could be considered as subsurface hot springs. Microbial communities of such boreholes present near the natural hot springs could have an influence of discrete environmental factors. In a long-term study of microbial communities inhabiting deep thermal subsurface (borehole, depths of 500 to 4000 m) has shown that they could harbor both stable and variable fractions of the microbial communities (Frank et al. 2016). Some other parameters such as the thermal water overflowing from the hot spring is either subsequently tapped by constructing a pool around the hot spring (stagnant water) or it freely flowing over the ground (flowing water) also might be influencing the microbial composition of hot springs as water pools are often used by human for various activities that may lead to the contamination of the source water of the hot

spring. Such anthropogenic activities also have a major impact on such hot spring aquatic ecosystems. This is more relevant in the Indian context as most of the springs are used for a bath in belief to cure diseases that may lead to unwanted but evident inoculation of pathogens into the spring. The influx of such non-indigenous members into the natural hot springs ecosystems significantly disrupts the structure and function of natural microbial assemblages, leading to reduced species diversity, increased heterotrophy and rises in the numbers of potentially virulent/toxic microbes (Derso et al. 2015).

Hot spring ecosystems are valuable microbial sources for various biotechnological applications; however, in India, they are experiencing unprecedented levels of impact of anthropogenic disturbances as a consequence of human activities (Poddar and Das 2017). Considering the impacts of all such factors on the natural microbial compositions of hot springs, there is a need for more comprehensive studies. Such studies could evaluate the impacts of various factors on the structure and function of Indian hot springs microbial communities. Although few of the Indian hot springs was addressed for microbiological investigations, however, research efforts still seek their exploration in a way that will help to understand the influences of seasonal variations, environmental conditions and anthropogenic activity on intrinsic properties of hot springs. Therefore, with a more research understanding and better public awareness as well as long-term environmental impact assessments possibly will give a more robust view of the microbial profile and the influence of various factors on microbial communities. In the present study, we have performed a detail microbiological survey of Unkeshwar hot spring by characterizing microbial communities using various samplings from the hot spring along with some nearby natural as well as anthropogenically impacted sites. Microbial communities of water, mat, and sediments of main hot spring were profiled for a more comprehensive understanding of the microbial diversity and ecology of the hot spring. Furthermore, taxonomic as well as functional analyses using different

methods, sequencing technologies were also performed to gets more and bias-free insights into the hot spring ecology. Comparative accounts of these microbial communities with nearby pristine (boreholes) and anthropogenically distrusted sites (bath tank) as well as with other Indian hot springs were also interpreted. The overall aim was to gain insights into the microbial diversities of Unkeshwar hot spring and to understand the influences of various environmental factors on its compositions.

2.2 Materials and Methods

2.2.1: Chemicals and reagents

All the chemicals and reagents used in the present investigation were of high purity and analytical grade. Taq DNA polymerase, PCR buffer, magnesium chloride and dNTP's were obtained from Banglore Genei Pvt. Ltd. India. Other chemicals and reagents were procured from Hi-Media, Merck, Fisher Scientific and Sigma. Chemicals such as chloroform, phenol, isoamyl alcohols were purchased from Merck Ltd, India, were of Molecular Biology grade.

2.2.2 Description of the site

Unkeshwar is a small village lying between [190 34’ to 190 40’] N Lat., and [780 22’ to 780 34’] E long from mean sea level positioned on the south-East corner of Maharashtra State in peninsular India. The hot water spring located in this village called as Unkeshwar hot spring is a terrestrial hot water spring. With its climatic conditions, attitude similarities and by its geographical position occupies major portion of South East Deccan Continental basalt of India (Fig. 2.1). Unkeshwar hot spring is a unique, terrestrial hot spring on Deccan basalt, amongst presently active systems falls within the Deccan volcanic province of India (Varun et al. 2012). Being rural and totally undeveloped area, very little is known about the geological description about this spring. In total, there are three (two minor) hot springs are present in close vicinity, namely; Mukhya kund, Surya kund and Sarasbhang kund (Temple

Kund). Mukhya kund, also called as Ugam kund is the main hot spring and is present in the form of a small tank with a surface area in about 2.13 m in diameter. This hot spring is the natural outlet of groundwater discharge having a temperature of waterfall above the mean of annual air temperature of that region. In close proximity to the main hot spring around 100 m towards the north, a hot water borehole (depth of 400 m) with sealed wellhead is also present which was previously drilled for some scientific studies (Karve 1995). Therefore in total four hot springs are present in this region including the borehole.

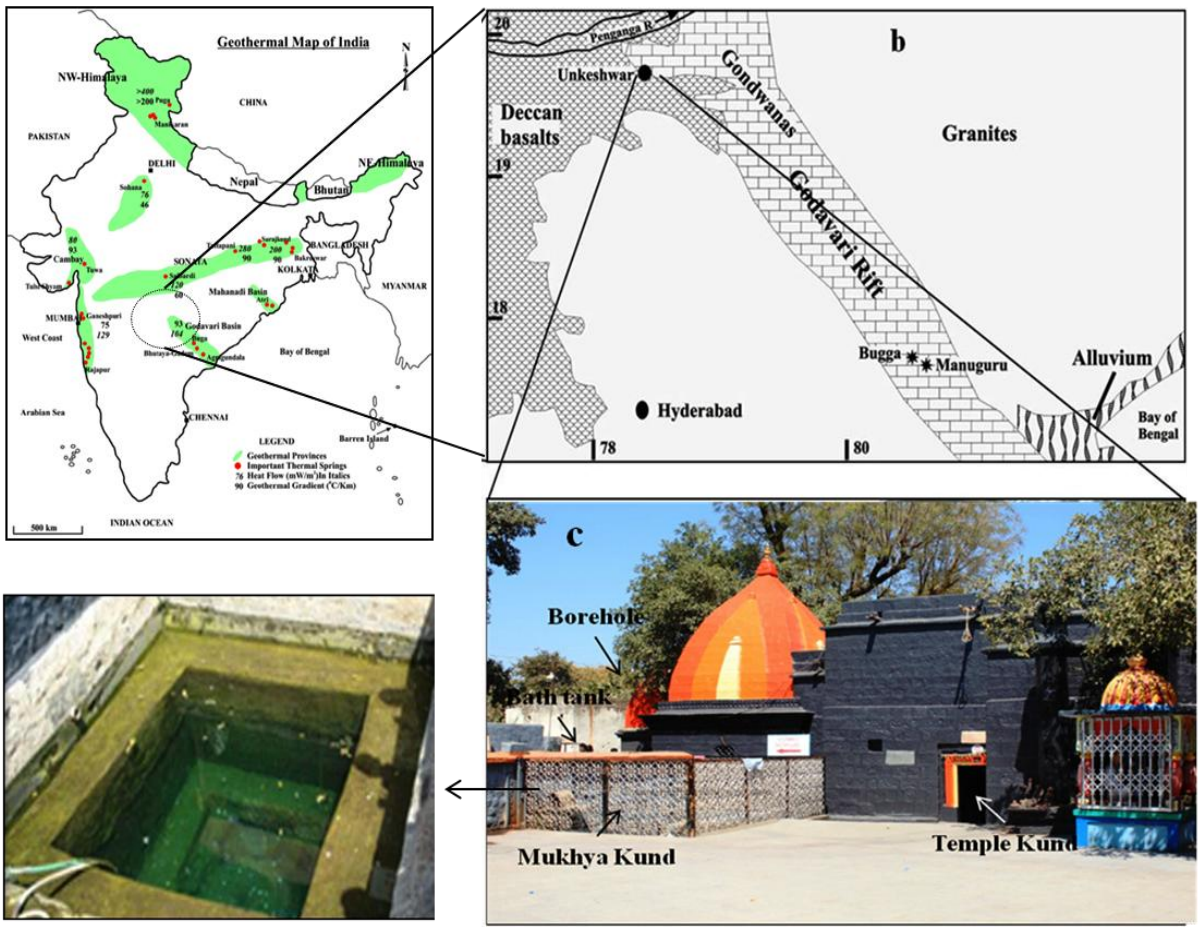

Fig. 2.1: Location of Unkeshwar hot spring, a: A map of the geothermal provinces of India; b: Godavari Rift valley in the Deccan Basalt region; c: Location of the hot spring and other sampling sites at the Unkeshwar.

2.2.3: Microbial diversity analyses: an experimental plan

To get insights into the structure and function of microbial communities of Unkeshwar hot springs, a comprehensive experimental design was chartered (Fig. 2.2). Different samples such as sediment, mat, and water were collected from the main hot springs during various sampling visits. In addition, water, sediment samples of bath tank, discharged water and from the nearby borehole was also collected.

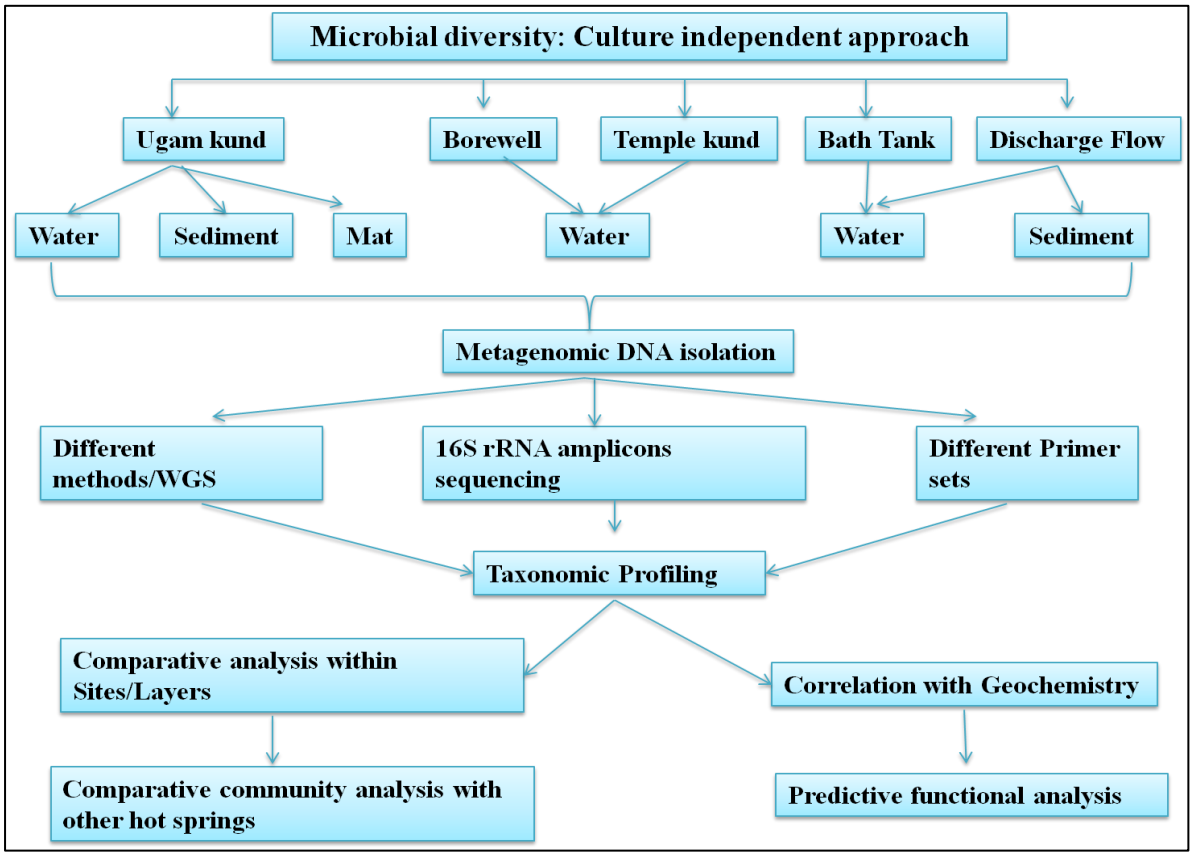

Fig. 2.2: An experimental design of microbial community analysis

2.2.4: Sampling and physicochemical analyses

For a more comprehensive study, repeated samplings trips were made from 2012 to 2016 in various seasons, majorly in summer and winter seasons. Detail about the sampling strategies along with the description of the sampling site, type of the sample is shown in table 2.1. During each sampling trips, various samples such as water, sediment, and microbial mat were collected in sterile containers and transported to the laboratory, stored at 4ºC for further

analysis. Around 6-10 liter of water samples and more than 100 gram of sediments samples were collected for the physicochemical as well as microbiological analysis.

Table 2.1: Sampling details of Unkeshwar hot springs

S. No	Sample code	Sampling site	Sample name	Quantity	Sampling date
1	12UM	Mukhya kund (water)	UMHK1	100 ml	Nov 2012
2	14UM	Mukhya kund (water)	GMHK1	5L	Nov 2014
3	15UM	Mukhya kund (water)	GMHK2	2L	Dec 2015
4	15KS1	Mukhya Kund (sediment)	GMHKS1	50 gm	Dec 2015
5	15KM1	Mukhya kund (mat)	GMHKM1	20 gm	Dec 2015
6	16KS2	Mukhya Kund (sediment)	GMHKS2	50 gm	Dec 2016
7	15DW2	Bath tank (water)	GDW2	4L	Dec 2014
8	15DW2	Bath tank (water)	GDW2	4L	Dec 2015
9	15FW1	Discharged outflow water	GDFW1	2L	Dec 2015
10	15FS1	Discharged outflow sediment	GDFS1	500 gm	Dec 2015
11	14UK	Borehole (water)	GMK1	6L	Nov 2014
12	15UK	Borehole (water)	GMK2	8L	Apr 2015
13	15UK2	Borehole (water)	GMK3	16L	Dec 2015
14	14CK1	Temple kund (water)	GCK1	4L	Nov 2014
15	GCS1	Temple kund (sediment)	GCS1	500 gm	Nov 2014

During collection of water samples, temperature and pH of the water was measured at the site using a digital thermometer and pH meter. The analysis of other physicochemical parameters

such as total dissolved solids, organic matter content and ionic concentration of different elements was performed at a certified chemical testing laboratory (Accurate Analytical Laboratory Pvt. Ltd., Pune, India) fallowing the standard methods of water examinations according to American Public Health Association (APHA, 2012).

2.2.5: Metagenomic DNA isolation and sequencing

2.2.5.1: Isolation of total community DNA

Total community DNA was isolated from different samples collected from hot spring and other sites. For sediments and mat samples, DNA isolation was done directly by taking appropriate quantities of samples (1-2 gm) samples. While in case of water samples, around 6 litres of each water sample was filtered through 0.22 µm filter membrane (Millipore, USA), the filter membrane sliced into small pieces and used for DNA extraction using DNA PowerSoil® isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's suggested protocol. The extracted DNA samples were further processed for the quality check to be used for sequencings.

2.2.5.2: Quantitative and qualitative analysis of DNA

The quantitative analysis of the DNA was done using Nano-drop spectrophotometer and Qubit®. 2.0 Fluorometer. A NanoDropTM 2000 Spectrophotometer was used to check the purity and quantity of nucleic acids present in the extracted DNA. While Qubit®. 2.0 Fluorometer was used to check the exact concentration of dsDNA present in the extracted DNA and neglects the single nucleotides and quantifies only long DNA strands. The integrity of the isolated DNA samples was checked on 1% agarose gel electrophoresis and visualization by a gel documentation system (Protein Simple). The quantified DNA from all samples was subsequently used for library preparations.

2.2.5.3: Sequencing methods and library preparations

In this study, we performed sequencing analysis using two approaches; the first approach was based on amplicon (16S rRNA) and second was the whole metagenome (shoutgun) approach. The details are provided in table 2.2.

Table 2.2: Sequencing details of the samples

Sr. No	Sample ID	Sequencing primer/methods	No. of reads	Platform
1	12UM	Whole metagenome (WMG)	1,360,637	Illumina
2	12UM	V3-V4 (16S rRNA)	848,096	Illumina
3	14UM	V3-V4 (16S rRNA)	894,924	Illumina
4	14UK	V3-V4 (16S rRNA)	1,646,350	Illumina
5	14CK1	V3-V4 (16S rRNA)	533,714	Illumina
6	14DW1	V3-V4 (16S rRNA)	2,16,962	Illumina
7	15UK	V3-V4 (16S rRNA)	9,68,797	Illumina
9	15UM	V3-V4 (16S rRNA)	443,824	Illumina
10	15KS1	V3-V4 (16S rRNA)	749,342	Illumina
11	15KS1	Whole metagenome (WMG)	5,982,247	Illumina
12	15KM1	V3-V4(16S rRNA)	527,825	Illumina
14	15DW2	V3-V4(16S rRNA)	476,552	Illumina
15	15FW1	V3-V4(16S rRNA)	285,629	Illumina
16	15FS1	V3-V4(16S rRNA)	288,101	Illumina
17	15FS1	Whole metagenome (WMG)	6,445,989	Illumina
18	16KS1	V3-V4(16S rRNA)	6,41,002	Illumina
19	16KS1	Whole metagenome (WMG)	4,142,104	Illumina
20	16KS1	Whole metagenome (WMG)	329, 222	MinION

2.2.5.3.1: 16S rRNA amplicon sequencing

The variable regions of 16S rRNA gene are frequently used in phylogenetic classifications such as genus or species in diverse microbial populations. Here, we have used DNA samples for sequencing the variable V3-V4 region of the 16S rRNA gene using the primer sets; Pro341F (5'-CCTACGGGNBGCASCAG-3') and Pro805R (5'-GACTACNVGGGTATCTAATCC-3') (Takahashi et al. 2014). For amplification of this region, PCR was performed using a reaction mixture of 5 µl, 5x Phusion® HF Reaction Buffer (New England Biolabs (NEB), UK), 0.4 µl of 10mM each dNTP (NEB, UK), 0.2 µl HF DNA polymerase (2000 U/ml) (NEB, UK), 2 µl of each primer (10 pM) and 10 ng of template DNA. The reaction volume was adjusted to 25 µl using nuclease-free water and plate was sealed. PCR amplification was performed in a thermal cycler using the program: PCR Reaction: 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 72°C for 30 sec, extension at 72°C for 5 sec followed by 4°C hold. After amplification for the desired region, PCR products were run on 2% agarose gel using SYBR® Safe DNA gel stain (10µl/100ml) and then Purified using invitrogen™ PureLink™ Quick Gel Extraction and PCR Purification Combo Kit. Amplicons were sequenced using paired-end (2×251 bp) sequencing on an Illumina MiSeq sequencer (Scigenom, India) using the MiSeq reagent kit. Raw reads obtained from sequencing were used for bioinformatics analysis.

2.2.6: Bioinformatics analysis

2.2.6.1: Fastq quality checking and preprocessing of reads

Quality parameters for the raw sequences such as base quality score distributions, average base content per read and GC distribution in the sequence reads were checked using the FASTQ Quality Filter, Phred score greater than 30 (>Q30; error-probability >= 0.001). After quality checking of the sequence of 16S rRNA V3-V4 region of bacteria, these paired-end sequences contain some portion of a conserved region, spacer, and V3-V4 region. As a first

step, we remove the spacer and conserved region from paired-end reads. After trimming the unwanted sequences from original paired-end data a consensus V3-V4 region sequence is constructed using a FLASH program. A multiple filters such as conserved region filter, spacer filter, and mismatch filter are performed to take further only the high-quality V3-V4 region sequences for various downstream analyses. While making consensus V3-V4 sequence, All consensus reads were formed with 0 mismatches with an average contig length of ~260 to >410. The resulting good quality sequences were then used to merge paired-end illumina reads that are overlapping into single longer reads using SeqPrep QIIME program (Kuczynski et al. 2012). Chimeras were removed using the de-novo chimera removal method UCHIME implemented in the tool USEARCH. After chimera removal, the resulting non-chimeric sequences were taken up for picking the Operational Taxonomic Units (OTUs) from all the samples.

2.2.6.2: OTUs and taxonomy classification

Chimeric sequences removed using the program UCHIME (Edgar et al. 2011) and non-chimeric sequences were further analyzed using the QIIME (Qualitative Insight Into Microbial Ecology) pipeline version 1.9.1 (Kuczynski et al. 2012) with default parameters. Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using swarm algorithm (Mahe et al. 2014). The OTUs were checked for singleton OTUs (OTUs has single read) and singletons were removed. The representative sequence was identified for each OTU and aligned against SILVA core set of sequences using Python Nearest Alignment Space Termination (PyNAST) program (DeSantis et al. 2006; Caporaso et al. 2010). Further, we aligned this representative sequences against reference chimeric data sets. Then, taxonomy classification was performed using RDP classifier and SILVA OTUs database (Pruesse et al. 2007). The sequences do not have any alignment against taxonomic database are categorized as ‘unknown’.

2.2.6.3: Analysis of alpha diversity

In this section, we analyzed the microbial diversity within the samples by calculating Shannon, Chao1 and observed species metrics. The Chao1 metric estimates the species richness while Shannon index is used to estimate the observed OTU abundances, and accounts for both richness and evenness (Kuczynski et al. 2012). The observed species metric is the count of unique OTUs identified in the samples. The rarefaction curve for each of the metric is generated; the metric calculation was performed using QIIME software.

2.2.6.4: Analysis of beta diversity

We have performed an explicit comparison of microbial communities between the samples. At first, we have generated the distance matrix using both weighted and unweighted UniFrac approach. Sequence abundances were taken into account in Weighted UniFrac for comparing microbial diversity. A jackknife test was performed to construct a consensus UPGMA (Unweighted Pair Group Method with Arithmetic Mean) tree for different pairs of samples. The resulted consensus UPGMA trees built using weighted and unweighted UniFrac distance matrix for different pairs of samples.

2.2.6.5: Statistical analyses

To determine the influence of physicochemical parameters on the observed diversity of hot spring communities, multivariate principal coordinate analysis (PCoA) of 21 physicochemical parameters was carried out using paleontological statistics (PAST) v3.14 software (Hammer et al. 2001). All measured physicochemical parameters were checked for normality and normalized abundances of major taxonomic groups at phylum and genus level were used for canonical correspondence analysis (CCA). From all the samples OTUs with the relative abundance of > 0.01% were used as community matrix for CCA analysis. Separate plots were generated for both the taxonomic rank (phylum and genus). The significance of CCA models was tested using 999 permutations. All the statistical analyses were performed

either using QIIME or PAST and STAMP software (Hammer et al. 2001; Parks and Beiko 2010; Kuczynski et al. 2012).

2.2.6.6: Mining for metabolic potentials

The metabolic function prediction analysis was done using Tax4fun software (Aßhauer et al. 2015) to explore the functional gene content in the hot spring metagenome based on the 16S rRNA sequencing data represented in Silva database. Tax4Fun predicts a taxonomic profile from SILVA based community profile. It uses its own pre-computed association matrix, KEGG organism functional profiles and 16S rRNA copy numbers obtained from NCBI genomes. Tax4Fun gives the proteins with their KO IDs and their predicted relative abundances based on the OTU taxonomic assignments done using QIIME. Further, an empirical pathways profile was created using Tax4Fun output by retrieving the complete list of pathways associated with each protein from the KEGG database and dividing the abundance from Tax4Fun output among them, the proteins having no pathways listed in KEGG entry were retained in the profile. Further, a class and subclass distribution of the predicted pathways was calculated using the pathways profile in a similar way by retrieving the class of each pathway from KEGG, again all pathways, which were unclassified in KEGG, were retained in the final profile. At each step in this analysis, we would also use keywords to extract all proteins associated with a certain pathway or all pathways associated with certain class or sub-class for further in-depth analysis. The post-processing of Tax4Fun results was done using in-house scripts written in Perl (all the scripts created and used in the process have been made available on github at https://github.com/ncim-ncl/metgenomics.

2.2.7: Whole metagenome sequencing analyses

2.2.7.1: De novo metagenome assembly

Based on the quality reports of fastq files, we trimmed the sequence read where necessary to retain only high quality sequence for further analysis. The *De novo* metagenome assembly

was carried out for samples by assembling contigs from the reads. Further contigs were linked by the assembly algorithm to create scaffolds. N50 contigs were kept as a good assembly and misassemblies were removed from the result. Assembly was performed with default k-mer length (21-size) using de-bruijn graph method. In-house PERL and Python code were used to parse the fastq files for the downstream analysis.

2.2.7.2: Taxonomy analysis

Taxonomic profiling for metagenomics sample was performed using NCBI taxonomy datasets. The taxonomy tree was generated based on a neighbor-joining method using MEGAN software. The tree at phylum level and species level allow us to view the hierarchy of comparative taxonomic abundance in samples based on contig abundance. Rarefaction curves were generated by comparing the species abundance between the samples based on a number of leaves in the taxonomy and number of sequence occurred. The curve is made for all taxa include bacteria, archaea, eukaryote, viruses, unclassified and other sequences.

2.2.7.3: Functional annotation

As a result of de novo assembly, contigs in FASTA format were generated for samples. The contigs were queried to BLASTX program with an optimum e value of $1e^{-10}$. The gene or protein functions of all the contigs from BLASTX output was parsed using in-house PERL script. The complete functional annotation summary along with contig ID, gene function and sequences of a sample were generated. Further, functional annotation was carried out by doing KEGG analysis based on taxons.

2.2.7.4: Metabolic pathway annotation

KEGG pathway analysis was performed for each contig sequences by assigning KEGG Orthology (KO) numbers obtained from known reference hits. Further, enzyme and pathway information was assigned to contigs based on KO. The pathway classification is represented as a rooted tree. Each leaf from the tree is representing different pathways. The numbers of

sequences corresponding to each pathway were calculated.

2.2.7.5: Statistical analyses

All the statistical analyses were performed either using QIIME or PAST and STAMP software (Hammer et al. 2001; Parks and Beiko 2010; Kuczynski et al. 2012).

2.2.8: Nanopore MinION sequencing

The Nanopore MinION sequencing was performed using 1D Native barcoding genomic DNA (with EXP-NBD103 and SQK-LSK108) protocol as prescribed by the (Oxford Nanopore Technologies, Oxford, UK) with certain modifications. In brief, the DNA sample was fragmented by transferring 1µg of DNA sample to a Covaris g-tube and subsequently verifying the fragmentation quality and quantity. Further, a required amount of fragmented DNA sample subjected to end repair step and the resulting end-prepped DNA was used for library preparation using Native Barcoding Kit 1D (EXP-NBD103) and Ligation Sequencing Kit 1D (SQK-LSK108) (Oxford Nanopore Technologies, Oxford, UK). The library was then prepared for loading using Library Loading Bead Kit (EXP-LLA001), (Oxford Nanopore Technologies, UK) and sequenced using the flow cell (FLO-MIN 107, Oxford Nanopore Technologies, Oxford, UK). WIMP (What's in my Pot?) analysis was done for the output using the EPI2ME server provided by Oxford Nanopore. Raw reads obtained from both the technologies were filtered to remove and trim low quality reads using Trim_Galore (version 0.4.4). The good quality sequences were subjected to de novo assembly using MetaVelvet (version 1.1.01). The assembled contigs were analyzed using MG-RAST for taxonomical and functional annotations.

2.2.9: Comparative metagenomic analyses

A comparative analysis of microbial diversity was performed based on the sequencing technologies such as Illumina and Nanopore to understand the sequencing differences for microbial community analysis studies. Further, a comparative account of present hot spring

with other previously studied Indian hot springs was also performed. Sequencing data of other hot springs was obtained from the databases such as Sequence Read Archive (SRA) of National Centre for Biotechnology Information (NCBI) and other sources. The analysis was performed using the bioinformatics pipeline as mentioned earlier.

2.2.10: Submission of raw sequences in database

The raw sequence data of the samples generated in the study were submitted to Sequence Read Archive of NCBI under accession number SRX1499016, SRX1499015, SRP067549, https://www.mgrast.org/mgmain.html?mgpage=project&project=637ba934736d6770383538 3533,https://www.mgrast.org/mgmain.html?mgpage=project&project=637ba934736d677038 3538.

2.3: Results and discussions

2.3.1: Physicochemical analysis of water samples

During sampling the measured temperature of natural hot spring was found in the range of 47ºC - 50ºC and borehole water has a little higher temperature (52ºC -55ºC). The pH of the hot spring and borehole water sample was found 7.2 and 6.9 respectively. Though temperature and pH range of hot spring and borehole found in the similar range, other physicochemical factors were varied between hot spring and borehole samples. Parameters such as phosphorus, nitrates, sulphates, chlorides, calcium, magnesium and potassium were present relatively in higher concentrations in hot spring samples than the borehole (Table 2.3). In one of the samples of hot spring (15UM), total solids (TS), total volatile solids (TVS), volatile acids (VA) as well as total organic carbon (TOC), total nitrogen (TN) were present in higher concentrations relative to all other samples. Borehole water samples of 2014 and 2015 showed less variation in concentrations of various elements. Overall, it was found that this hot spring has higher concentrations of various elements such as sulphur, magnesium

and total organic carbon, which could have an influence on the microbial community composition.

Table 2.3: Physicochemical analysis of hot spring and borehole water samples

Sr. No	Test parameters (Unit)	Natural spring water		Borehole water	
		14UM (2014)	**15UM (2015)**	**14UK (2014)**	**15UK (2015)**
1	Temperature (°C)	47	50	52	55
2	pH	7.2	7.2	6.9	6.9
3	Electrical Conductivity (µmhos/cm)	409	472	339	352
4	Volatile dissolved solids at 550°C (mg/L)	144	200	157	146
5	Total volatile solids at 550°C (mg/L)	153	216	166	154
6	Total dissolved solids at 105°C (mg/L)	420	584	460	428
7	Total solids at 105 °C (mg/L)	436	616	472	440
8	Volatile acids as acetic acid /L (mg/L)	8.47	14.11	3.529	3.529
9	Total Organic carbon (TOC) (mg/L)	1.55	3.098	1.55	1.55
10	Total Kjeldhal Nitrogen as N (mg/L)	49.25	35.57	64.81	35.17
11	Total phosphorus (PO_4^{---}) (mg/L)	0.2034	0.2546	0.0254	0.0523
12	Nitrates as (NO_3^-) (mg/L)	29.5	26.25	2.125	1.0
13	Sodium as sodium chloride(NaCl) (mg/L)	153.51	185.024	162.51	179.19
14	Carbonates (CO_3^{---}) (mg/L)	30.35	42.48	33.38	45.52
15	Chlorides (Cl^-) (mg/L)	61.52	59.59	29.99	29.61
16	Ammonia (NH_3) (mg/L)	24.4	20.39	8.16	24.48
17	Sulphates (SO_4^{--}) (mg/L)	52.5	73.75	40	45
18	Sulphates as (S) (mg/L)	17.5	24.58	13.33	15
19	Calcium (Ca) (mg/L)	34.48	65.25	27.96	13.05

20	Cobalt (Co) (mg/L)	0.0425	0.0	0.0	0.0
21	Boron (B) (mg/L)	0.054	0.15	0.204	0.114
22	Magnesium (Mg) (mg/L)	2.8284	5.09	0.567	2.26
23	Sodium (Na) (mg/L)	93.16	112.28	98.62	108.74
24	Potassium (K) (mg/L)	4.0	4.0	3.0	3.0
25	Iron (Fe) (mg/L)	0.0569	0.0854	0.0569	0.0284

2.3.2: Metagenomic DNA isolation and library preparation

Total community DNA was extracted from various samples such as water, sediment, and mat samples. In case of water samples, DNA isolation was standardized based on the quantity of water used for extraction of DNA using a filter membrane to the obtained higher yield of DNA. The DNA samples were quantitatively analyzed and processed for PCR amplification. The amplified products were checked on the agarose gel and then used for sequencing library preparations (Fig. 2.3).

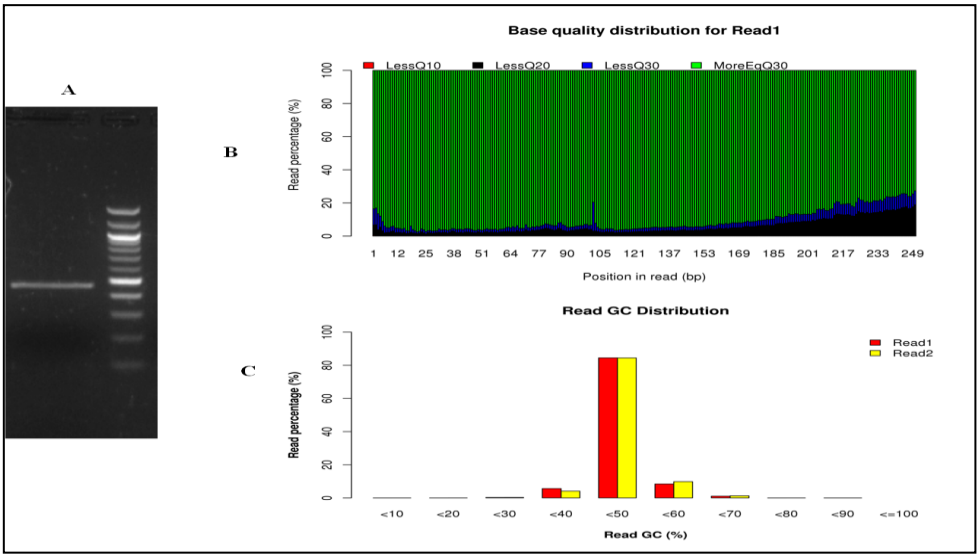

Fig. 2.3: Quality of the amplified product was accessed using agarose gel, a representative image (A) is shown here, quality of the raw sequences was determined based on base quality composition (B) and distribution and GC content (C)

2.3.3: Amplicon (16S rRNA) based microbial community analyses

Metagenomic studies are commonly performed by analyzing the prokaryotic 16S ribosomal RNA gene (16S rRNA), which is approximately 1,500 bp long and contains nine variable regions interspersed between conserved regions. Variable regions of 16S rRNA are frequently used in phylogenetic classifications such as genus or species in diverse microbial populations. In this study we have used V3-V4 for amplification based diversity analysis.

2.3.3.1: Insights into main hot spring water

A total of 1,360,637 raw reads were obtained, and 873,631 reads were considered for analysis (after filtering), from which a total of 6,684 OTUs were detected. Taxonomic classification showed the dominance of *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Cyanobacteria* (Fig. 2.4A).

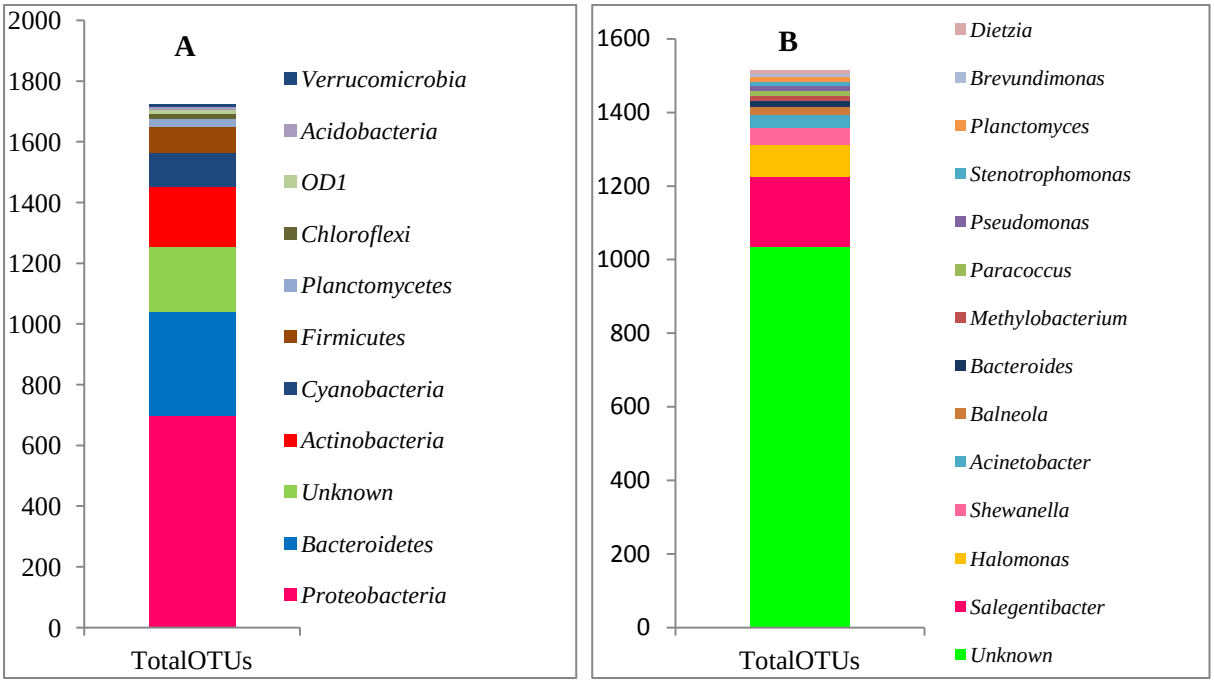

Fig. 2.4: Taxonomy classification of OTUs at the phylum level (**A**) and genus level (**B**) for the 12UM (water) sample of the hot spring. Only dominant phyla and genera are shown here.

A few OTUs were also assigned to *Archaea* belonging to the *Cenarchaeum* genus (phylum *Thaumarchaeota*). The rest of the OTUs were mapped to other and unknown phyla. Genus

level analysis showed the predominance of *Salegentibacter*, *Halomonas*, *Shewanella*, *Balneola*, *Methylobacterium*, *Bacteroides*, *Paracoccus*, and *Acinetobacter* (Fig. 2.4B). *Salegentibacter*, the member of the *Flavobacteriaceae* family play important roles in marine ecosystems and in biogeochemical cycles in interaction with other marine organisms (Bowman & McCuaig 2003). *Shewanella*, a gram-negative bacterium, can use a wide range of electron acceptors, including fumarate, trimethylamine N-oxide, dimethyl sulfoxide, nitrate, nitrite, thiosulfate, and sulphite.

2.3.3.2: Profiling of water, sediment and mat of main hot spring

The sediment, water as well as mat samples could harbour a distinct microbial community composition. To get an overall census of microbial diversity of this hot spring, we performed the diversity analysis from these three samples collected at the same sampling time point from the main hot spring. After quality filtration, denoising and demultiplexing, a total of 1164714 reads were obtained which were used for making consensus V3-V4 sequences (Table 2.4).

Table 2.4: Pre processing reads statistics of water, sediment and mat sample of hot spring

Sample Name	Total reads	Passed merged reads	Passed quality read	Total OTUs	Singletons removed	Final OTU count
15KS1	749342	528379	430657	275818	265335	10483
15KM1	527825	497462	310240	181688	171856	9832
15UM	443824	424738	423817	110800	102179	8621

These sequences were clustered for OTUs with a threshold of 97% of similarity which led to the identification of 106936 OTUs. A total of 42 phyla including Archea were detected among all three samples. Among these 42 phyla, 16 phyla were found in dominant and around 90% OTUs were assigned to them. However, a relative abundance of the phyla varied

among these three types of samples. Taxonomic analysis based on relative abundance revealed that *Bacteroidetes* (44.0%) followed by *Saccharibacteria* were the dominant members in the sediment (15KS1) sample; *Cyanobacteria* (48.1%) and *Firmicutes* dominated the mat (KM1) sample, whereas, in water (15UM) sample, *Proteobacteria* (36.3%), *Firmicutes* (34.7%) and *Actinobacteria* (11.1%) were found dominant (Fig. 2.5).

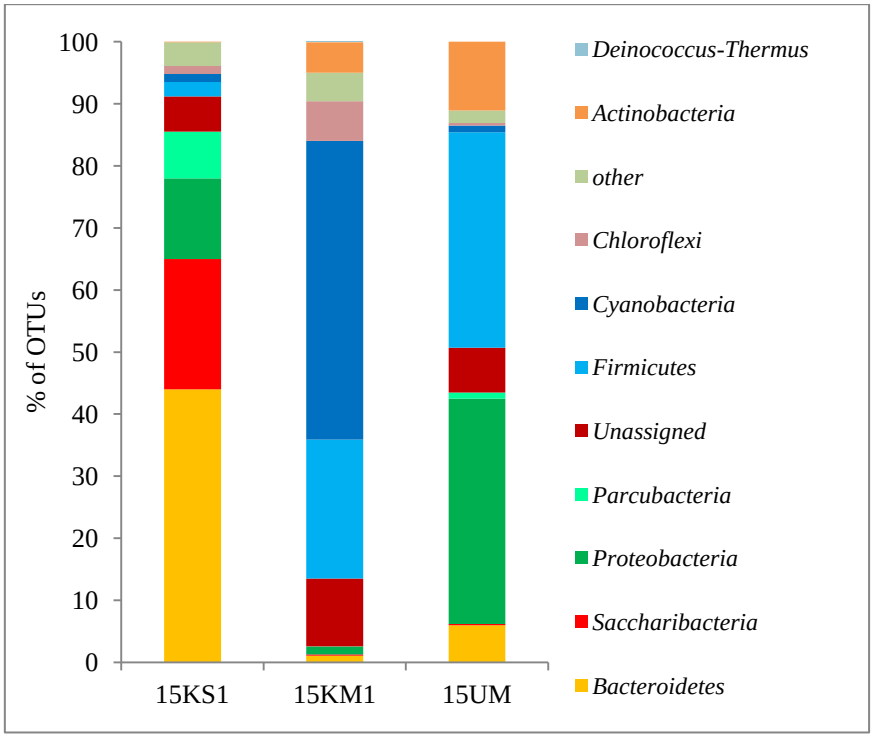

Fig. 2.5: Taxonomy classification of OTUs at the phylum level for the sediment, mat and water sample of the hot spring. Only dominant phyla are represented here.

Other phyla such as *Parcubacteria*, *Chloroflexi* and *Deinococcus-Thermus* represented a relatively minor portion of the total diversity. However, some other phyla exclusively found only in one or two samples, such as *Acidobacteria*, *Thaumarchaeota*, *Chlorobi*, *Verrucomicrobia*, *Planctomycetes*, *Deferribacteres* and *Fusobacteria* either were detected in sediment (15KS1) or Mat (15KM1) sample with varied relative abundance. Among all three samples, a predominance of *Firmicutes*, *Cyanobacteria*, *Proteobacteria* and *Bacteroidetes* was observed. Distribution of the total number of phyla as well as dominant phyla showed

that a total of 10 phyla (24.4% of total OTUs) were shared among all the three samples *i.e.* sediment, mat and water samples (Fig. 2.6).

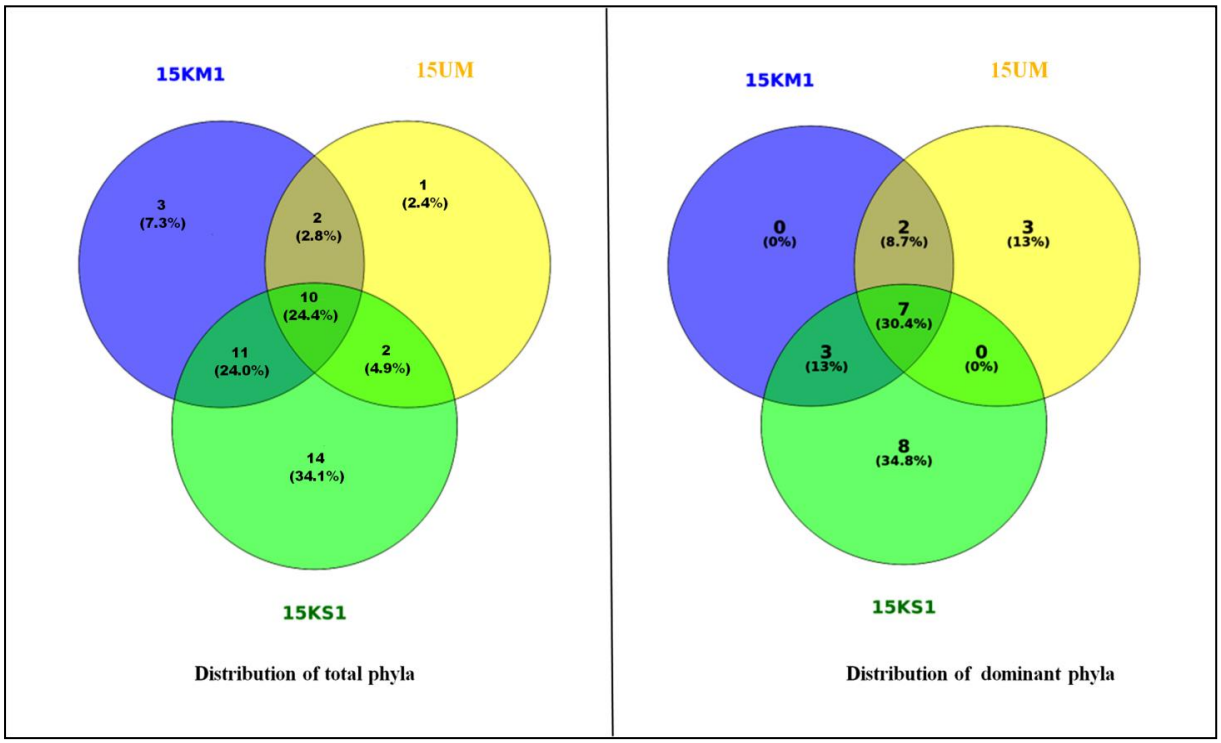

Fig. 2.6: Venn diagram demonstrating the distribution of unique and shared OTUs of total phyla and among the dominant phyla across sediment, mat and water samples

Interestingly, the sediment sample (15KS1) found to have the maximum number of phyla than the corresponding mat and water sample. A total of 14 phyla (34.1% of total OTUs) were exclusively found in sediment sample, which included most of candidate division phyla. These phyla belonged to *Spirochaetae*, candidate division TM6, Candidate division WS6, candidate division SM2F11, Candidate division OP3, *Omnitrophica*, *Microgenomates*, Candidate division GOUTA4, *Elusimicrobia*, *Gracilibacteria*, Archaea; *Woesearchaeota* (DHVEG-6), *Lentisphaerae*, Archaea; *Diapherotrites* and candidate division OC31. Among the sediment and mat sample, around 11 phyla were shared, which includes, *Parcubacteria*, *Acidobacteria*, Archaea; *Thaumarchaeota*, *Chlorobi*, *Chlamydiae*, *Planctomycetes*, WCHB160, *Armatimonadetes*, *Hydrogenedentes*, *Fibrobacteres*, candidate division TA06.

Most of these shared phyla were also found in several previously studied hot spring mat and sediment samples. Interestingly, only a few OTUs of two phyla were shared among mat and water samples, indicating that the microbial communities of the mat were significantly different than the corresponding water samples, but might be originating from sediment samples. While in the case of sediment and a corresponding water sample, OTUs belonging to only two phyla; *Fusobacteria* and *Tenericutes* were shared. In case of mat sample, three of phyla; *Gemmatimonadetes*, *Aquifer* and candidate division SHA-109 found exclusively, whereas OTUs of Miscellaneous Crenarchaeotic Group (archaea) only found in a water sample. When we compared the OTUs assigned to only dominant members, it was observed that 6 bacterial phyla; *Cyanobacteria*, *Firmicutes*, *Chloroflexi*, *Proteobacteria*, *Bacteroidetes*, *Saccharibacteria*, and unassigned OTUs were shared among the three sample. The OTUs of *Planctomycetes*, *Acidobacteria*, and *Parcubacteria* were shared among sediment and water samples. In case of sediment sample, out of total 14 phyla, which were exclusively found in this sample, eight phyla; *Thaumarchaeota* (archaea), *Chlorobi*, *Verrucomicrobia*, *Chlamydiae*, WCHB1-60, *Spirochaetae*, candidate divisions TM6 and WS6 were represented with a higher number of OTUs. A heat map of all the phyla found among these three samples is shown in figure 2.7. The map shows that, most of phyla belong to candidate division were not detected either in mat or water samples. Overall, it was observed that the sediment sample harboured the highest number of phyla than the corresponding mat and water samples. However, most of these phyla were represented by only few numbers of OTUs. The analysis inferred that, sediment microbial communities were more diverse than the water and mat microbial communities.

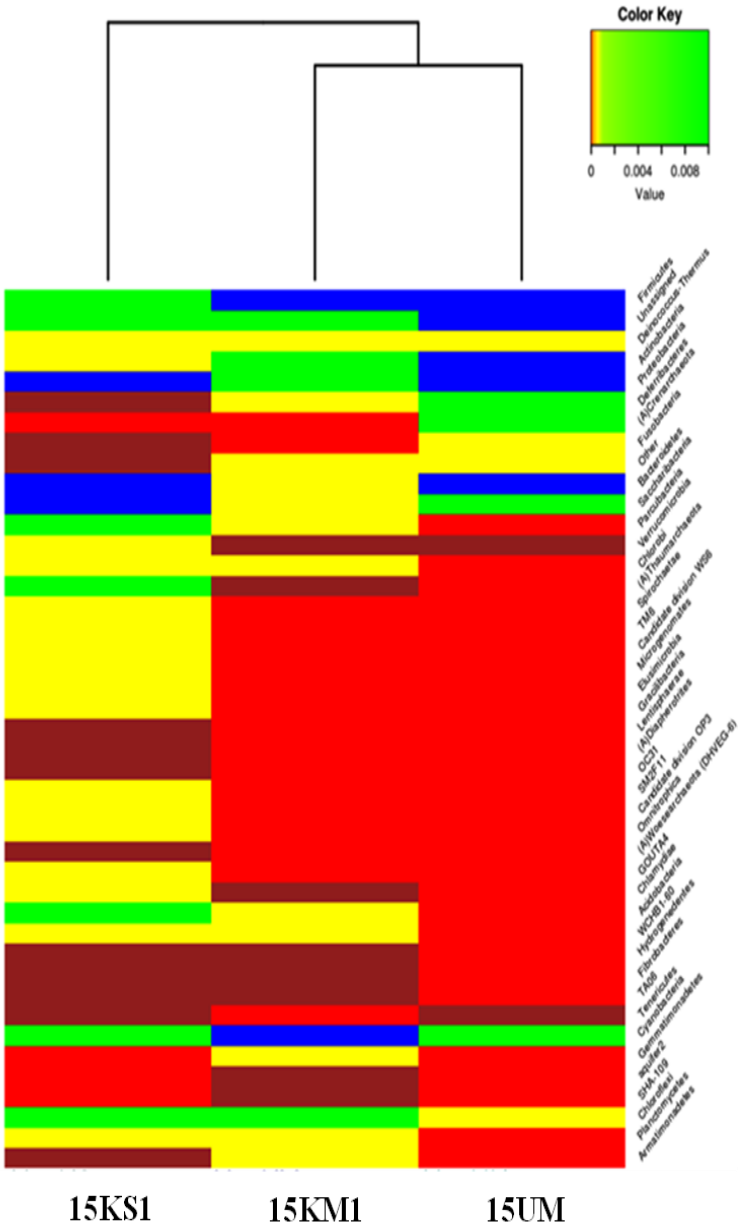

Fig.2.7: A heat map showing the abundance and distribution of OTUs at the phylum level among the three samples corresponding to sediment, mat and water samples of the hot springs.

At the genus level of taxonomic classification, most of the OTUs were found to be unassigned in sediment and mat samples possibly indicating that, these are unknown members of the community and represent high diversity. Apart from unassigned OTUs, few of the OTUs were assigned to genera such as *Bdellovibrio*, *Blastocatella*, *Cellvibrio*,

Chryseobacterium, *Chryseolinea* and *Sphingobacterium*. In case of mat sample, few other genera such as *Arthrobacter*, *Bacillus*, *Blastocatella*, *Marmoricola*, *Nocardioides*, and *Rhodococcus* were detected, which were found to be common members of various other normal environments. Some of the OTUs from the water sample were assigned to genera such as *Corynebacterium*, *Dolosigranulum*, uncultured members of *Halomonadaceae*, *Halomonas*, *Lachnospiraceae* NK4A136 group, uncultured members of *Lachnospiraceae*, *Shewanella* and *Staphylococcus* (Fig. 2.8).

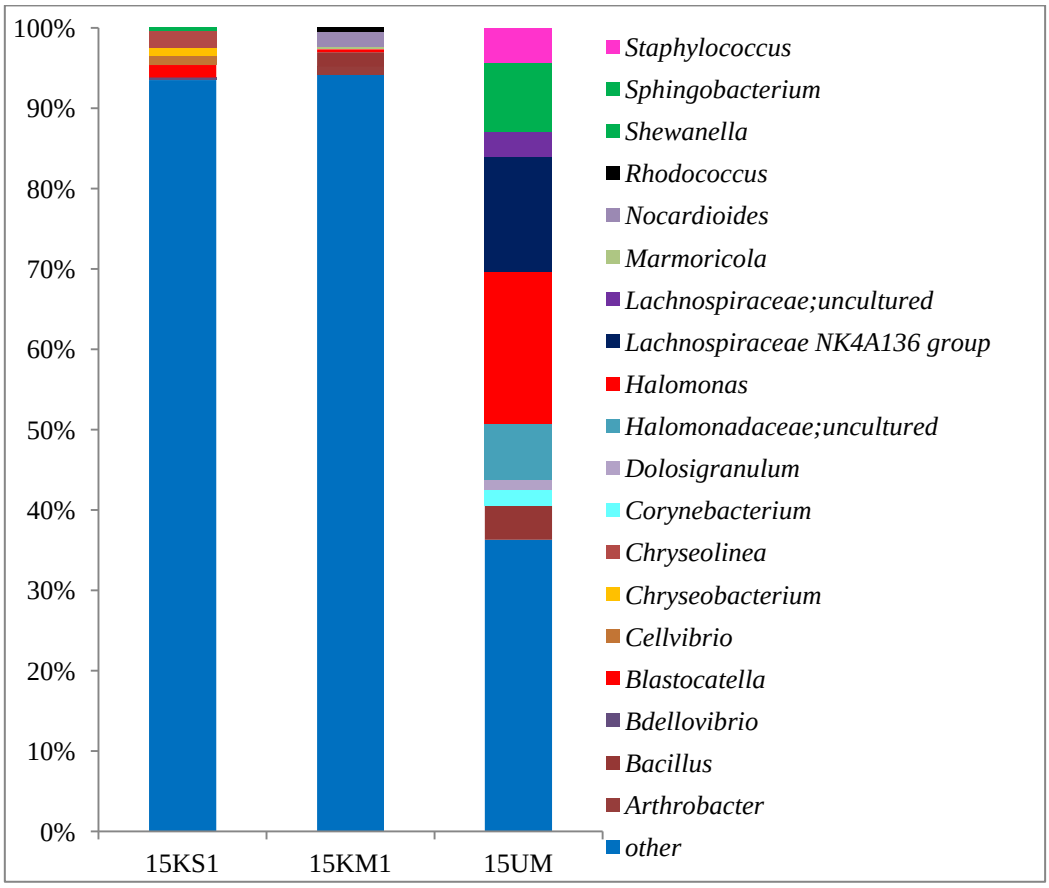

Fig.2.8: Taxonomy classification of OTUs at the genus level for the sediment, mat and water sample of the hot spring. Only dominant phyla are represented here.

Overall, it could be inferred from the analysis that, the distribution and relative abundance of microbial phyla (archeal and bacterial) was significantly different in all three samples. However, in all three samples predominance of *Firmicutes*, *Proteobacteria*, *Bacteroidetes*,

Cyanobacteria, and *Saccharibacteria* was observed. The differences in the relative abundances of microbial community members might be due to various environmental conditions. As we found that mat microbial communities majorly dominated by photosynthetic bacteria *i.e. Cyanobacteria* and *Chlorofexi* indicating that they play a major part in the productivity. While in the case of a sediment sample, the microbial community was found to be more established with various diverse members including some candidate division, which might be playing some role in biogeochemical cycling. In case of a water sample, being more exposed to external sources, found to have an impact of external factors on community members. In this sample, occurrence of the some of the genera, which are also the common inhabitant of various other environments such as *Staphylococcus*, *Bacillus*, and *Clostridium* possibly, indicate that they might have transferred from other sources into the hot spring.

2.3.3.3: Comparative profiling of natural hot spring and borehole sites

In this study, microbial communities of water samples collected from Unkeshwar hot spring and its associated borehole were characterized. Water samples collected in the month of November of 2014 and 2015 were analyzed using 16S rRNA amplicons (V3-V4 region) sequencing and in depth characterization. After quality filtration, denoising and demultiplexing, a total of 3953895 reads were obtained which were used for making consensus V3-V4 sequences. A total of 736468 (14UM), 1485341 (14UK), 424738 (15UM) and 850625 (15UK) consensus reads were formed with 0 mismatches with an average contig length of ~270 to 360 (Table 2.5). These sequences were clustered for OTUs with a threshold of 97% of similarity which led to the identification of 66986 OTUs. The Taxonomic distribution of these OTUs has shown that 95.9% OTUs were assigned to bacteria, 0.1% to archaea and 3.9% remained unassigned.

Table 2.5: Pre processing reads statistics of hot spring and borehole samples

Sample name	Total reads	Passed merged reads	Quality reads	Total OTUs	Singletons removed	Final OTU count
14UM	894924	736468	701966	418535	402378	16157
14UK	1646350	1485341	1443187	635315	609008	26307
15UM	443824	424738	423817	110800	102179	8621
15UK	968797	850625	821802	345004	329103	15901

2.3.3.3.1: Composition and diversity of Archaea

The overall archaeal OTUs were assigned to three different archaeal phyla *i.e*, *Euryarchaeota*, *Crenarchaeota* and *Thaumarchaeota* including some uncultured members. *Crenarchaeota* was predominant (44.6% of total OTUs assigned to archaea) followed by *Euryarchaeota* (40.0% of total OTUs assigned to archaea) and *Thaumarchaeota* (15.35%). At the lower taxonomic rank, members of *Crenarchaeota* and *Euryarchaeota* were varied between the hot spring and borehole samples. In the hot spring sample (15UM), a high number of sequences assigned to the uncultured members of *Crenarchaeota i.e.* Miscellaneous Crenarchaeotic Group (MCG) and to the candidatus *Nitrososphaera*. While in the case of borehole sample (15UK), more sequences were assigned to the uncultured marine group II (*Euryarchaeota*). In addition, borehole samples also showed the presence of other members such as *Methanobacteriales*; genus, *Methanobacterium*, *Methanobrevibacter*, *Methanosaeta* and *Nitronococcus*.

2.3.3.3.2: Composition and diversity of Bacteria

Bacteria domain was the most predominant group found in all the samples. The taxonomic distribution at phylum level showed the presence of 22 and 12 bacterial phyla in the hot spring samples of 2014 (14UM) and 2015 (15UM) respectively. In case of borehole samples, presence of 21 bacterial phyla was observed in each sample *i.e.* 14UK, 15UK samples.

Among all the samples, hot spring sample of 2015 recorded with lowest number of phyla (12 phyla). Overall composition showed that the members of *Deinococcus-Thermus* (0.2%–36%), *Proteobacteria* (18.4%–37.4%), *Firmicutes* (5.7%–35.7%), *Bacteriodetes* (6.1%–14.1%), *Synergistetes* (7.4%–10.1%) and *Actinobacteria* (4.2%–11.1%) were predominant (Fig. 2.9).

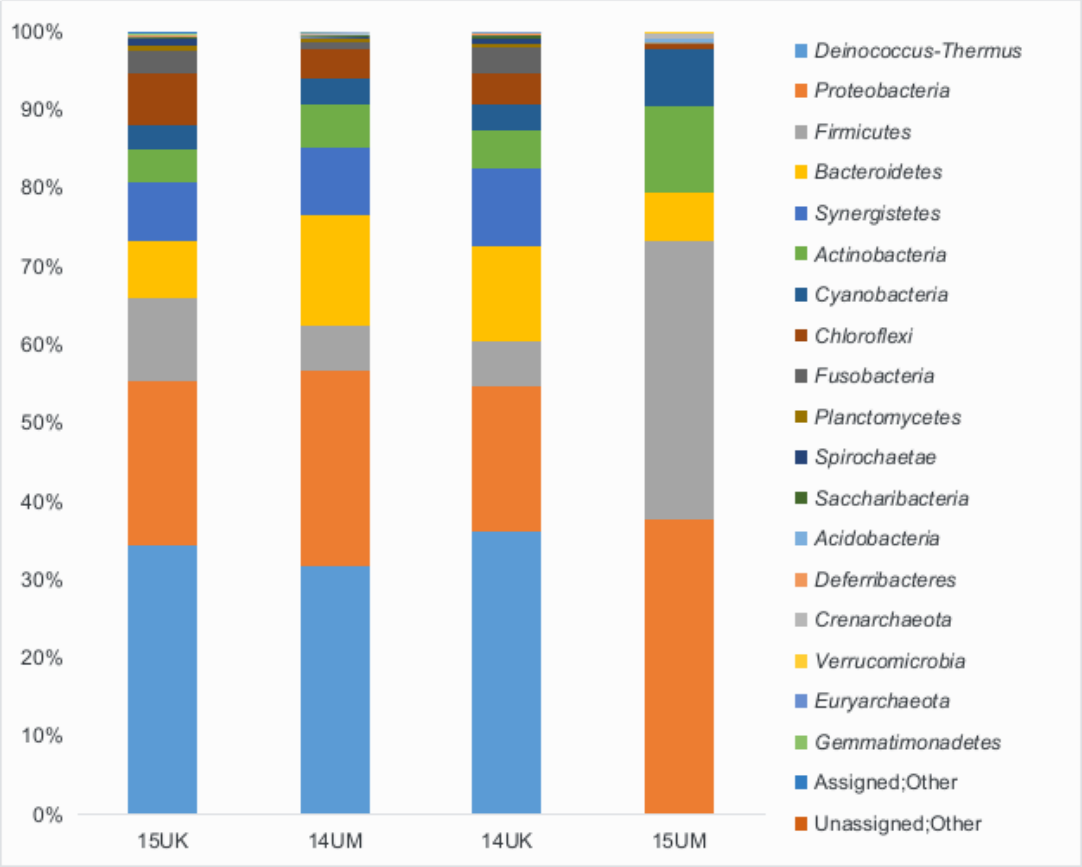

Fig. 2.9: Taxonomy classification of OTUs at the phylum level of hot spring and borehole water samples. Only dominant phyla are represented here.

Bacterial phyla such as phototropic members, *Cyanobacteria* (0.7%-6.6%) and *Chroroflexi* (0.1%-3.2%) were relatively present in lower abundance. In dominant phyla, the distribution of OTUs assigned to lower taxonomic members such as genera was greatly varied. For instance, *Deinococcus-Thermus* mostly represented by the uncultured members of *Meiothermus* (99.9% of OTUs assigned to *Deinococcus-Thermus*). While in case of

Proteobacteria and *Actinobacteria*, OTUs were found to assign to taxonomically different genera under different families (Fig. 2.10).

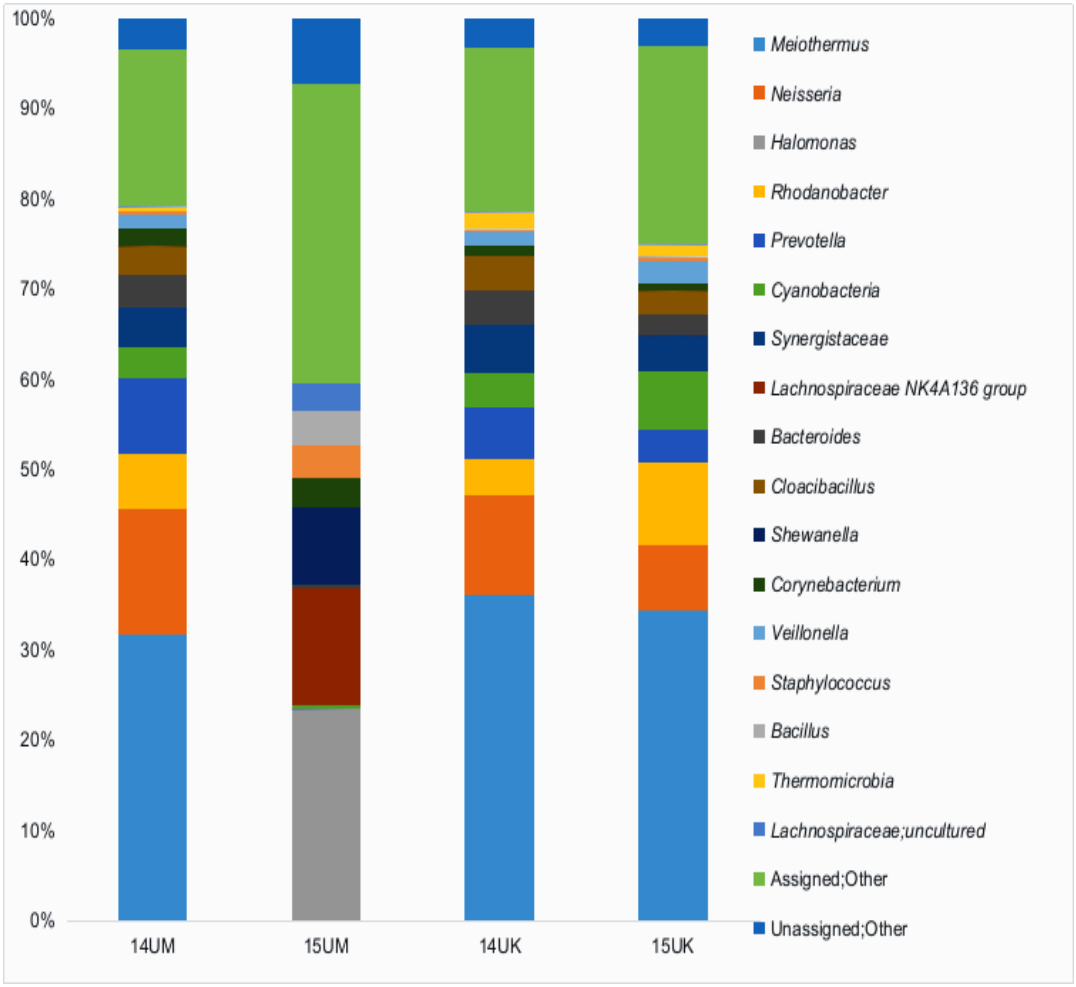

Fig. 2.10: Taxonomy classification of OTUs at the genus level of hot spring and borehole water samples. Only dominant phyla are represented here.

Among other minor community members such as *Fusobacteria*, *Planctomycetes*, *Spirochaetes*, *Acidobacteria*, *Deferribacteres*, *Gemmatimonadetes*, *Verrucomicrobia*, *Lentisphaerae*, *Tenericutes* and *Thermotogae* were present with <1% of total OTUs. Some candidate phyla (OTUs <1%) such as *Saccharibacteria*, *Gracilibacteria*, *Hydrogenedentes*, *Aminicenantes*, SHA-109, *Atribacteria* were also detected (Fig. 2.11).

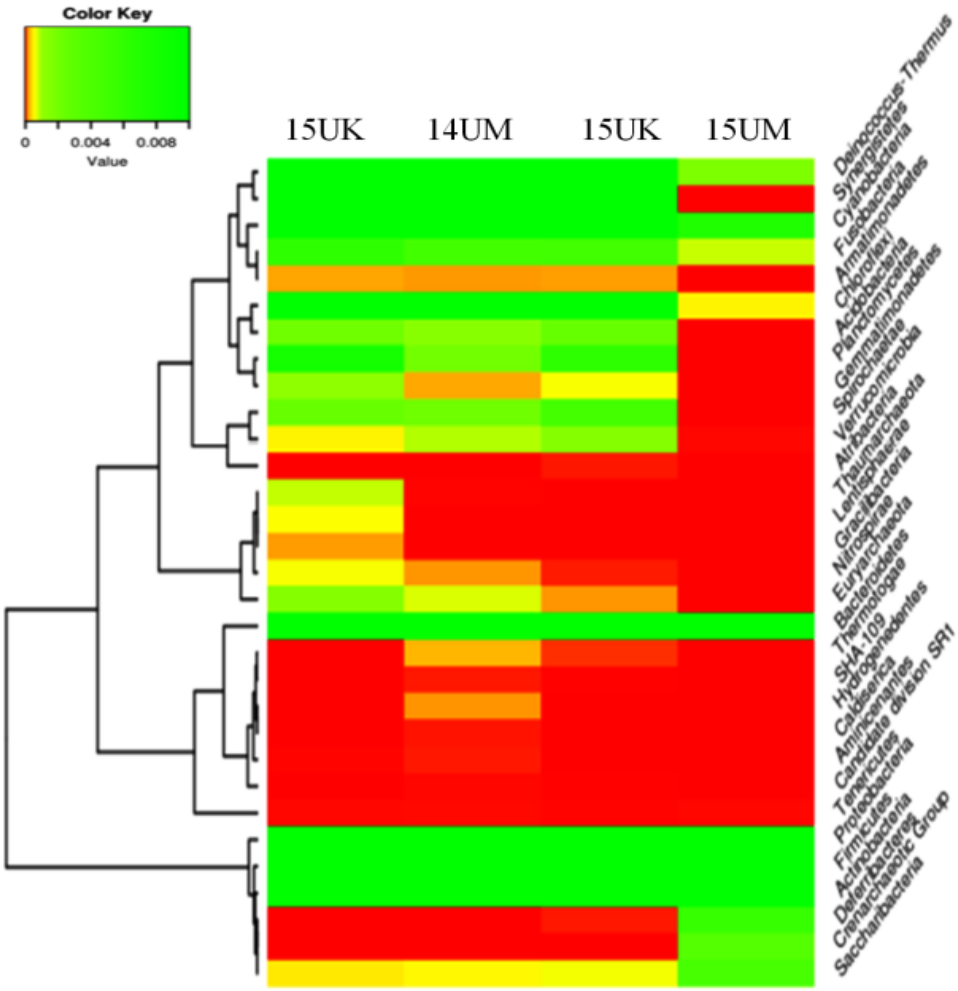

Fig. 2.11: A relative abundances of phyla among borehole and hot spring samples

In *Proteobacteria*, OTUs were assigned to five subdivisions *i.e.* alpha, beta, gamma, delta and epsilon *Proteobacteria* with highest dominance of *Gammaproteobacteria* (11.8% of total OTUs) and *Betaproteobacteria* (10.5% of total OTUs). However, both of these subdivisions varied in their abundances among the samples at lower taxonomic rank. For example, in *Gammaproteobacteria*, order *Xanthomonadales* (4.2%-9.3%) was present in all samples except in the 15UM sample, which had showed the dominance of *Oceanospirillales* (23%) including genera like *Halomonas*, *Alcanivorax* and *Shewanella*. Other members of the *Gammaproteobacteria* such as order *Chromatiales*, genus, *Acidiferrobacter*; order *Pasteurellales*, genus, *Photobacterium* were present in low abundance in all the samples.

Betaproteobacteria was majorly represented by the order *Neisseriales* in all the samples. Members of order *Burkholderiales*, genus, *Polaromonas*, *Lautropia*, *Variovorax*, *Ramlibacter*, *Cupravidus*, *Tepidomonas*, *Polynucleobacter*, order *Oxalobacteraceae*, *Undibacterium*, order *Methylophilaceae*, genus, *Methylobacillus*, orders *Nitrosomonadales* and *Rhodocyclales* were present in low abundance. Other subdivisions of *Proteobacteria* have almost similar distribution among all the samples. *Alphaproteobacteria* was represented by the order *Rhodobacterales*, genus, *Paracoccus*, *Defluviimonas*. Order such as *Desulfovibrionales* and *Bdellovibrionales* from *deltaproteobacteria* were present in most of the sample with slight variation in their abundances. These members include the sulphate reducing bacterial genera such as *Desulfovibrionaceae*, *Bilophila*, *Desulfovibrio*, *Desulfurellales*, *Desulfuromonadales*, *Nitrospina* and *Geobacter*. While in case of *Bdellovibrinales*, genus *Peredibacter* and some other uncultured species, which are also known as predatory bacteria, were present.

Phylum *Synergistetes* including genera such as *Cloacibacillus* and *Fretibacterium* was present in all the samples except in the 15UM sample. In contrast, members of *Firmicutes* were present in high abundance in this sample (15UM). Firmucutes of this sample has the dominance of *Bacteroidales* and *Clostridiales* including genera such as *Geobacillus*, *Gracilibacillus*, *Halobacillus*, *Gemella*, and *Planomicrobium*. Similarly, 15UM sample also found to content high dominance of the Actinobacteria expect the order *Frankiales* with genera such as *Geodermatophilus*, *Nakamurella*, *Acidothermus*, *Fodinicola* were present in the borehole samples. Although Unkeshwar hot springs has a moderate level of temperature which could support a high diversity of phototrophic bacteria, they have been observed in low abundances in both the sites. Among the *Cyanobacteria* and *Chloroflexi*, *Cyanobacteria* wer dominant as compared to *Chloroflexi*. Majorly, the detected genera were found to belong to *Synechococcus*, *Xenococcus*. In case of *Choroflexi*, diverse group members such as order

Anaerolineales, genus, *Longilinea*, *Ornatilinea*, orders *Ardenticatenales*, *Caldilineales*, *Chloroflexales* genus, *Roseiflexus* observed in borehole samples.

2.3.3.3.3: Alpha, beta diversity patterns

An overview of microbial diversity present in each sample was estimated using alpha diversity indices such as Shannon, Chao1 and observed species matrices. Random sequences were picked up to normalise the sequencing depth of for all the samples, then the metric calculations were performed using QIIME (Table 2.6).

Table 2.6: The alpha diversity indices of hot spring and borehole water samples

Site	Sample Name	Shannon H	Chao1	gini index	Number of OTUs
Hot spring	14UM	4.22	497.0	0.97	477
	15UM	4.66	238.0	0.96	238
Borehole	14UK	4.21	615.74	0.96	610
	15UK	4.37	463.63	0.96	455

The rarefaction curves based on Shannon metric showed that all the samples have reached near saturation (Fig. 2.12), except one sample of natural hot spring (15UM), which was found to be least sampled

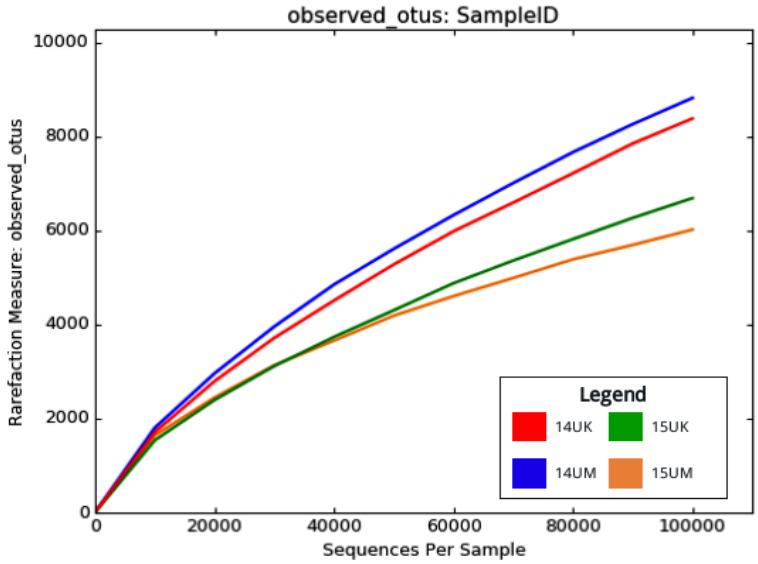

Fig. 2.12: Rarefaction analysis of OTUs borehole and hot spring samples

Beta diversity was documented for analysing the microbial community compositions among all the samples. Using PCoA with weighted UniFrac, PC1 explains 96.6% of the variation, while PC2 explain 2.9%. However, the sample (15UM) differs greatly from the other samples as observed an outlier in PCoA (Fig. 2.13) may be due to its lower richness than other samples as estimated by Cho1 analysis.

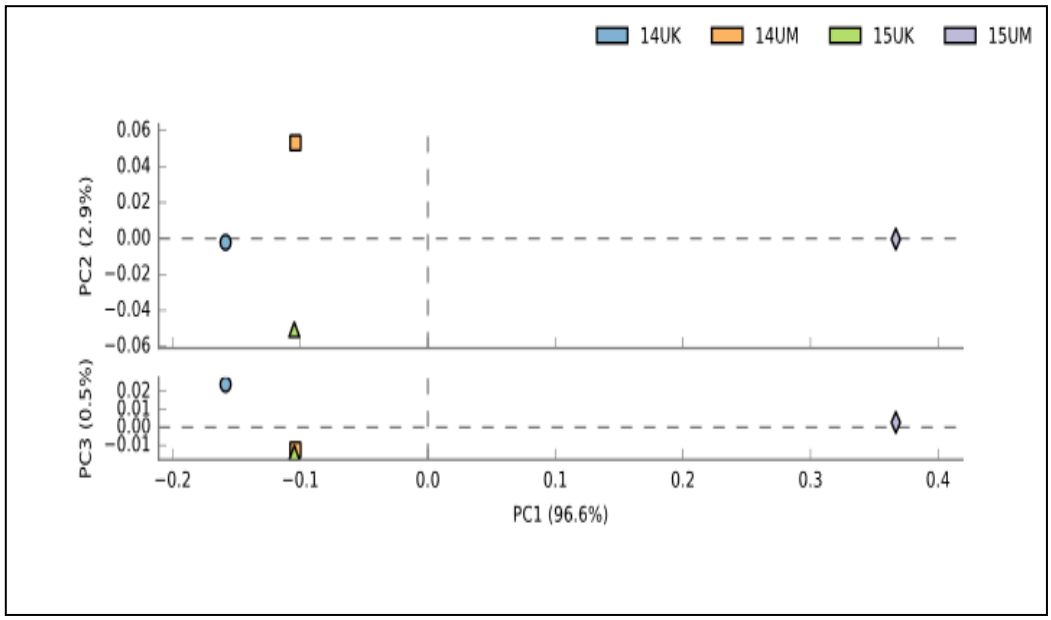

Fig. 2.13: Microbial communities of borehole and hot spring samples clustered using PCoA of the weighted Unifrac distance matrixes

In general, among the two annual samples, samples of 2014 of both the sites revealed with almost similar type of microbial compositions. However, in 2015 the sample of natural hot spring (15UM) has shown fluctuation in microbial composition of some of the microbial phyla (Fig. 2.14A). In the 15UM sample, a relative abundance was decreased for *Deinococcus-Thermus* (31.7% to 0.2%), *Bacteriodetes* (14.1% to 6.1%), *Synergistetes* (8.7% to 0.0%), *Chloroflexi* (2.5% to 1%) and an increase for *Firmicutes* (5.5% to 35.5%), *Proteobacteria* (25.0% to 37.5%), *Actinobacteria* (5.5% to 11.5%) from 2014 to 2015. Among the increased members of *Proteobacteria*, genera such as *Halomonas* and *Shewanella* were found with high abundance. Similarly, members of actinobacterial such as *Kocuria*,

Micrococcus, *Nocardiodes* and *Brachybacterium* were also observed with increased abundance. However, very little variations were observed from the borehole microbial communities in these both year samplings (Fig. 2.14B).

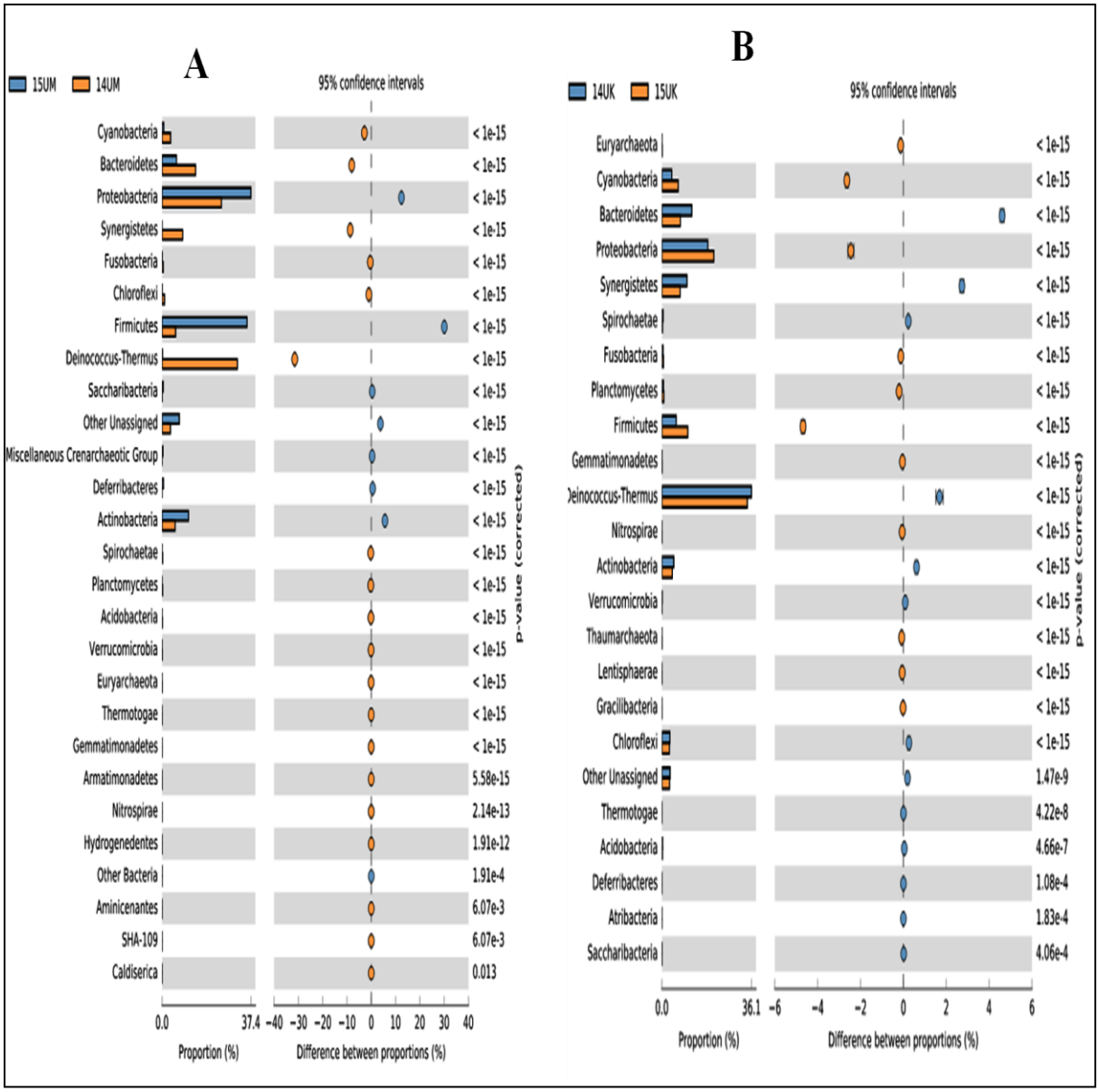

Fig. 2.14: Overall distribution at the phylum (A) and genus (B) level of hot spring and borehole samples

For instance, *Deinococcus-Thermus* and *Synergistetes* were barely present in 2015 in the hot spring sample, while in the borehole they decreased only by 1.7% and 2.1% respectively. Overall, dominance of *Deinococcus-Thermus*, *Proteobacteria*, *Firmicutes*, *Bacteriodetes*,

Synergistetes, and *Actinobacteria* was observed in both the microbial communities. However, greater dissimilarity in community composition was observed between hot spring samples collected in 2014 and 2015 as compared to borehole samples. A relative decrease in *Deinococcus-Thermus* (31.7% to 0.2%), *Bacteriodetes* (14.1% to 6.1%), *Synergistetes* (8.7% to 0.0%), and *Chloroflexi* (2.5% to 1%) and an increase in *Firmicutes* (5.5% to 35.5%), *Proteobacteria* (25.0% to 37.5%) and *Actinobacteria* (5.5% to 11.5%) was observed from 2014 (14UM) to 2015 (15UM) samples (Table 2.7).

Table 2.7: A relative abundances of dominant phyla among hot spring and borehole samples

Phylum	**14UM**	**15HK2**	**14UK**	**15UK**
Deinococcus-Thermus	31.5%	0.20%	36.0%	34.3%
Proteobacteria	14.0%	37.0%	18.0%	21.0%
Bacteriodetes	9.0%	6.0%	12.0%	7.0%
Firmicutes	6.0%	36.0%	6.0%	10.0%
Actinobacteria	5.4%	11.0%	4.7%	4.3%
Synergistetes	8.6%	ND	10.1%	7.3%

Other important factors such as exposure to rainwater and human activities possibly might have contributed to such changes in geochemical and microbial compositions in hot spring than its associated borehole microbial communities. The study inferred that microbial communities inhabiting the Unkeshwar hot spring occurring hot springs are more prone to environmental disturbances than the boreholes.

2.3.3.3.4: Correlation with physicochemical factors

A correlation among the microbial community members and physicochemical factors was analysed using CCA, a multivariate analysis. Some of the community members were positively correlated with few of the physicochemical parameters, while inverse correlations were also observed among other members and factors (Fig. 2.15).

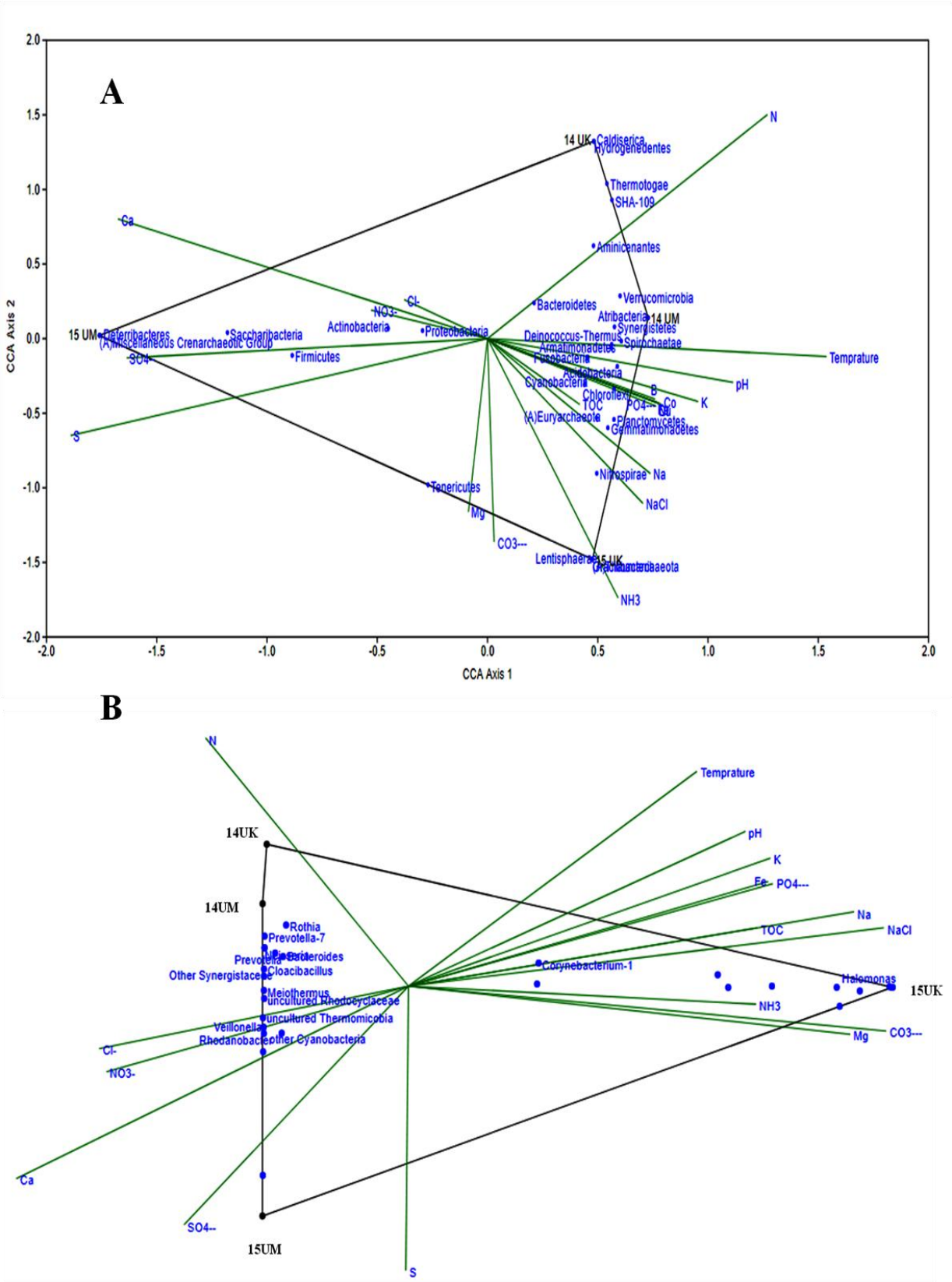

Fig. 2.15: Canonical correspondence analysis (CCA) of microbial phyla (>0.1% OTUs assigned) in relation to physicochemical variables.

In case of temperature, members of *Nitrospirae*, *Fusobacteria*, *Spirochaetes*, *Acidobacteria*, *Deferribacteres*, *Verrucomicrobia*, *Lentisphaerae*, *Tenericutes* and *Thermotogae* were found to positively correlate with the temperature of both the natural hot spring and borehole. Other inherent geochemical factors were found to have an impact on different members of the microbial community. As most of the variables of three samples including two annual samples of borehole (14UK, 15UK) and one from natural hot spring (14UM) were found on the same axis on the right side of the plot, CCA axis showing little variations among these three sites (Fig. 2.15). Only the 15UM sample present on the left side of the plot revealing that it is very different from other samples. Phyla like *Proteobacteria*, *Firmicutes*, *Deferribacteres*, Candidates phylum; *Saccharibacteria*, and *Crenararcheatia* (archaea) were positively correlated with sulphate and nitrate content. Among the *Proteobacteria* members such as *Desulfovibrio*, *Desulfurellales*, *Desulfuromonadales*, *Syntrophobacterales* were found to be associated with samples containing higher concentration of the sulphate. While, members of *Nitrospina*, *Saccharibacteria* and archaeal members of MCG were positively correlated with nitrate content. In case of *Actinobacteria*, members were found to be positively correlated with chloride and calcium ions, however, the relation of such ions with *Actinobacteria* was not understood, and perhaps maybe due to that actinobacterial would require it for their growth as these bacteria form spore which are dormant. Among the phototropic bacteria, *Cyanobacteria* and *Chloroflexi* were positively correlated with the phosphorus content of hot springs.

2.3.3.4: Mining for functional metabolic potentials

The functional diversity of springs was studied using Tax4Fun and the inferences were analyzed to understand microbial survival strategies and adaptation in extreme niches. The tool Tax4Fun works on R software and gives an output of the predicted functional profile based on the taxonomic diversity profile created using SILVA taxonomy.

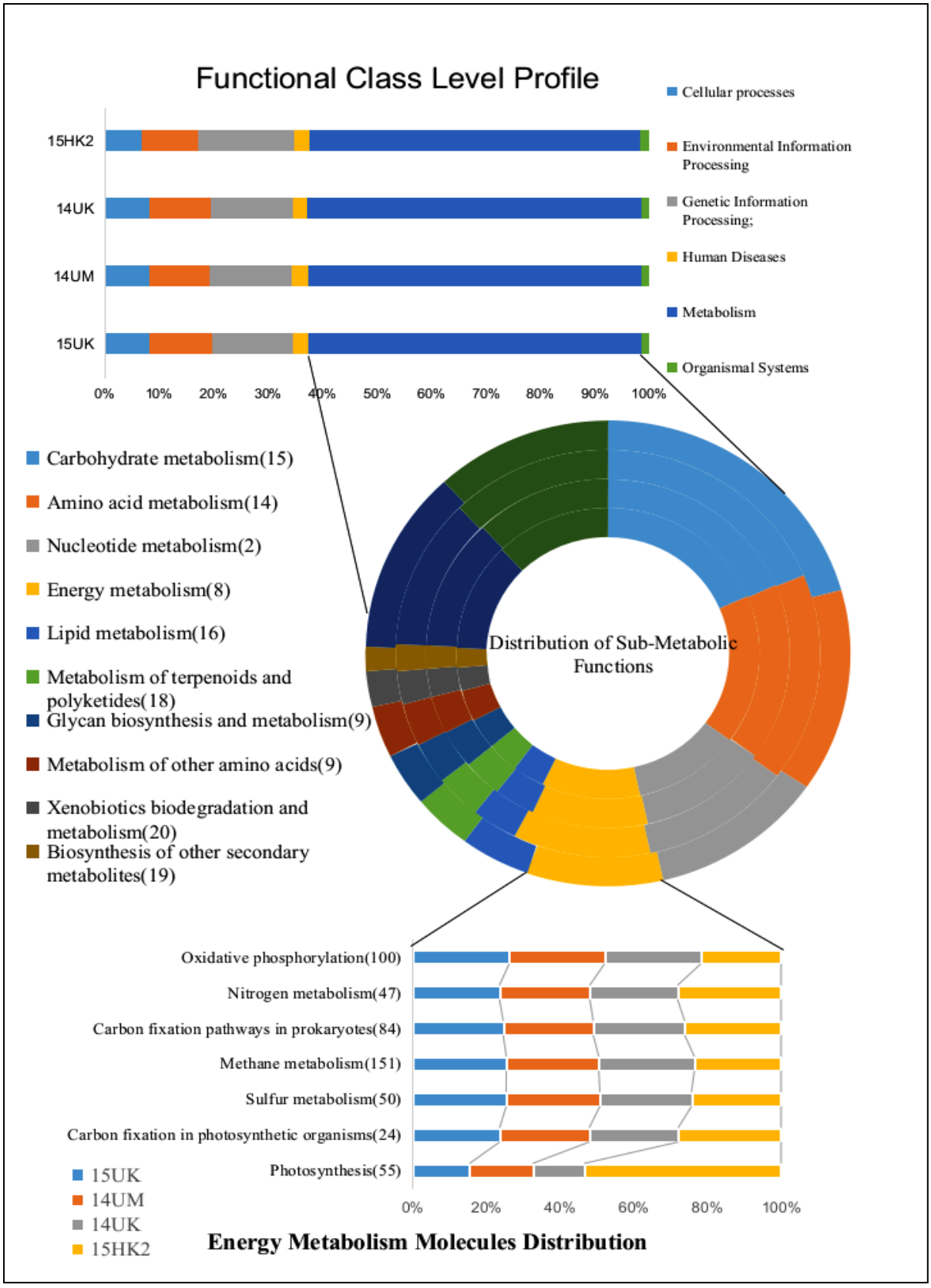

Fig.2.16: Functional analysis based on KEGG pathway of hot springs and borehole samples and subsystem level distribution of metabolism

Tax4Fun predicted the presence of 6581molecules from the taxonomic profiles. Further using our own in-house scripts, identified 321 pathways from 3626 molecules from the KEGG database, the remaining 2955 molecules did not have any known pathways in KEGG. If the molecule was known to be present in multiple pathways then the functional profile values were divided among the pathways. Further 321 pathways were classified according to their class level functions, which indicated that all four samples had a similar distribution of functions and metabolism being the most abundant followed by genetic information processing and environmental information processing (Fig. 2.16). Carbohydrate metabolism was found to be most frequent, followed by amino acid metabolism, Co-factor and vitamins metabolism, nucleotide metabolism, and energy metabolism. Detailed analysis of KEGG pathways at subsystem level was carried out. Interestingly it was found that almost 20 pathways were assigned to xenobiotic degradation (Fig. 2.17).

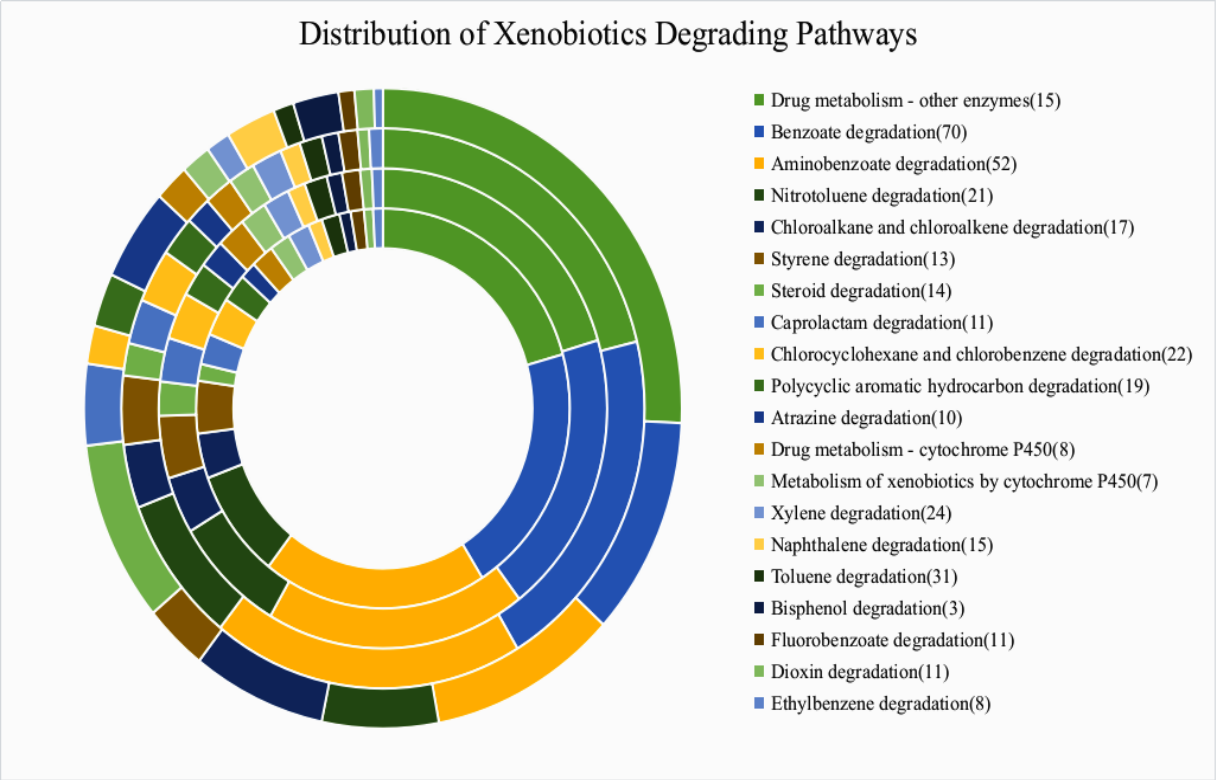

Fig. 2.17: KEGG based functional metabolism at subsysten level of xenobiotics degradation

Number of genes associated with degradation of benzoate, aminobenzoate compounds, chlorocyclohexane, polycyclic aromatic hydrocarbons (PAH) compounds, xylene, toluene, naphthalene and with many other compounds have been predicted from this analysis (Fig. 2.17). In particular, high numbers of molecules were detected for intermediates metabolites of some PAHs such as benzoate/amino-benzoate degradation (Fig. 2.18).

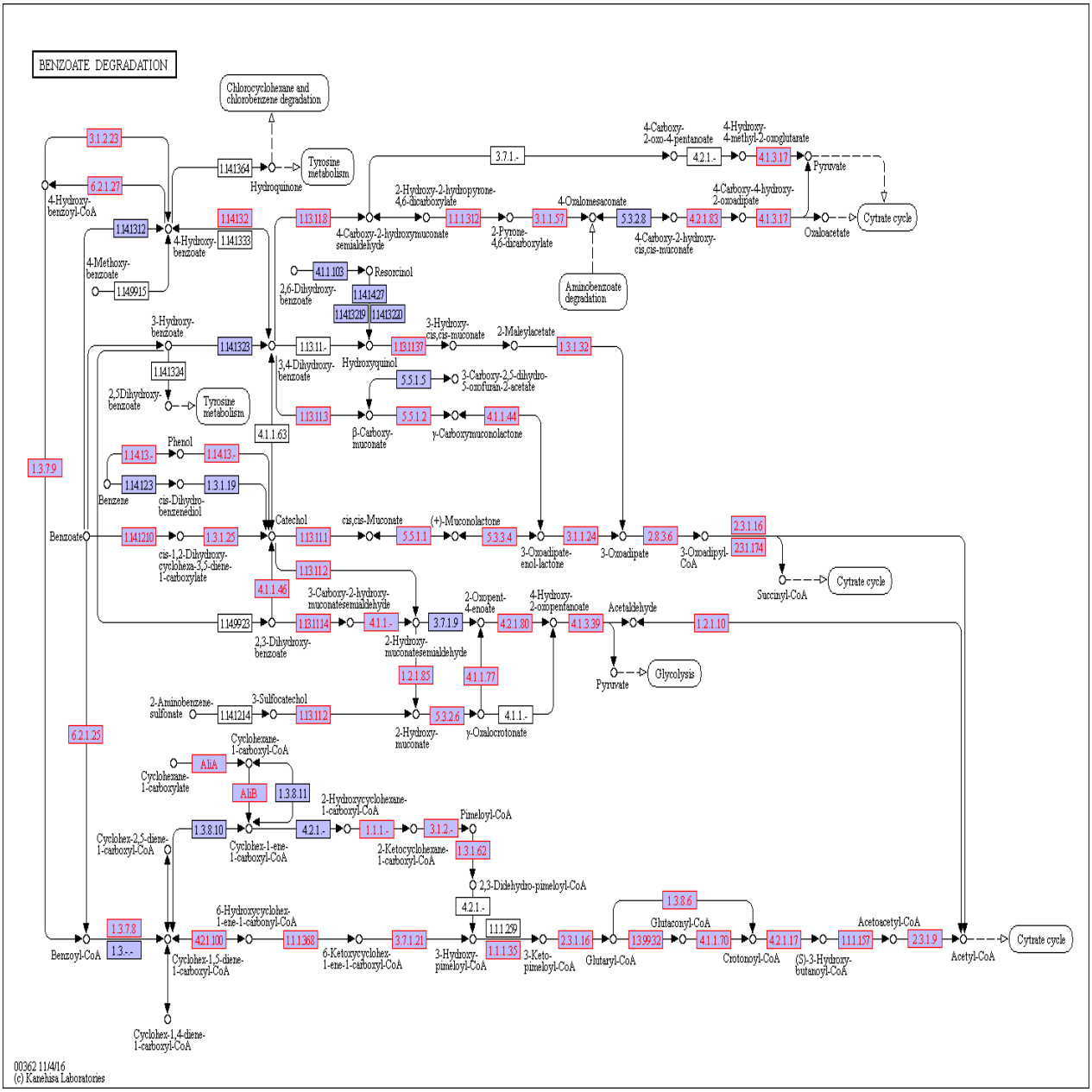

Fig. 2.18: A metabolic pathway map of benzoate degradation showing the observed KOs (highlighted in red).

It is also interesting to observed genes associated with herbicide degradation such as atrazine, dioxins, caprolactam, and many other potential pollutants. Atrazine, toxic pollutant used as herbicides for the control of the weed. In the analysis, few genes were also mapped for degradation of atrazine (Fig. 2.19).

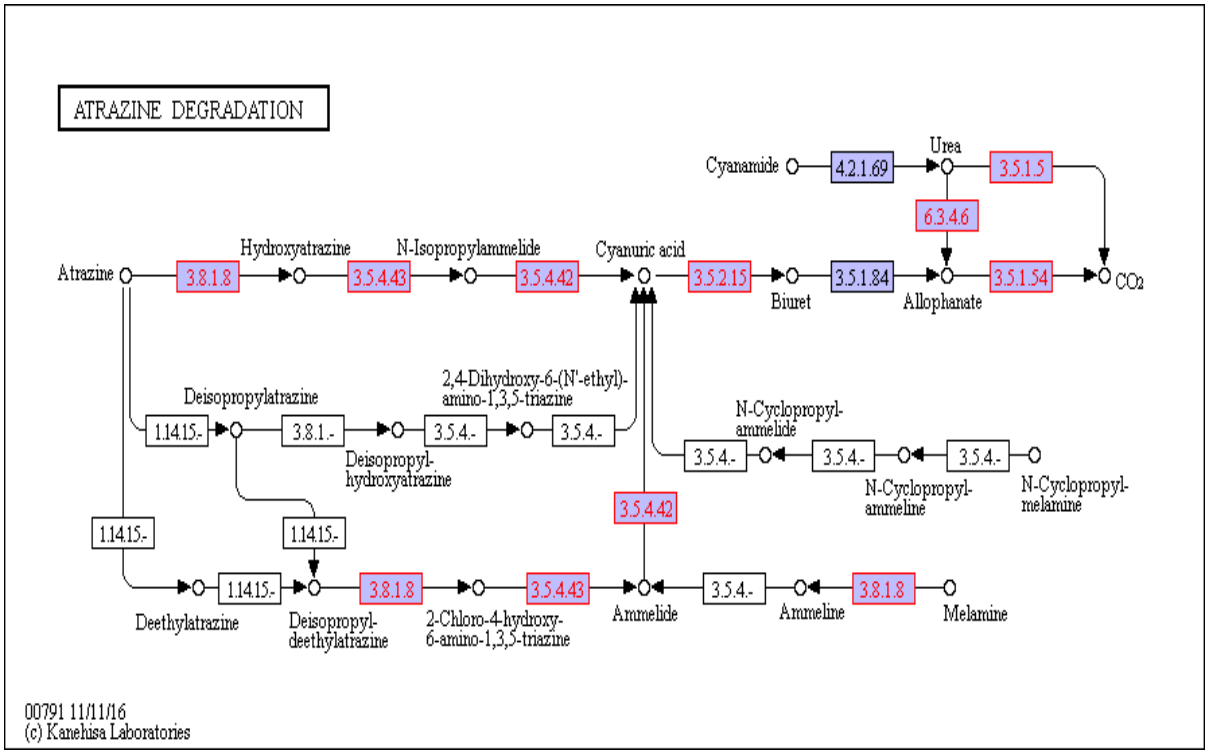

Fig. 2.19: A metabolic pathway map of atrazine degradation showing the observed KOs (highlighted in red).

Similarly, pathways were also detected assigned to energy utilization and among which most are part of sulfur metabolism and methane metabolism. This could indicate that the majority of microorganisms were Chemolithotrophs and have a key physiological role in biogeochemical cycles such as sulfur cycling. Also, the correlation between these energy metabolism molecules and environmental factors showed that sulfur metabolism was positively co-related with higher sulfur/sulfate content.

2.3.3.5: Comparison with other Indian hot springs

Unkeshwar hot spring has a moderate temperature ranges, therefore host a high bacterial diversity. To get an insight into the overall diversities of various Indian hot springs, a comparative analysis was performed. It has been reported that the composition of the microbial population residing in hot springs often influenced by the geographical conditions and geochemistry of a particular hot spring. Previously studied Indian hot springs such as Atri (temperature 45ºC - 60ºC, pH, 7.4) and Taptapani (temperature 42ºC; pH 7.2) (Sahoo et al. 2015), Bakreshwar (66ºC - 70ºC; pH 9.1- 9.3), (Choudhari et al. 2017), Manikaran (90ºC-96ºC; pH 7), (Bhatia et al. 2015a), Yumthang (temperature 39ºC, pH 8-9) (Panda et al. 2016), Puga valley (50ºC - 60ºC, pH 7) (Gupta et al. 2017) and Soldhar (90ºC - 95ºC, pH 7.8) (Sharma et al. 2017) were selected for the analysis (Fig. 2.20B). An overview of microbial diversities present in each hot spring was estimated using alpha diversity indices such as Shannon, Chao1 and observed species matrices. Random sequences were picked up to normalize the sequencing depth of for all the samples, and then the metric calculations were performed using QIIME. The rarefaction curves based on observed species indices showed that all the samples have reached near saturation (Fig. 2.20A). Using PCoA with weighted UniFrac, beta diversity between the samples was documented. PC1 explains a 28.87%, of variation, PC2 explained 19.63% and PC3 explained 13.87% of the variation among the microbial diversities of these hot springs (Fig. 2.20C). The PCoA plots could cluster these hot springs based on the variation. Among these eight hot springs, Unkeshwar, Puga valey, Soldhar, Taptapani, and Yumthang were clustered together (cluster I), while Bakreshwar and Manikaran have formed second cluster, whereas Atri was found to be an outlier in the plot. This could be explained based on the temperature profile and relative abundances of phyla present in each hot spring. The five hot springs including Unkeshwar clustered together, although they were not from the same geographical regions but having a moderate

temperature ranges. Therefore the phylum distribution was found more similar in these hot springs. Manikaran and Bakreshwar, being the highest temperature hot springs, were clustered together and represented the predominance of few thermophilic phyla. Atri although has a moderate temperature, was found to be outlier may be due to other geological conditions.

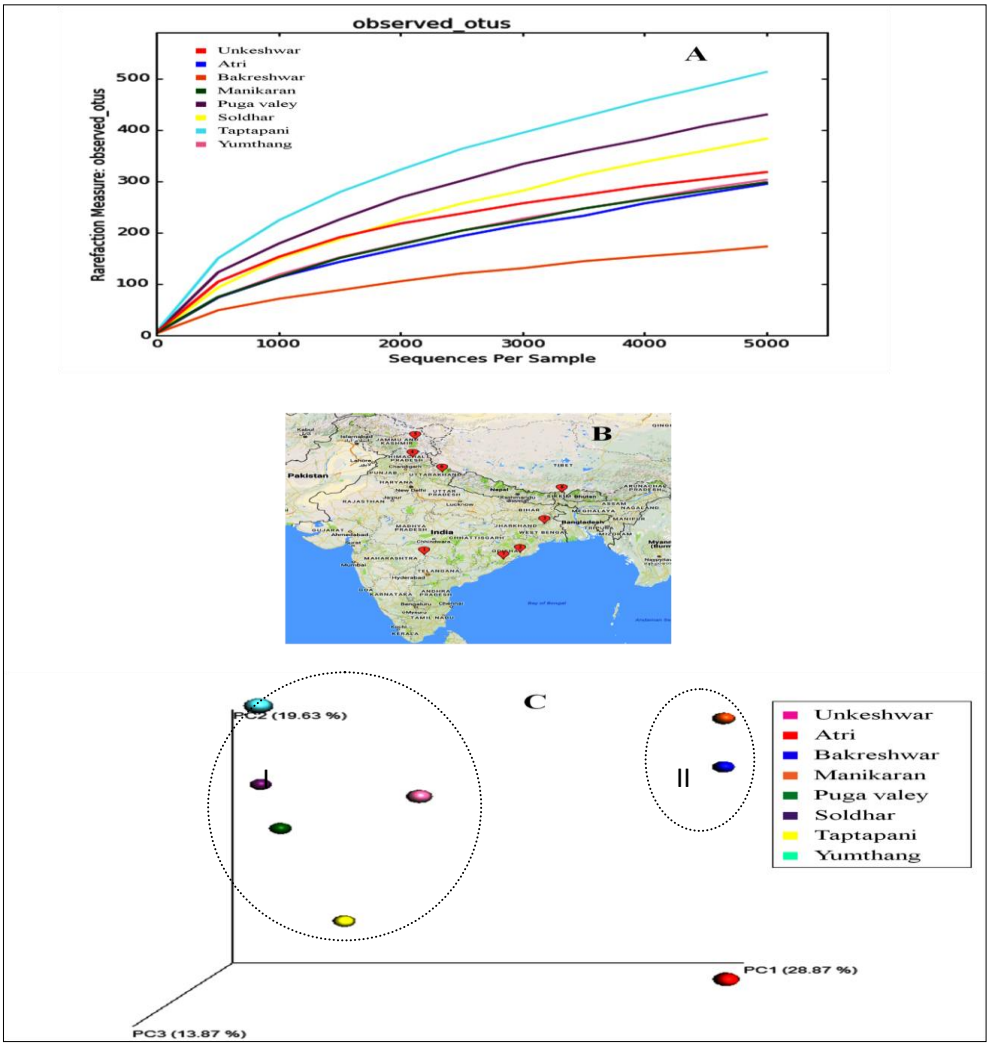

Fig. 2.20: A comparative account of alpha and beta diversity analysis of selected Indian hot springs

Overall it could be inferred that the temperature is one of the important factors which controls the microbial compositions of the hot springs. Here, all the analysed hot springs were found to be dominated by *Proteobacteria* and *Firmicutes*. However, the compositions of the other

dominant groups were found distinct in each of the hot spring. The microbial communities of Bakreshwar and Manikaran hot springs were dominated by *Deionococcus-Thermus* (72% in Bakreshwar, 17% in Manikaran), *Firmicutes* (17% in Bakreshwar, 44% in Manikaran). In addition, Manikaran hot spring also host a high proportion of hyperthermophilic phyla such as *Aquificae* (39%) may be because of its highest temperature as compared to Bakreshwar hot spring. The occurrence of such thermophilic and hyperthermophlic phyla is obvious in these hot springs, as both of these hot spring has the highest temperature range (Bakreshwar, 70ºC), Manikaran, 95ºC) than all other hot spring evaluated in this study. Hot springs, which formed another cluster (those having moderate temperature ranges) were dominated by *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, and *Bacteroidetes* (Fig. 2.21).

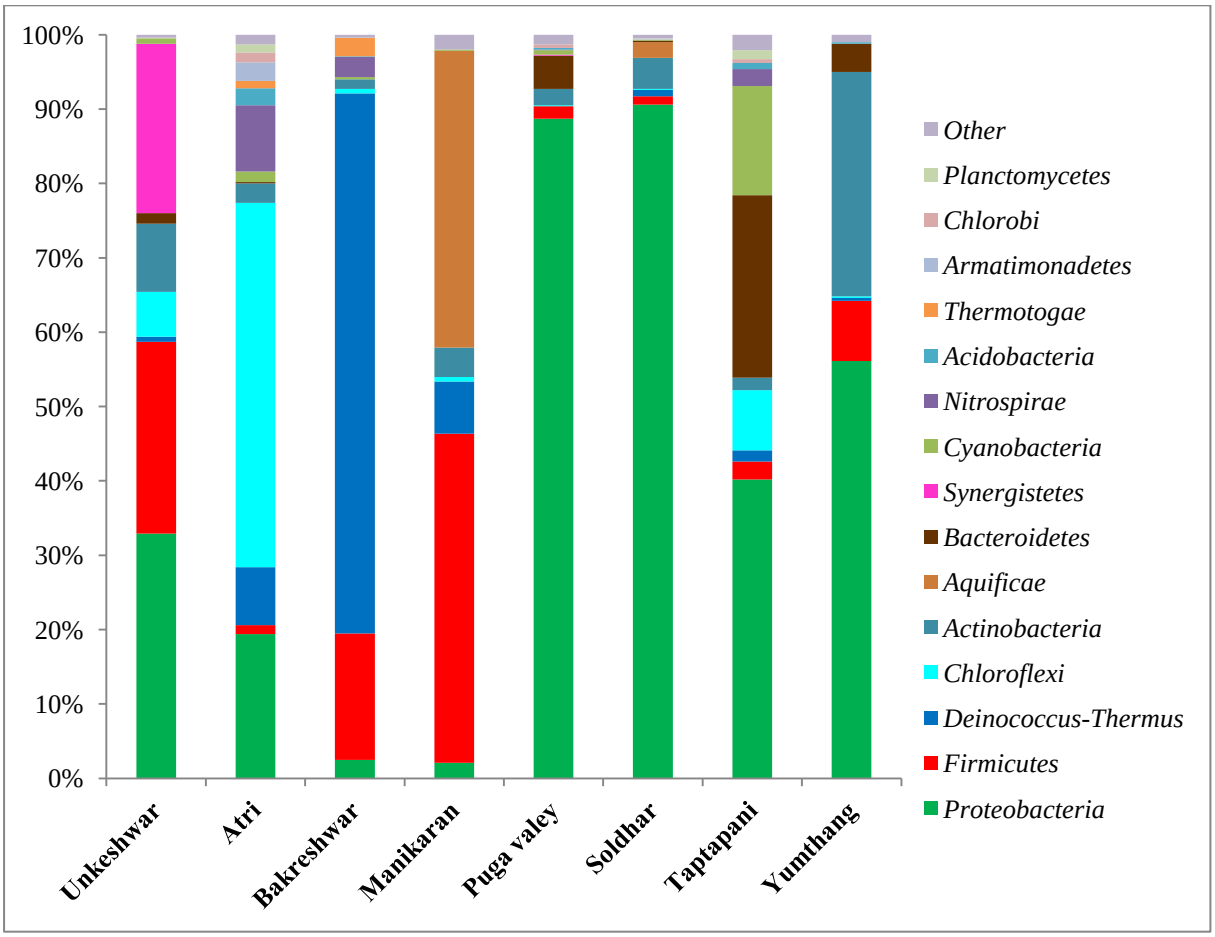

Fig. 2.21: Taxonomy classification of OTUs at the phylum level of selected Indian hot spring. Only dominant phyla are represented here.

At the genus level, a similar trend of relative abundance was observed. In Bakreshwar, highest abundance of the thermophilic genera such as *Thermus* (72%) and *Caldicellulosiruptor* (16%) was recorded. In Manikaran, genera like *Bacillus* (32%) and *Hydrogenobacter* (39%) were found as dominant genera. Interestingly, in cluster I, Unkeshwar hot spring had shown the highest abundances of *Neisseria* (22%) and *Synergistes* (27%), both of these genera were not detected in any other hot spring. While in the case of other genera, each hot spring has shown distinct abundances indicating that the microbial composition of each of the hot spring is different maybe because of the geographic location and temperature conditions. Therefore it could be inferred that the dissimilarities/similarities found among the various taxonomic groups in different Indian hot springs were may be due to differences in the geochemical conditions, mostly because of the temperature conditions.

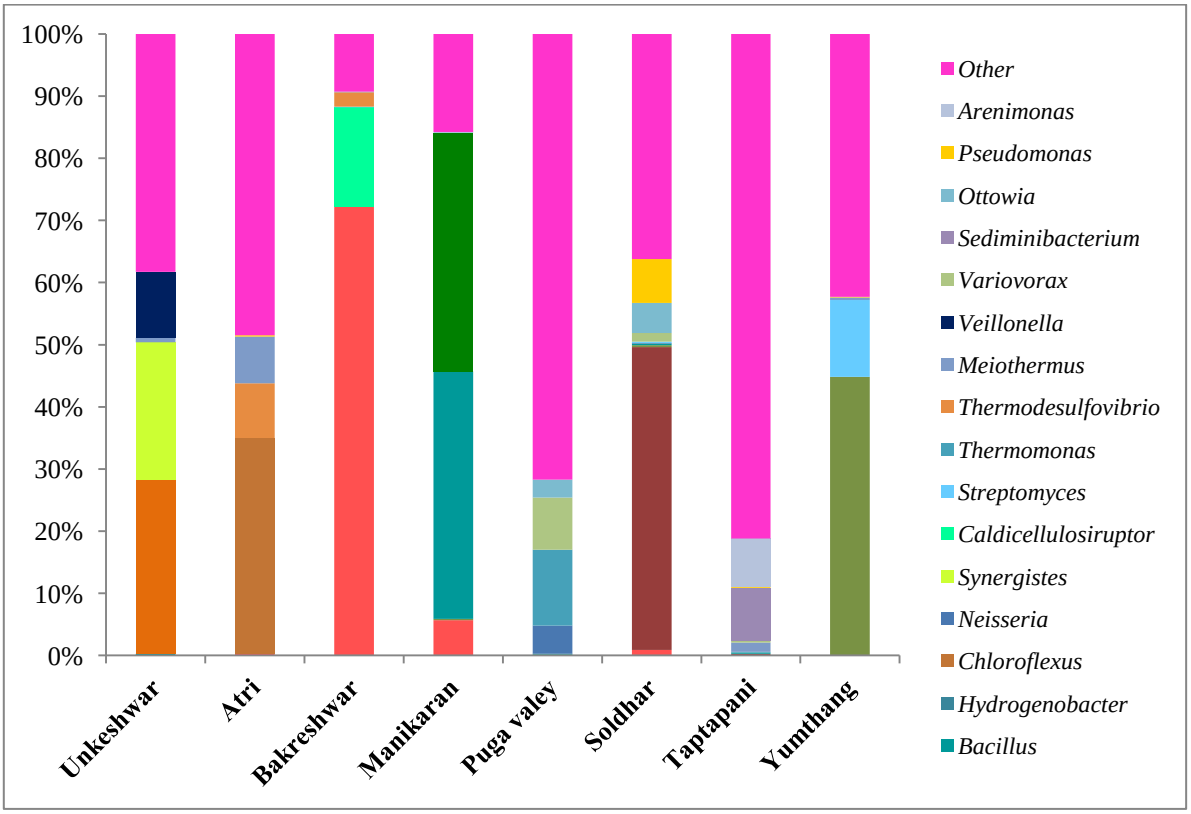

Fig. 2.22: Taxonomy classification of OTUs at the genus level of selected Indian hot spring. Only dominant phyla are represented here.

2.3.4: Whole metagenome (shotgun) sequencing analyses

2.3.4.1: Insights into structure and functions of water

Whole metagenome shotgun sequencing of 12UM sample was performed using the Illumina HiSeq 2500 sequencer (Illumina, USA). Whole metagenome shotgun sequencing revealed a total of 848,096 reads with 50.87 % G+C content. After trimming and assembly, a total 34,123 contigs were obtained. Taxonomic profiling was performed using NCBI taxonomy datasets for 21,424 reads and revealed 41 phyla including bacteria and archaea. The contig sequences presented by *Actinobacteria* (56%), *Verrucomicrobia* (24%), *Bacteroides* (13%), *Deinococcus-Thermus* (3%) and *Firmicutes* (2%) as shown in figure 2.23.

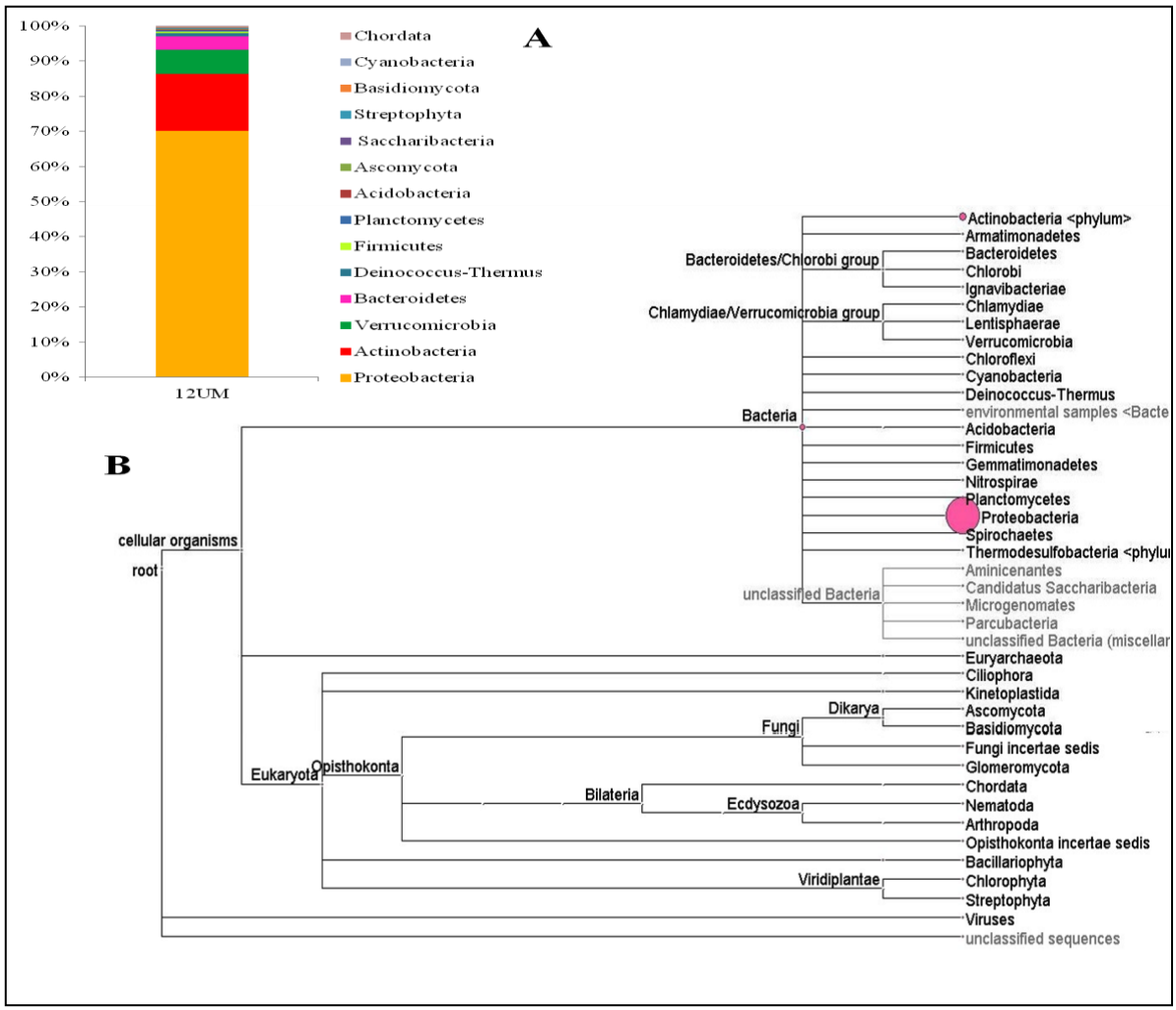

Fig. 2.23: Taxonomic profiling showing the Phylum abundance and phylogenetic relationship among the community members

At the phylum level, dominant bacterial phyla were *Actinobacteria*, *Bacteroides*, *Deinococcus-Thermus, Firmicutes* and *Planctomycetes*. Bacterial genera like *Rhodococcus, Microbacterium, Propionibacterium, Flavobacterium, Deinococcus, Caulobacter, Brevundimonas, Methylobacterium, Paracoccus, Roseomonas, Novosphingobium, Sphingomonas, Achromobacter, Acidovorax,* and *Aquabacterium* were also dominant. At the species level, *Opitutus terrae* (33%), *Rhodococcus erythropolis* (17%), *Cellovibrio mixtus* (10%) were found dominant species (Fig. 2.24A).

Prediction of open reading frames (ORFs) in the assembled contigs was done using Glimmer-MG tool (Kelley et al. 2012) and the complete functional annotation along with contig ID, gene function and sequences were carried out. For functional annotation, the contigs of 12UM were queried to BLASTX program with an optimum e value of 1e-10. The gene or protein functions of all the contigs from BLASTX output was parsed using in-house PERL (Practical Extraction and Report Language) script. Further, functional annotation was carried out by doing KEGG (Kyoto encyclopedia of genes and genomes) analysis based on taxons. The protein functions of each contig having the highest alignment score from BLASTX results were considered for functional assignment. Pathway annotation was done using KEGG pathway analysis was performed for each contig sequences by assigning KEGG Orthology (KO) numbers obtained from known reference hits. Around 65% contig sequences were assigned to metabolism, 20% unclassified sequences, 9% organismal system, 3% environmental information processing, 1% cellular processes and 1% human diseases (Fig. 2.24B). Sample read counts of KEGG annotation were metabolism (1990), organismal systems (283), environmental information processing (81), human diseases (37), genetic information processing (36), cellular processes (11) and unclassified (616). KEGG pathway analysis had shown the higher number of sequences were contributed to metabolism shows the larger edge size.

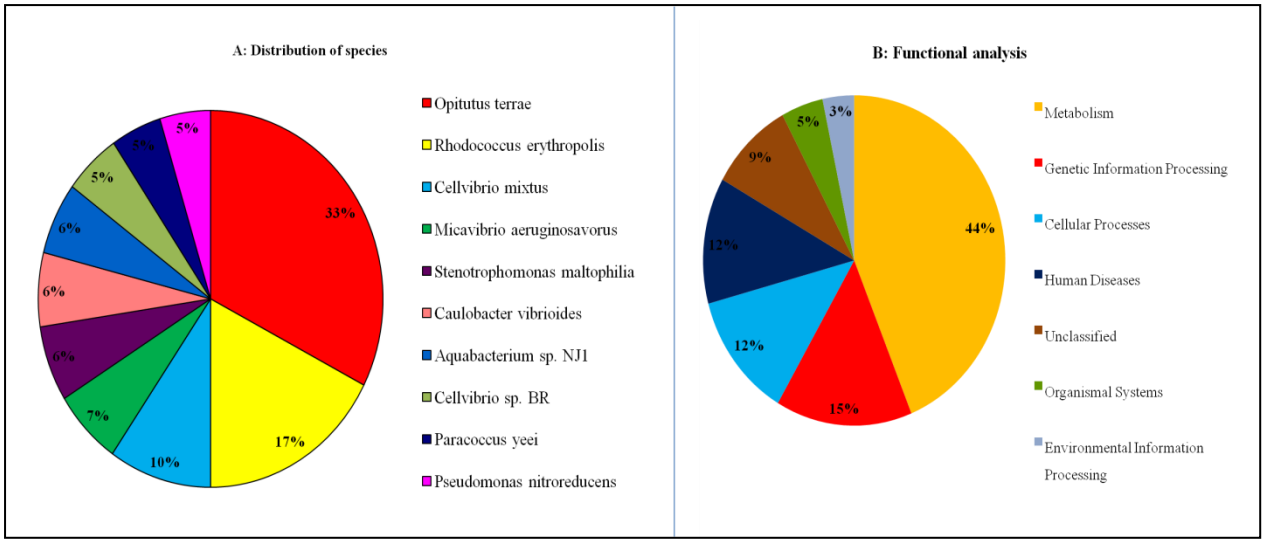

Fig. 2.24: Species level abundance (A) and functional profiling (B)

2.3.4.2: Insights into structure and functions of sediment samples

For a more comprehensive analysis using the whole metagenome sequencing analysis, we also analyzed the microbial diversity of sediment samples collected in two consecutive years *i.e.* 2015 (15KS1) and 2016 (16KS1). The results revealed that both samples showed an almost similar relative abundance of phyla composition. In these samples, a predominance of *Proteobacteria* (25-21%), *Bacteroidetes* (7.7-3.8%), *Firmicutes* (6.8-4.2%), *Actinobacteria* (4.4-4.3%) and *Verrucomicrobia* (2.4-.2.3%) was observed (Fig. 2. 25A). However, in 2016 sample (16KS1), a decrease in relative abundance of *Proteobacteria* (4%), *Bacteroidetes* (2.9%) and an increase in *Firmicutes* (2.6%), and *Actinobacteria* (0.1%) was observed. At the genus level, most of the OTUs were assigned to unclassified members of bacteria and eukaryota. Among the assigned OTUs, genera such as candidatus *Solibacter*, *Xanthomonas*, *Cytophaga*, *Nitrospira*, *Clostridium*, *Flexibacter*, *Verticillium*, and *Pseudomonas* were found with the highest OTUs (Fig. 2.25B). However, in 2015 sample (15KS1); *Xanthomonas*, *Cytophaga*, and *Flexibacter* have the higher relative abundance than the 2016 sample.

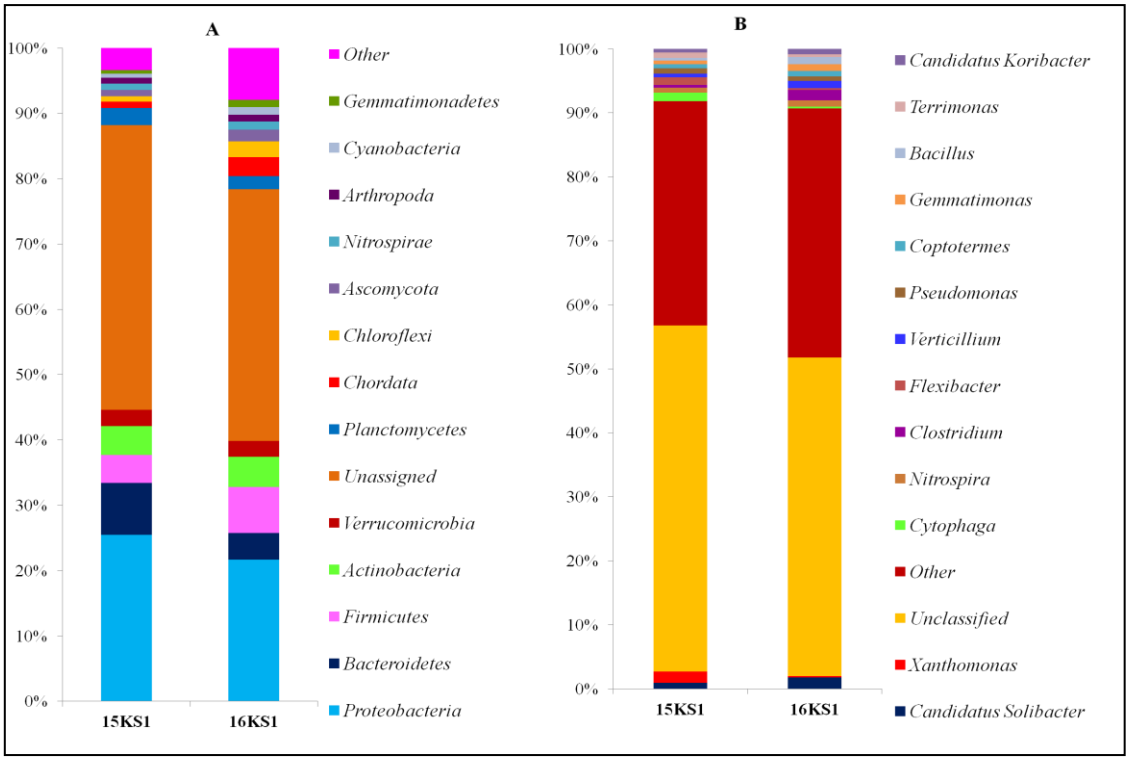

Fig. 2.25: A comparative analysis of sediment samples of main hot spring at phylum level (A) and genus level (B) collected in 2015 and 2016.

Functional analysis was performed using pathway annotation using KEGG pathway analysis as described for a water sample. In this case, most of the contig sequences (58%) were assigned to assign to metabolism followed by genetic information processing (18%), environmental information processing (17%) and organismal systems (6%) as shown in figure 2.26A. In the metabolism, highest sequences were assigned to carbohydrate metabolism (52%) followed by amino acid metabolism (35%), (Fig. 2. 26B). Some of the sequences were also assigned to xenobiotic metabolism and biosynthesis of secondary metabolism. Further cauterization of carbohydrate and xenobiotic metabolism has shown some differences in the relative abundances of sequences assigned to metabolic pathways (Fig. 2.26C and D). In carbohydrate metabolisms, highest metabolism of pyruvate, glycolysis, citrate cycle (TCA cycle) and glyoxylate and dicarboxylate metabolism was observed as compared to 16KS1 samples. Similarly, in xenobiotic metabolism, a relatively

higher number of sequences were assigned to benzoate degradation, nitrotoluene degradation chlorocyclohexane, and chlorobenzene degradation and aminobenzoate degradation in 2015 sample (15KS1). Such observations probably indicate that the microbial communities of 2015 samples were more metabolically active than the 2016 samples.

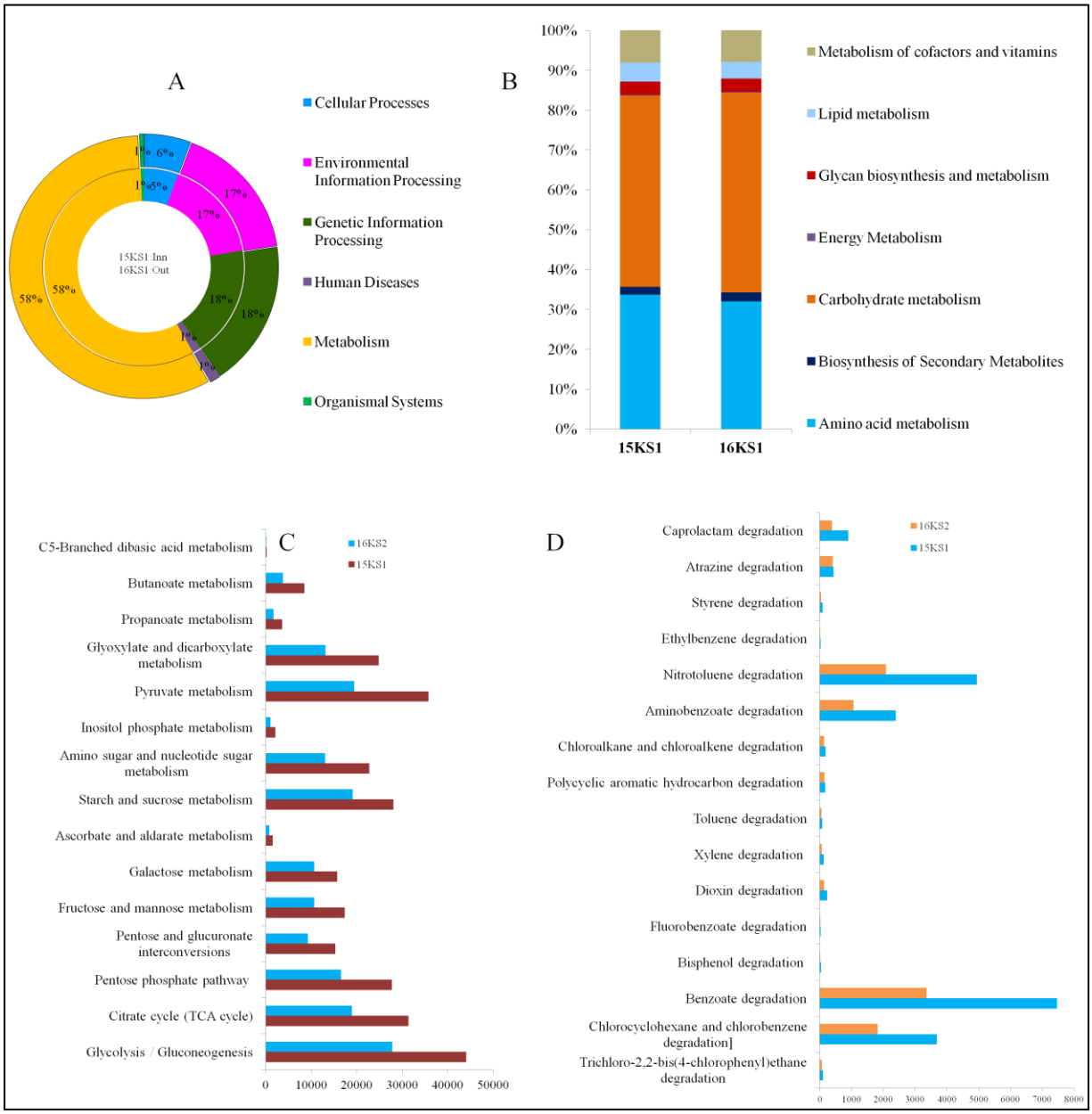

Fig. 2.26: Functional analysis of water sample microbial communities at Subsystem level 1 (A), Subsystem level 2; metabolism (B), detail pathway annotation of carbohydrate metabolism (C) and xenobiotic metabolism (D).

2.3.4.3: Profiling using Illumina and Nanopore sequencing

With recent advances in genomics and sequencing technologies, microbial community analyses using different sequencing platforms are being performed for validation of data and for bias free analysis. Hot springs microbial communities were also studied using various sequencing techniques and platforms. Here, we performed the sequencing analysis of sediment sample (15KS1) using two different sequencing techniques and compared the taxonomical and functional attributes of the microbial communities.

2.3.4.3.1: Microbial diversity

Overall microbial composition was found similar in analyses of both the techniques; however the relative abundance of few phyla was varied. The archaeal OTUs were assigned to five different archaeal phyla *i.e, Euryarchaeota, Crenarchaeota, Thaumarchaeota, Korarchaeota and Nanoarchaeota* (Fig. 2.27A). The most abundant was *Euryarchaeota* followed by *Crenarchaeota* and *Thaumarchaeota*. However, in case of Illumina sequencing, *Euryarchaeota* reads were found more as compared to the MinION nanopore analysis. In addition, Illumina also showed the presence of other phyla such as *Korarchaeota and Nanoarchaeota* in higher abundant as compared to the MinION nanopore sequencing. In case of bacterial analysis, predominance of *Proteobacteria, Firmicutes* and *Actinobacteria* was observed in Illumina sequencing analysis. While in case of MinION nanopore sequencing, *Firmicutes, Bacteriodetes* were predominantly observed (Fig. 2.27B). However, predominance of *Proteobacteria* and *Actinobacteria* was found to be similar in both the techniques. At the genus level, difference in relative abundances of a few predominant genera was observed. In Illumina sequencing analysis, *Bacillus, Pseudomonas* and *Mycobacterium* were observed as the predominant genera. In MinION nanopore sequencing, *Burkholderia, Brucella* and *Pseudomonas* were found as predominant genera.

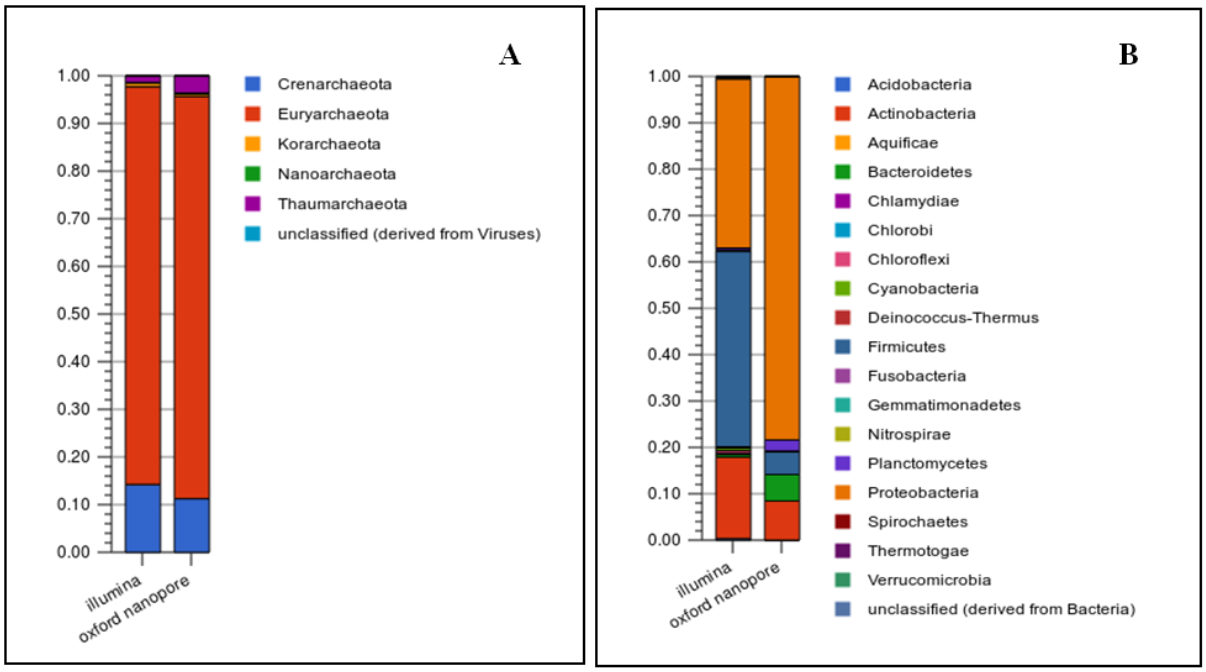

Fig. 2.27: A comparison of the distribution of archaeal (A) and Bacterial (B) phyla in 15KS1 sample analysed by Illumina and Nanopore sequencing techniques.

2.3.4.3.2: Functional analysis

The prediction of the ORFs in the assembled contigs was done using a Glimmer-MG tool and the complete functional annotation along with contig ID, gene function and sequences. Pathway annotation was done using KEGG pathway analysis per- formed for each contig sequence by assigning KEGG Orthology (KO) numbers obtained from known reference hits. In total, 57- 60% contig sequences were assigned to metabolism, 15-18% was assigned to environmental information processing and 16-17% was to the genetic information processing (Fig. 2.28).

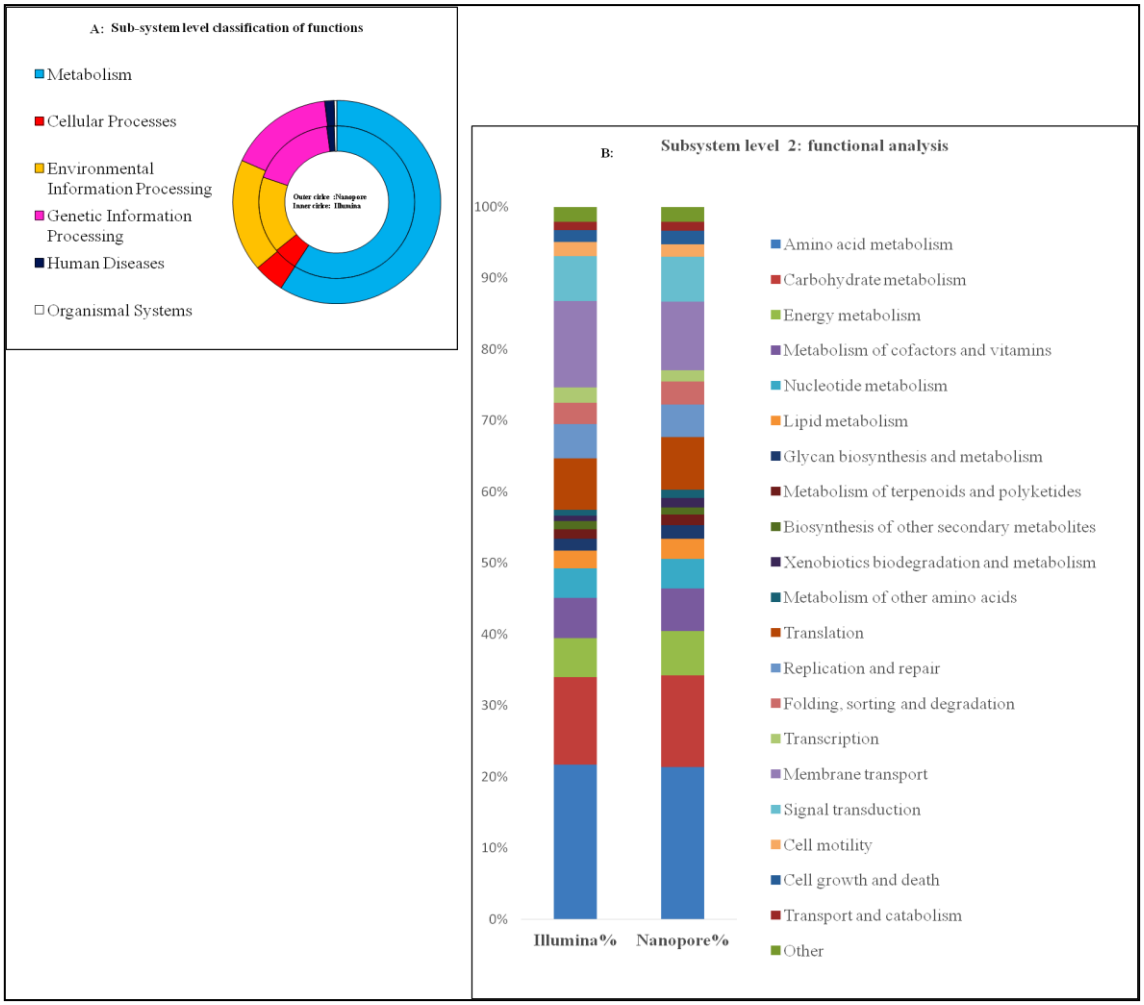

Fig. 2.28: A comparative account of functional analysis (15KS1) at subsystem levels Illumina and Nanopore

2.3.5: Aanthropogenic impact on natural hot spring diversity

2.3.5.1: Profiling of water samples

Most of the Indian hot springs believed to have some medicinal properties, because of which these have become a tourist spot and many people takes bath in hot spring water to cure some skin diseases. Such anthropogenic activities might have an influence on the natural microbial communities of hot spring. Here, we have performed a comparative analysis of microbial communities of natural hot spring (15UM), its associated bath tank (15DW) as well as discharged water outflow (15FW) to get insight regarding changes in the overall microbial composition due to the disturbances caused by anthropogenic factors. We found that the

relative abundances of some of the microbial species has changed in when it goes to the bath tank and subsequently discharged as an outflow (Fig. 2.29).

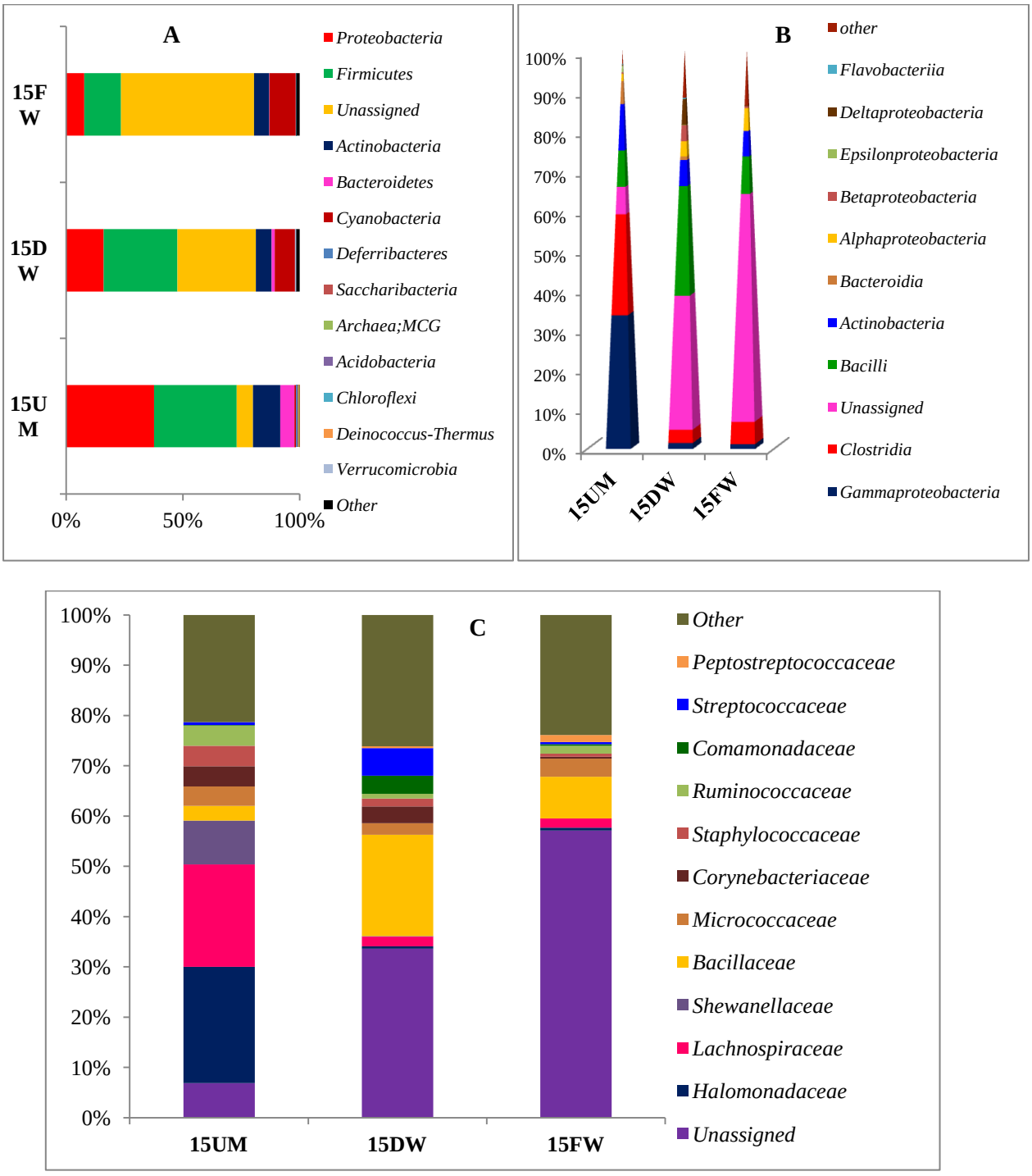

Fig.2.29: Taxonomy classification of OTUs at the phylum (A), class (B) and family (C) level of hot spring, bath tank and discharged water samples. Only dominant phyla are represented here.

At the phylum level, a relative decrease in *Proteobacteria* (37.6% - 16% - 7.7%), *Firmicutes* (35.4% - 31.6% - 15.7%), *Actinobacteria* (11.7% - 6.8% - 6.5%), *Bacteroidetes* (6.1% - 1.3% - 0.1%), *Deferribacteres* (0.6% - 0% - 0%), *Saccharibacteria* (0.50% - 0%- 0%), *Deinococcus-Thermus* (0.2% - 0% - 0%) and an increase in unassigned OTUs (6.9%- 33.6%- 57.1%), *Cyanobacteria* (0.7%- 8.7% - 11.2%) and *Acidobacteria* (0.0% -0.1%- 0.2%) was observed (Fig. 2.29A). At lower level of taxonomic classification, a similar tread was found, where a decrease in the members of *Gammaproteobacteria* (33.5% - 1.5% -1.2%), *Epsilonproteobacteria* (1.6%- 1%-0%), *Clostridia* (25.3%- 3.3% - 5.6%), *Bacteroidia* (5.8% - 0.9% - 0%), *Flavobacteriia* (0.2% - 0.3% - 0%) and an increase in *Alphaproteobacteria* (1.8% - 3.8% - 5.7%), *Betaproteobacteria* (0.4% - 4%- 0.5%), unassigned OTUs (6.9% - 33.6% - 57.1%), *Bacilli* (9.1% - 27.5% - 9.4%), *Deltaproteobacteria* (0.3%- 6.5%- 0.2%), *Flavobacteriia* (0.2%- 0.3%- 0%) was observed (Fig. 2.29B). At the family level, some of members such as *Halomonadaceae* (23.1% - 0.5% - 0.6%), *Lachnospiraceae* (20.4% - 1.9% - 1.8%), *Shewanellaceae* (8.7% - 0.1% - 0%), *Micrococcaceae* (3.9% - 2.3% - 3.6%), *Corynebacteriaceae* (4% - 3.3% - 0.4%), *Staphylococcaceae* (4.1% - 1.6% - 0.6%), *Ruminococcaceae* (4% - 0.9% - 1.6%) and an increase in members of the *Bacillaceae* (2.9% - 20.2% - 8.3%), *Comamonadaceae* (0.1% - 3.6% - 0.3%), *Streptococcaceae* (0.6% - 5.5% - 0.4%), *Peptostreptococcaceae* (0% - 0.4% - 1.4%) was found (Fig. 2.29C). Further at the genus level, differences in proportion in abundances were found among hot spring, bath tank and discharged water samples (Fig. 2.30). Overall based on such analysis it could be inferred that anthropogenic activities over the hot springs could have influence on the structure and function of natural microbial communities. A decrease in natural microbial population and increase in some other non-indigenous microbial flora could be a serious threat for hot spring microbial communities.

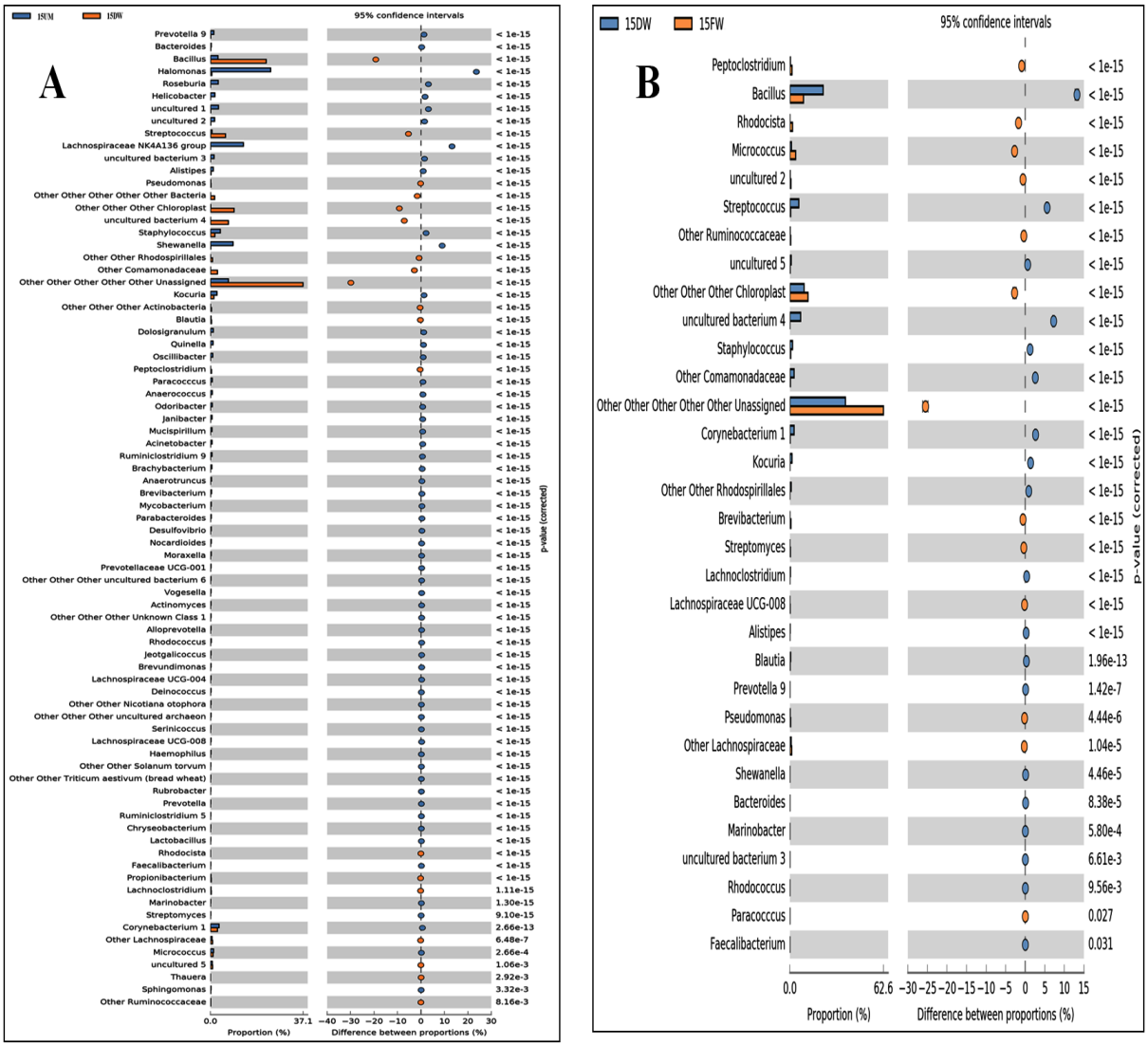

Fig. 2.30: Overall distribution at the genus level in hot spring: bath tank (A) and bath tank: discharged water (B) samples.

2.3.5.2: Profiling of sediment samples

Microbial communities of the sediment samples of natural hot spring (15KS1) and the discharged sediment (15FS1) samples were compared. In this analysis, the overall predominance of *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Verrucomicrobia*, and *Planctomycetes* was found. However, the relative abundances of these predominant phyla varied among both the samples. The natural population found to decrease in discharged sediment samples. Here, a decrease in *Proteobacteria* (29.27 to 15.2%),

Bacteroidetes (9.1 to 6.1%), *Verrucomicrobia* (2.8 to 1.8%) and *Planctomycetes* (3 to 1.4%) was observed. However, an increase in a relative abundance of *Actinobacteria* (3.6 to 11.9%) and *Firmicutes* (4.9 to 7.2%) was observed in the discharged sediment sample (Fig. 2.31A).

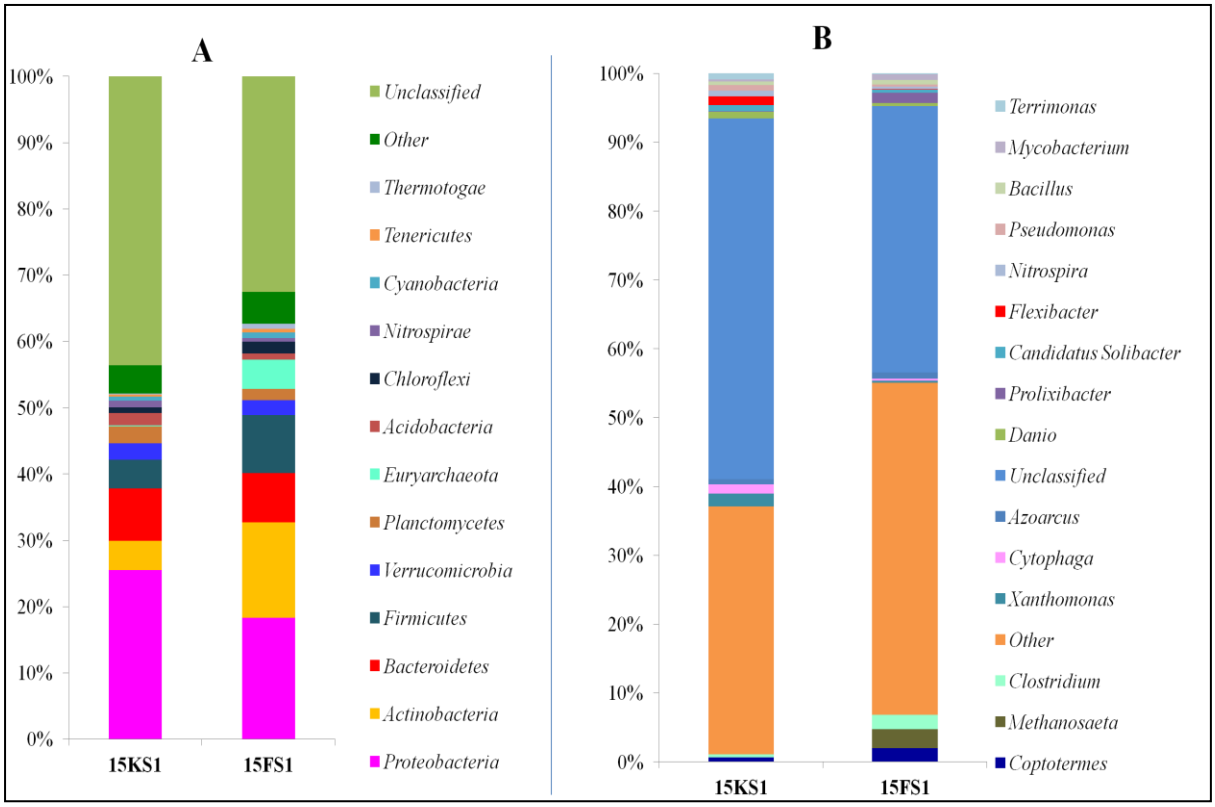

Fig. 2.31: Phylum level (A) and genus level (B) diversity analysis of sediment samples

At the genus level, the highest dominance of *Methanosaeta* (Archea), *Clostridium*, *Xanthomonas*, *Cytophaga*, *Prolixibacter*, Candidatus *Solibacter*, *Flexibacter*, *Nitrospira*, *Pseudomonas*, *Bacillus*, *Mycobacterium*, and *Terrimonas* was found with varied abundance in each sample (Fig. 2.31B). In this case, surprisingly, in the discharged sample (15FS1), a high number of OTUs were found to assign to an archaeal genus, *Methanosaeta* within *Methanosaetaceae* family. Like other species in this family, *Methanosaeta* can metabolize acetate as their sole source of energy; however, their increase in abundance in the discharged sample is not understood. Other genera such as *Clostridium*, *Prolixibacter*, and *Mycobacterium* were found with increased abundance in the discharged sample. The study

indicates that the microbial natural population of the sediment samples has changed after anthropogenic activities.

In function based analysis, a similar observation was made. In the discharged sample (15FS1), a relatively high number of contigs were assigned to environmental information processing and genetic information processing at the subsystem level of KO annotation (Fig. 2.32A).

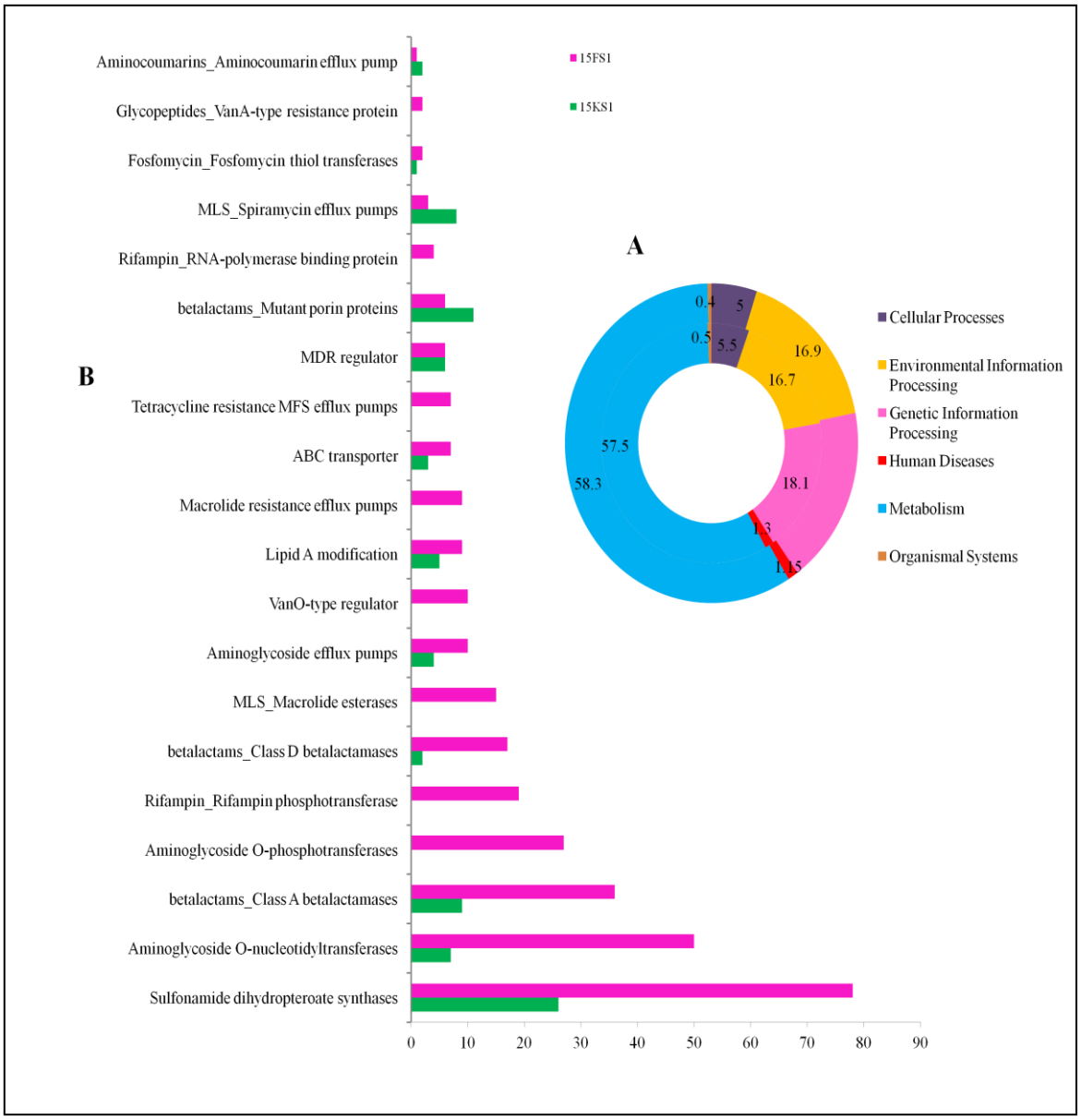

Fig. 2.32: Functional analysis of sediment samples at subsystem level 1 (A) and profile of abundance of antibiotic resistance genes among natural and discharged samples (B)

Further, we have annotated pathways for antimicrobial resistance among both of these samples, we found a higher number of pathways and genes for the mechanism of antibiotic resistance in the discharged sample than the sediment sample of the natural hot spring. Here, a relative increase in the genes for sulfonamide dihydropteroate synthases, aminoglycoside o-nucleotidyl transferases, and class-A beta-lactamases was observed. These genes are involved in the antibiotic resistance mechanisms for a different class of antibiotics (Fig. 2.32B). Additionally, few of the genes such as aminoglycoside o-phosphotransferases, rifampin phosphotransferase, macrolide esterases, macrolide resistance efflux pumps, tetracycline resistance efflux pumps and some other were exclusively observed only in the discharged sediment sample (15FS1). Overall, these observations indicate that the bathing activities and other anthropogenic disturbances may lead to unwanted but evident inoculation of antibiotic-resistant bacteria in the hot spring. Therefore some preventative measures must be taken to conserve such metabolically active and diverse microbial communities of natural hot springs in India.

2.4: General discussion

Microbiological research of hot springs is important for the understanding of microbial diversity, ecology, and activities of microbes in the ecosystems. Various methods are being used to explore the microbial communities and diversities of hot springs and understanding their correlation with geochemical factors. In the present study, we have characterized the microbial communities associated with the Unkeshwar hot spring, situated in the central region of India. To gets more insights into the microbial composition and influences of various environmental as well as human activities on microbial composition, diverse sampling strategies were designed. Various other samples along with the main hot spring such as from the nearby boreholes, bath tanks, discharged water samples were analyzed. In

the case of main hot springs, diversity analysis was performed for water, sediment, and microbial mat samples. In addition, physicochemical analysis of water samples was also performed to correlates the impacts of associated geochemical on the overall composition of the hot springs microbial communities.

The physicochemical analysis revealed that Unkeshwar hot spring has a moderate level of temperature (50ºC- 60ºC) and circumneutral pH (6.9 - 7.2). Most of the other geochemical factors such as sulphate, magnesium, potassium, and bicarbonates were found to be higher as compared to other hot springs present in central India (Badhai et al. 2015; Saxena et al. 2017). A successive sampling after a year has shown fluctuations in the concentrations of some of the geochemical elements. We found an increase in concentrations of sulphate (52.5 to 73.75 mg/L), sodium (93.1 to 112.2 mg/L), calcium (34.5 to 65.25 mg/L) and magnesium (2.8 to 5 mg/L) in the 2015 samples. Comparatively, such annual variations were not observed in the borehole samples indicating that the hot springs being present on the surface might be getting other influx in it through rainwater or other activities. It was also noticed that in 2015 there was a significant increase in water level due to high rainfall as compared to the previous year (2014).

Microbiological analysis showed the overall predominance of *Proteobacteria*, *Firmicutes* and *Cyanobacteria*. However, among the three different samples *i.e.* sediment, mat and water, the distribution of microbial phyla (archeal and bacterial) was distinct. Such differences in the relative abundances of community members might be due to environmental conditions associated with each type of sample. Similar types of studies done previously over the other ecosystems such as soda lakes has also shown such distinct microbial communities among sediment and water samples (Poul et al. 2016). However, over the hot springs, such studies were not much reported; one of study based on lipid profiling was performed over the hot springs (Mrozik et al. 2014). The sediment microbial communities were most diverse

than the corresponding water and mat samples. Unexpectedly, water microbial communities have shown the occurrence of few bacteria genera, those generally also found common in various other environments. This could have happened due to the more exposure to external sources. Detection of *Staphylococcus*, *Bacillus*, and *Clostridium* possibly, indicate that they might have transferred from other sources into the hot spring. On the notice of occurrences of such bacterial genera in Unkeshwar hot spring, we studied the microbial diversities of the borehole, which is closely associated with the hot spring. In this case, some more interesting observations were recorded by a comparative analysis between hot spring and borehole microbial communities. The borehole, being a closed system and at the subsurface level, might be having similar geochemical conditions to that of hot spring. Microbial communities of both hot spring and borehole were therefore observed with some differences in annual samples. Overall, the archaeal population was found in very low abundance in both the sites, majorly three different phyla, *Euryarchaeota*, *Crenarchaeota*, and *Thaumarchaeota* were detected. Although these phyla were known to occur in various hot spring environments, some of the members identified from the present hot springs were not previously found to be associated with hot springs. For example, members of *Crenarchaeota* which are sulphur dependent and chemolithoautotrophic in nature were known to inhabit hot spring environments (Song et al. 2010; Delgado-Serrano et al. 2014). Surprisingly, in the present study, members of this phylum were represented by uncultured Miscellaneous Crenarchaeotic Group (MCG), a group generally associated with the deep oceanic subsurface (Parkes et al. 2005). However, one of the previous studies has hypothesized that members of this group may also dominate the sulphur and methane-containing environments (Kubo et al. 2012). Apparently, a relatively high abundance of MCG was found the sample (15UM) which has a relatively high concentration of sulphate indicating this group could also inhabit sulphur containing hot springs. Similarly, *Euryarchaeota* members, such as *Thermoplasmatales* and

Marine group II were identified in the present study, among them, Marine group II is known to inhabits marine, coastal or estuarine water systems (Bano et al. 2004; Zhang et al. 2015; Zhang et al. 2017). Until now no precise ecological record of this group was recorded which may possibly suggest that hot spring water systems could also be the habitats of this group. Other members of *Euryarchaeota* were mostly found to be reported from acidic hot springs and hydrothermally modified soils (Mardanov et al. 2011; Coman et al. 2013) including *Methanobacterium*, *Methanobrevibacter*, and *Methanosaeta*. Among which *Methanosaeta* has abilities of methane production using acetate and were identified from hot springs (Welte and Deppenmeier 2011; Chan et al. 2017). While *Methanobrevibacter* found to be surprising in hot springs, as they are in general reported as a host associated methanogenic archaea of human gut and animals (Eckburg et al. 2003; Samuel et al. 2007).

In both the sites, the microbial composition was dominated by bacterial phyla. The dominance of bacteria was also found in previous studies of other Indian hot springs, as well as some global hot springs (Poddar and Das 2017; Hussein et al. 2017). Interestingly, a remarkable dominance of *Deinococcus-Thermus*, genus *Meiothermus*, and its uncultured members was found in the present hot springs. Generally, members of *Deinococcus-Thermus* were detected in low abundance from other Indian hot springs having moderate temperatures (Badhai et al. 2015; Saxena et al. 2017, Sharma et al. 2017). Furthermore, it was also reported that *Deinococcus-Thermus* not found in dominance within the terrestrial hot springs, however, genus *Meiothermus* generally prefers moderate temperatures habitats (Wang et al. 2013). Nevertheless, they have been found to be associated with some of the hot springs such as Tibetan hot springs from China (Wang et al. 2013), China (Wang et al. 2013; Jiang et al. 2016), Iceland (Tobler and Benning 2011), Tunisia (Sayeh et al. 2010) and Spain (López-López et al. 2014). Based on such observations, it could be inferred that the dominance of *Deinococcus-Thermus* might also depend on other geochemical factors along with the

temperatures. Among other thermophilic members, *Spirochaetes*, *Acidobacteria*, *Deferribacteres*, *Verrucomicrobia*, *Lentisphaerae*, *Tenericutes* and *Thermotogae* were detected with very low abundance as these members generally inhabit the high-temperature habitats. In a moderate level of temperature hot springs, these bacterial found with low abundances, also evidenced from other studies of different hot springs (Badhai et al. 2015; Sahoo et al. 2017).

Among other bacterial phyla, *Proteobacteria*, *Bacteriodetes*, *Firmicutes*, *Synergistetes*, and *Actinobacteria* were the dominant groups in both the natural hot spring and borehole samples. These phyla generally found to be associated with moderate temperature hot springs. Previous studies of such microbial community analysis of hot springs from India (Badhai et al. 2015; Saxena et al. 2017; Sharma et al. 2017). Malaysia (Chan et al. 2015, 2017). China (Song et al. 2013), Kenya (Kambura et al. 2016) has shown similar observations. Among the members of *Proteobacteria*, genera such as *Halomonas*, *Rhodanobacter*, *Shewanella*, and *Alcanivorax* were present in high abundance. Among them, *Shewanella* and *Alcanivorax* were previously known from hot spring environments (Ghosh et al. 2003; Ghilamicael et al. 2017). However, *Halomonas* generally are not found to be associated with hot springs, but positively correlated with the physicochemical conditions of one of the sample (15UM) of the hot spring.

While among the genera with low abundances, *Ramlibacter* and *Tepidomonas* (*Betaproteobacteria*) were of special significance. Recent studies have shown that members of these genera could also inhabit hot spring environments (Saxena et al. 2017; Panda et al. 2016). In general, species of *Ramlibacter* are cyst forming chemoautotroph and were isolated from dry environments (Heulin et al. 2003). Interestingly, species of *Tepidomonas* were reported with the prediction of their possible role in biogeochemical cycling in hot springs environments. Genome sequencing of the species of this genus revealed that the genes related

to biogeochemical cycling were present in the genome of *Tepidomonas* (Saxena et al. 2017). From the *Deltaproteobacteria*, two important group members *i.e.* sulphate reducing bacteria (SRB) and predatory bacteria (*Bdellovibrio*) were found to present. In *Deltaproteobacteria* highest sequences were assigned to *Desulfovibrio*; a sulphate reducing bacteria commonly found in geothermal environments (George et al. 2008; Fadhlaoui et al. 2015). Other members of *Deltaproteobacteria* include Bdellovibrio-and-like organisms (BALOs), which are also known as predatory bacteria were also identified in this study. Previously, BALOs were reported from the high temperature Indian hot spring (Sangwan et al. 2015), however, their occurrence in moderate temperature hot springs is notable. Importantly, until now the ecological role of these bacteria in the environments is not thoroughly understood (Davidov and Jurkevitch 2004) suggesting that they must have some ecological functions in the hot spring ecosystems. Other members of *Deltaproteobacteria* were found to be associated with the biogeochemical cycling. For example, *Nitrospina*, a member of nitrite-oxidizing bacteria (NOB) was found to be key players in nitrogen cycling of the hot springs environments (Lebedeva et al. 2005). Whereas, *Geobacter* are Fe (III)-reducing bacteria were the model organisms to study the biochemical mechanisms of Fe-reduction (Emerson et al. 2012). Surprisingly, phototropic bacteria were found with low abundance (3-5%) as compared to other dominant phyla, though group members of such bacteria were known to dominate the moderate level temperature (>75ºC) of the hot springs (Wang et al. 2014). Based on annual samplings, borehole microbial communities were found more stable. In main hot springs, the relative abundance was changed in successive annual samplings for few of the microbial phyla. For example, *Rhodanobacter* (*Proteobacteria*) was present with the highest dominance in the natural hot springs annual sample of 2014 (14UM), has disappeared in 2015 (15UM). On the other hand, two genera, *Halomonas* and *Shewanella* were found with increased abundance in this sample (15UM) of the natural hot spring. In addition, fluctuations in the

concentrations of some of the geochemical factors were also observed in the natural site, which could also have contributed to such changes in abundance of community members in natural hot spring. For example, a positive correlation between the relative increased concentration of sodium chloride (NaCl) and sodium ions and high dominance of *Halomonas* was observed in the 15UM sample. It was known that *Halomonas* could be able to proliferate under a broad range of temperatures and salinities (Harrison et al. 2015; Boltyanskaya et al. 2007). Similarly, *Shewanella* with the abilities to use nitrate, nitrite, thiosulphate, and sulphite as an electron acceptor (Moser and Nealson 1996) was found predominant in natural hot spring. Some of the thermophilic members of *Shewanella* were identified and isolated from hot springs (Ghosh et al. 2003). However, members of denitrifying bacteria such as *Rhodanobacter* were detected mostly from the deep subsurface environment (Green et al. 2012), in this study also they found dominant in the borehole water samples. Another interesting bacterial phylum, *Synergistetes* found to be widespread in the environments and also found to be associated with animals. Different members of *Synergistetes* have been isolated from wastewater of anaerobic digesters, natural springs, natural seawater and sulphur mats (Jumas-Bilak and Marchandin 2014), however, they have been rarely found associated with the hot springs. Based on such comprehensive analysis, we could finally infer that Unkeshwar hot springs microbial communities are more prone to environmental variations than the associated borehole microbial communities.

In general, microbial communities prevailing in hot springs found to be stable over the time (Miller et al. 2009; Everroad et al. 2012). Nevertheless, terrestrial hot springs present on the earth surface might be influenced by various environmental factors (Hubalek et al. 2016; Chan et al. 2017). In India, terrestrial hot springs present on the surface are more prone to environmental variations and also are frequently influenced by human activities in a belief of their natural healing properties. On the contrary, boreholes present in such geothermal

regions are less vulnerable to such interruption as they have partially subsurface nature (Fredrickson and Balkwill 2006; Frank et al. 2016). Though the surface factors found to have an influence on microbial compositions, at the root level, geochemical factors might be controlling the overall microbial compositions in this hot spring. A correlation analysis between microbial members and physicochemical factors revealed both positive and negative correlations of microbial members with various physicochemical factors. An increase in abundance of the sulphur dependent group of *Crenarchaeota* was positively correlated with increase sulphate concentrations in the natural hot spring (15UM) sample. However, phototrophic bacteria were negatively correlated with sulphite concentrations, may be due to fact that *Cyanobacteria* could not survive with high levels of sulphite concentrations (Lacap et al. 2007). Therefore, overall their low abundance might be due to the sulphate content of the hot springs. Some other phyla *Planctomycetes*, *Lentisphaerae,* and *Nitrospirae* were found to be positively correlated with nitrogen concentrations. Members of some of these bacterial phyla are known to have an important role in nitrogen cycling (Henry et al. 1994; Strous et al. 1999; Kalyuzhnyi et al. 2010). Various other physicochemical factors such as total organic carbon, pH, different ionic concentrations of potassium, sodium chloride were also known to influences the abundances of certain groups members like *Acidobacteria*, *Fusobacteria*, *Synergistetes*, *Armatimonadetes* (Naether et al. 2012; Liu et al. 2014). When we compared the microbial communities of various previously studied Indian hot springs, it also revealed that the different geographic conditions have a greater impact on the microbial composition of each hot spring. In most of the cases, a temperature of hot spring was found to drive the phylum diversity in hot springs of India. Irrespective of its geographical distribution, hot springs with high temperature such as Bakreshwar and Manikaran had shown the predominance of thermophiles belonging to *Deinococcus-Thermus*, *Aquificae* and *Firmicutes*. While in the case of other moderate temperature hot springs including

Unkeshwar hot spring were found to be predominated by *Proteobacteria*, *Bacteroidetes*, *Actinobacteria,* and *Cyanobacteria*. Such observations were found similar as observed in many global studies that summarized that the thermophilic bacteria belonging to phyla *Aquificae, Deinococcus-Thermus*, *Thermodesulfobacteria*, *Thermotogae, Proteobacteria* and *Firmicutes* are abundant in hot springs having temperature above 70°C, whereas mesophilic bacteria of *Cyanobacteria*, *Chloroflexi* and *Proteobacteria* group mostly occupy hot springs with moderate to low-temperature profile (Poddar et al. 2017, Wang et al. 2013).

As mentioned earlier, that the few other factors also found to responsible for the changes in the hot spring microbial diversity. During our several visits to the Unkeshwar hot spring, we found that various people visit the hot spring for taking bath in the hot spring water in a bath tank. As a consequence of such anthropogenic interference, the natural microbial composition might be getting changed. Therefore, to analyze the impact of such activities, we profiled and compared the microbial analyses of hot spring along with bath tank and discharged water samples. The results indicated that the relative abundances of the natural flora of hot spring have changed as when it passed into the bath tank and subsequently discharged as an outflow. A few of the studies have also made a similar observation about the impact of such activities and rain water influx in modifying the microbial community structure and function in the hot spring ecosystems (Brigg et al. 2014; Wang et al. 2014).

Further, we have compared the microbial communities analyzed using different sequencing techniques. In both the techniques, a relative abundance of few bacterial phyla, as well as genera were found to be different. However, it was observed that Nanopore analysis has resulted in identifying the maximum number of phyla. Based on various comparative analyses, we inferred that natural microbial communities of Unkeshwar hot spring have a greater impact of physicochemical factors, geographic locations and to some extent of human activities. However, the natural population till found to present in the hot springs and

represent the microbial species with high metabolic potentials. Predictive functional analysis, ORF and KEGG based pathway annotation revealed the predominance of metabolism-related functions in this hot spring. In metabolism, highest numbers of pathways were assigned to xenobiotics degradation, thus indicating the presence of organisms having bioremediation potential. Metabolic pathways for the biodegradation of benzoate/aminobenzoate compounds, chlorocyclohexane, poly cyclic aromatic hydrocarbons (PAH) compounds, etc were detected. Usually, thermophilic communities were not been studied for environmental applications. However, some of the recent reports have shown that thermophiles also possess genes and enzymes for the degradation of aromatic hydrocarbons (Saxena et al. 2017). A few of the metabolic pathways related to the degradation of dioxins, caprolactam, and ethylbenezene were also detected. Dioxins are a diverse range of compounds and are highly toxic and have a serious impact on reproductive and developmental problems in humans (White and Birnbaum 2009). Carpolactam is a hazardous air pollutant and different microorganisms capable of its degradation have been isolated from different environments (Baxi and Shah 2002). Such observation could suggest that the thermophilic communities of hot springs might have the metabolic potential for utilization of such compounds. Pathways were also mapped for atrazine degradation, which is widely used as an herbicide for the control of broadleaved and grassy, weeds (Satsuma 2009). Degradation of atrazine was previously studied using soil bacteria (Zhang et al. 2012). Overall analysis showed that Unkeshwar hot springs harbor microbial population having certain metabolic potential which could have application in bioremediation processes of some toxic pollutants. In a similar study reported from Atri and Taptapani hot spring of Odisha, where a high number of sequences were assigned to metabolisms (Sahoo et al. 2015). In addition, in the present study, a considerable number of molecules were also assigned to subsystem level of energy metabolisms such as sulphur and methane metabolism. Sulphur metabolism is an important physiological function in hot

springs (Badhai et al. 2014; Panda et al. 2016). The sulphate reducing microorganisms have an important role in the degradation of organic matter in anoxic environments were also reported from the hot springs of Malaysia (Chan et al. 2015). Overall the study showed that natural microbial communities of Unkeshwar hot springs have a high metabolic potential, which could be used for various applications.

2.5: Conclusion

The present study gives a broad description of Unkeshwar hot spring's community structure and functions based on the culture-independent approaches of amplicons (16S rRNA) as well as whole metagenome sequencing techniques. The work provides a comprehensive understanding of microbiomes of these hot spring locations and the impact of physical and chemical characteristics as well as human innervations on the microbial community members of the hot spring. Overall, microbial community was found to be dominated by *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Deinococcus-Thermus*, *Cyanobacteria*, and *Saccharibacteria*. A water sample has shown interesting observation regarding overall microbial composition, therefore such samples were further investigated along with other natural and anthropogenically impacted water from the same location within a concessive time period. The bacterial community in the hot spring showed more significant variation than that in the nearby borehole possibly indicating that microbial community in the Unkeshwar hot spring is more prone to environmental disturbances. A comparative analysis of a few Indian hot springs including Unkeshwar revealed that physicochemical conditions of the hot springs have a major impact on the microbial community compositions. Functional analysis revealed that the hot springs have high metabolic capacities in terms of metabolisms of xenobiotic compounds and methane, indicating that both their natural role in biogeochemical cycling and also in the degradations of toxic pollutants. Overall, microbial communities from natural

hot springs and associated other sites of a geothermal region will possibly be helpful for knowing the impact of human interferences, rain influx, and other abiotic factors on microbial communities of hot springs. Furthermore, such aspects could also found useful while estimating microbial communities from the other terrestrial hot springs and also while bioprospecting form such systems.

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CHAPTER 3

Microbial diversity and metabolic potentials using cultivation approach

Abstract

Thermophilic microorganisms inherently exhibit great biotechnological potential primarily due to their unique and stable macromolecular properties. However, due to limitations of cultivation methods, a majority of such extremophiles remains uncultivated and unexplored. The advent of multiple cultivation conditions and specialized culture media could possibly aid to access the unexplored microbial portion of hot springs. In the presents study, culture-based microbial diversity of Unkeshwar hot spring was examined and compared to the previously characterized Indian hot springs microbial communities. Unkeshwar hot spring contained high culturable microbial diversity as reflected by the molecular and phylogenetic analysis of the cultured isolates. Imputed functional analysis using in-silico analysis has lead to the identification of metabolically potential fraction of a cultured microbial communities present in this hot spring. Assessment of such metabolic potentials in terms of production of important enzymes, heavy metal tolerance properties and genome sequence based functional annotations had shown the higher activities. However, the impact of anthropogenic disturbances was also evidenced on the microbial community members as recorded through antimicrobial resistance among some of the isolates. Overall, the present study revealed the advantages of cultivation methods using a comprehensive multiple isolation approaches for exploring untapped and unique bacterial diversity, and also utilities for various biotechnological and environmental applications.

Key words: Hot springs, Microbial cultivations, Imputed functions, enzymes, Heavy metals, antibiotics resistance

3.1: Introduction

Since the late nineteenth century, microbiology has relied on the acquisition of pure bacterial cultures, which have been central to allied studies, including taxonomy, ecology, and pathology. As microbiology moves into the 20-21st century, the use of culture-independent approaches gaining more importance and its use has increased significantly (Austin 2017). These advanced techniques give a wider view and insight into the diversity, adaption and ecological significance of a microbial habitat of any environment. Secondly, using such techniques, the microorganism may be studied regardless of whether or not they may be grown in the laboratory. Therefore, a majority of microbiological investigation of hot springs habitats were being performed using these culture-independent techniques (López-López et al. 2013). However, culture-based methods are necessary to decipher microbial physiology and till forms an integral part of any microbiological research. Different views regarding the use of culture-independent versus culture-dependent approaches for environmental microbiology investigations are being made. However, the combination of both techniques is necessary in recent times, as omics-based techniques drive new ecological hypotheses, and pure cultures are needed to test such hypotheses (Gutleben et al. 2017). Besides, culture-based methods are also useful for the isolation and identification of taxonomically interesting groups of bacteria with high biotechnological potential (Prakash et al. 2013; Grivalský et al. 2016). The metabolic potential of an isolated organism can be more readily exploited through genome manipulation for use in different applications (Pontes et. al. 2007; Pham and Kim 2012). However, until now only 1% of bacterial species are cultured by using current microbial cultivation techniques. To date, among the known bacterial species, more than half of the known phyla have not yet been isolated in culture (Lagier et al. 2015). Various culture techniques are being used to culture the bacteria from the host environments. These techniques were originally used by environmental microbiologists to explore minority

populations (Puspita et al. 2012; Lagier et al. 2015). Recently, microbial cultivation using high throughput culture-based methods is being used for the isolation of previously uncultured microbes (Vartoukian et al. 2010; Stewart 2012; Lagier et al. 2015).

Hot spring microbiology research is more fascinating as thermopiles have certain adaptive measures which may provide clues to evolutionary pathways in microorganisms. In addition, they also possess great biotechnological potentials primarily due to their thermally stable macromolecules which find application in biotechnology and industrial processes (Podar and Reysenbach 2006). For instance, the remarkable discovery of thermophiles and subsequent use of thermo-stable enzymes for molecular techniques have dramatically changed the field of biotechnology (Brock and Freeze, 1969). Therefore, hot springs microbiology needs to be studied using both the culture-independent as well as culture-dependent approaches. Using microbial cultivation methods, research efforts still seek isolation and exploitation of novel and uncultured microbial species from hot springs environments. Majorly studies focus on culturing of an uncultured fraction of microbial communities to provide an access to the hidden metabolic diversity (Grivalský et al. 2016). Among the more than 300 Indian hot springs, few were studied so far for microbiological analysis (Singh et al. 2016). A few culture-based studies were also performed, mostly from Himalayan geothermal belt; such as Soldhar and Ringigad (Kumar et al. 2004; Pandey et al. 2015), Himachal Pradesh (Kumar et al. 2014a, b; Sahay et al. 2017), and from one other region, Tulsi Shyam from Gujarat (Kikani et al. 2015). However, still more comprehensive studies are necessary in order to gain access to the microbial community composition, understanding their ecology and for bioprospecting for various biotechnological applications.

In this chapter, we surveyed the bacterial diversity present in Unkeshwar hot springs using varied culture conditions/media. The study involves the identification and isolation of previously unexplored microbes from hot springs and for the future preservation and

bioprospecting. Additionally, integration of sequence-based tools of imputed functional analysis, from which metabolic functions of community members can be readily inferred. Bioprospecting for different industrially important enzyme production was also evaluated. Microbes also play an important role for environmental applications such as removal of toxic heavy metals, chemical compounds from the contaminated sites. Therefore, cultures isolates were evaluated for such properties. It was also observed that most of the Indian hot springs are treated as holy places, used for a holy bath, which may cause anthropogenic disturbances to the natural microbial communities. Therefore antibiotic resistance patterns among community members were evaluated in correlation with the heavy metal tolerance. Furthermore, comparative analyses were performed with the previous metagenomics studies to analyze the results of both such culture-dependent and independent methods, which may be helpful for designing future studies.

3.2. Material and methods

3.2.1: Chemicals, reagent and culture media

All the chemicals and reagents used in the present investigation were of high purity and analytical grade. Taq DNA polymerase, PCR buffer, magnesium chloride and dNTP's were obtained from Banglore Genei Pvt. Ltd. India. Other chemicals and reagents were procured from Hi-Media, Merck, Fisher Scientific and Sigma. Chemicals such as heavy metal salts, Antibiotic disks, and culture media components were purchased from Hi-Media Ltd, India, while chemicals such as chloroform, phenol, isoamyl alcohols were purchased from Merck Ltd, India, were of Molecular Biology grade. PAH compounds were procured from the Sigma, India.

3.2.2: Sample collection and microbial cultivation

Sample collection was performed as described in chapter 2 (section 2.2.1 and table 2.1). Water sample of the main hot spring (UMHK) was utilised for isolation and cultivation of microbes. The aim of the study was to isolates taxonomically diverse bacteria from this hot spring water sample. As it was known that, most of the bacteria are difficult to culture and needs special conditions for their isolation, we have diversified culture conditions. Initially, isolation was performed using some regular nutrient media as well as culture conditions. Subsequently, some special culture conditions and various cultivation strategies were used during isolation mentioned as fallows.

3.2.2.1: Cultivation using solid media

Water sample was serially diluted and used for plating on various culture media. Varieties of culture media (from high nutrient content to low nutrient) were used. Different commercially available media as well as formulated in the laboratory using different media components were prepared for plating. The following are the media used for bacterial isolation; Nutrient agar, International Streptomyces Project (ISP) medium (ISP1-ISP7), Reasoner´s 2A agar (R2A), Starch Casein Agar, Streptomyces agar, Bushnell Haas Agar, Mueller Hinton agar, Thermus agar (peptone 0.5%, yeast extract 0.2%, beef extract 0.4%, NaCl 0.5%, agar 1.8%), Thermus agar dilutions (1:10; 1:100; 1:1000) and Nitrogen fixing growth (NFG) medium (glucose 1%, calcium chloride 0.01%, magnesium sulphate 0.01%, dipotassium phosphate 0.09%, monopotassium phosphate 0.01%, calcium carbonate 0.5%, ferric chloride 0.001%, sodium molybdate 0.0005%, agar 1.8%). Plating was carried out using standard spread plate technique and each media set plates were incubated at 37°C- 70°C with a 5°C increase in temperatures for each set of plates.

3.2.2.2: Cultivation using enrichment methods

To isolates diverse range of bacteria for biotechnological applications, different types of enrichments of the water sample were performed. Basically, enrichments were done based on various types of stress conditions such as temperature stress, nutrient stress and stress of toxic chemicals (Heavy metal, phenol and PAH compounds) with a methodology mentioned as fallows.

3.2.2.2.1: Temperature stress

Luria Bertani (LB) broth was used for this type of enrichment. Water sample initially pre-heated in a water bath at 50°C for an hour. From this, a 100 µL water sample was added to the flasks containing 100 ml LB broth. These flasks were incubated at an initial temperature of 45°C up to seven days. Thereafter, from the same flask 100 µL broths (as inoculate) was added to fresh LB broth and incubated at 50°C. This process was repeated several times still the temperature riches at 75°C with 5°C increments at each step. From each subsequent step, a 100 µL broth was used for plating (LB agar plates) using the standard spread plate technique for isolation.

3.2.2.2.2: Nutrient stress

For nutrient depletion enrichment, 100 µL water samples were added in 100 ml LB broth and incubated at 37°C up to 24 hours and growth was observed visually as turbidity in the flasks. Subsequently, the same broth was autoclaved and utilized repetitively through the same procedure up to 3-4 times and the final broth was used for plating.

3.2.2.2.3: Chemical stress

Enrichment was performed using phenol and PAHs as a chemical stress. For phenol enrichment, peptone water phenol medium (WP) containing (0.05% - 0.5% phenol) (Kanekar et al. 1998) was used. In this media, a 100 µL water sample was added and incubated at 37 ºC upto 7 days. From the same broth subsequent enrichments were performed after every 7 days

till 21 days. From all the enriched sets, plating was performed on nutrient agar using standard spread technique.

In case of PAH compound enrichment, 5ml water sample was inoculated in the MSM medium containing pyrene as carbon source. The composition of the MSM used was as follows (g l^{-1}): KCl (0.1), $NaNO_3$ (4.0), K_2HPO_4 (1.0), KH_2PO_4 (0.5), $CaCl_2$ (0.01), $MgSO_4 \cdot 7H_2O$ (0.5), $FeSO_4 \cdot 7H_2O$ (0.01), Yeast extract (0.1), and 10 ml of trace element solution containing (g l^{-1}): $CuSO_4 \cdot 5H_2O$ (0.5), H_3BO_3 (0.26), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.06), $MnSO_4 \cdot 7H_2O$ (0.5) and $ZnSO_4 \cdot 7H_2O$(0.7) (Potawory et al. 2015). The pH of the medium was adjusted to 7.0. The conical flasks were added with filter sterilised solution of pyrene prepared in acetone (HiMedia) with the final concentration of 100 ppm and flasks were incubated at 37 C and 150 rpm for 24 hours to evaporate the acetone present in the flasks. After total evaporation of acetone from the medium, 5 ml of water sample was inoculated in the flasks and covered with aluminium foil for creation of dark condition (for elimination of photo-degradation) and incubated at 37°C for 21 days. The 100 µl liquid broth was taken out after every 7 days of incubation for plating. For plating, Bushnell haas (BH) (HiMedia) agar plates were used as it is a recommended medium for studying the microbial degradation of hydrocarbons. While performing every experiment (direct and enrichment sets), incubated plates were observed for distinct colony morphologies after every 24 hours of incubation up to the 7 days of incubations. Based on differences in colony morphology, new visible colonies that appeared on plates were selected for isolation. Distinct morphotypes were selected from different plates and subsequently subcultured on respective media and stored as a stock in -80˚C in 20% glycerol.

3.2.3: Molecular characterization and phylogenetics

3.2.3.1: Genomic DNA isolation

From the glycerol stock, isolates were subcultured; purity of cultures was checked using morphological observations and Gram staining. Pure bacterial cultures subjected for genomic DNA isolation. The DNA isolation was carried out using a standard phenol/chloroform/isoamyl alcohol (25:24:1) method (Sambrook et al. 1989). Briefly, cell pellets are suspended in Tris EDTA buffer (TE buffer) in the 2 ml Eppendrof tubes. To the cell suspension, a 2µl Lysozyme (20 mg/ml) was added and kept at 37°C for 2 hours. After incubation, 3µl Proteinase K (10mg/ml) and 30µl 10% SDS was added and further kept at 55°C for 2 hours. Tubes were kept at room temperature, and added with a 100µl 5M NaCl solution and kept at 65°C for 2 minutes, subsequently a 80µl of 10% CTAB solution was added and kept at 65°C for 10 minutes. Extraction was carried out by adding equal volumes of (~800µl) Chloroform: Isoamyl alcohol (24:1) solution and centrifugation at 10,000 rpm for 5 minutes. The upper aqueous phase taken out and further extracted with equal volume of Phenol:Chloroform: Isoamyl alcohol (25:24:1) solution fallowed by centrifugation. From the aqueous phase, DNA was precipitated by addition of chilled Iso-propanol and 3M Sodium acetate and kept at -80°C for an hour. Tubes were taken out, kept at room temperature for 5 minutes and subsequently centrifuged at 12,000 rpm for 30 minutes at room temperature. DNA pellet was washed using chilled 70% ethanol and dried in vacuum concentrator (Speed Vac) and resuspended in 60µl 1X TE buffer. The purified DNA was visualized using agarose gel electrophoresis and the concentration of DNA was checked using spectrophotometer (Nanodrop 1000 UV, Nanodrop). DNA samples were stored at -20°C temperature until further use.

3.2.3.2: Amplification and sequencing of 16S rRNA gene

The isolated DNA was used for amplification of the 16S rRNA gene using a sets of universal bacterial primers 16F27 (5′CCAGAGTTTGATCMTGGCTCAG3′) and 16R1525 (5′TTCTGCAGTCTAGAAGGGGTGWTCCAGCC3′). The PCR reaction was carried out in a 50 µL reaction mixture containing 10 nM (each) primer (Eurofins), 200 µM (each) deoxynucleoside triphosphate (dNTP) (Genei), 1 U of Taq polymerase (Genei) in the appropriate reaction buffer, and 100 ng of DNA as a template. PCR conditions were 34 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min 30 s at 72°C. After amplification, all the PCR products were visualised using agarose gel electrophoresis fallowed by gel documentation. Purification of PCR products was done using either Polyethylene glycol-Nacl method or Exo-SAP method. In the Polyethylene glycol-NaCl method, ethanol along with Polyethylene glycol is used to precipitate the DNA. Sequencing for complete 16S rRNA gene was performed in the ABI 3500xl genetic analyzer (Invitrogen/Life Technologies) using internal primers to get appropriate overlaps of contigs (Weisburg et al. 1991). Detail protocol of cycle sequencing is as fallows

- A reaction mixture containing purified PCR product (50 – 100ng/µl), 3.2 µl primer with final 7.5 µl volume made with Nuclease free water was added to the ninety six well microtiter plate.
- In each well of the microtiter plate, a 2.5 µl big dye diluted solution was added. (Big dye is diluted as: 400µl big dye+ 400µl PCR water + 800µl big dye terminator buffer).
- Cycle sequencing was performed using ; 90°C for 5 min, followed by 25 cycles of 90°C for 10 sec, 50°C for 20 sec, and 60°C for 4 min, followed by final hold at 20°C thermal cycler conditions.
- Purification of the amplified products was performed by adding 12µl of master-mix I (10µl NF water + 2µl 125mM EDTA) and 52µl of master-mix II (2µl 3M Sodium acetate

+50µl 100% Ethanol) and incubation at RT for 15 minutes followed by centrifuge at 3000g for 30 minutes.

- Remove the plates, invert on tissue paper towel and rotate gently in the inverted position at 180g for few seconds.
- Give a subsequent twice wash by adding 100µl 70% ethanol in each well and centrifuge at 3000g for 5 min and repetition of the above step during each wash.
- Remove the plate from the centrifuge, keep for drying at RT for 1 hour, then add 10µl of HiDi formamide in each well and heat for 5 min at 95°C and give a Snap chill on ice.
- Run the 3500x genetic analyser (Invitrogen), obtained raw sequences and edit using ChromasLite software.

3.2.3.3: Identification and phylogenetic analysis

The 16S rRNA sequencing data of all the strains was edited using CHROMASLITE (version 1.5) and analyzed by Basic Local Alignment Search Tool (BLAST) with the closest cultured sequences retrieved from the National Center for Biotechnology Information (NCBI) database (Altschul et al. 1990) and EzTaxon server (Chun et al. 2007). The phylogenetic tree was constructed by a neighbor-joining method using Molecular Evolutionary Genetic Analysis (MEGA) 7 software (Tamura et al. 2013) of representative strains to determine the relationship among these strains. Representative sequences obtained in the present study were deposited in GenBank (NCBI).

3.2.4: Comparing culture-based diversity with metagenomic communities

The isolated fraction of microbial community was analysed for taxonomic diversity of bacteria. From the chapter 2, the same sample was also analysed using metagenomics sequencing for taxonomic diversity. Here, a comparison among these methods for the taxonomic status of bacteria was done. Additionally, the culture diversity was further compared with the metagenomic diversities of the hot springs present in other parts of India.

3.2.5: Metabolic-genetic based functional profiling

All the culture isolates were analysed for their taxonomic statues and diversity analysis. Further, using the 16S rRNA sequences of these isolates, imputed functional analysis was carried out using bioinformatics tools. Further, based on imputed function analysis, representative strains of each genus were evaluated for various bioprospecting activities.

3.2.5.1: Analysis of imputed functions

Imputed (predictive) functional analysis based on 16S rRNA sequences of all the 454 isolates was performed using the Tax4Fun software package (Aßhauer et al. 2015). It uses its own pre-computed association matrix, KEGG (Kyoto Encyclopedia of Genes and Genomes) organism functional profiles and 16S rRNA copy numbers obtained from NCBI database. An empirical pathways profile was created using Tax4Fun output by retrieving the complete list of pathways associated with each protein from the KEGG dataset and dividing the abundance from the Tax4Fun output. Then a class and subclass distribution of the predicted pathways was calculated using the pathways profile in a similar way by retrieving the class of each pathway from KEGG. Pathways profile, class and sub-class profiles were created using in-house scripts relying on KEGG REST API written in Perl and made available to the public on GitHub at https://github.com/ncim-ncl/metgenomics.

3.2.5.2: Assessment for industrially important enzymes

Based on the results of the imputed functional analysis, representative strains of bacterial genera were screened qualitatively for production of important enzymes such as cellulase, xylanase, amylase, and protease. The following media were used for screening activity of each enzyme; Nutrient agar containing 1% carboxymethylcellulose was used for cellulase screening; for xylanase, Xylan agar (0.2% KNO_3, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.3% $CaCO_3$, 0.001% $FeSO_4$, 0.08% xylan. 1.5% agar) was used; for amylase nutrient agar with 1% starch (1% yeast extract, 1% peptone, 1% beef extract, 0.5% NaCl, 1% starch, 1.8%

agar) and Casein agar (1% skimmed milk, 2% agar) was used for protease screening. The cultures were streaked on respective media plates and incubated at 37°C for 48 hours. The clear zone around the colony was considered as positive strains. However, for cellulase and xylanase clear zone was visualized after flooding the plates with an aqueous solution of 0.1% Congo red for 15 minutes and then washed with 1M NaCl solution and for amylase, plates were flooded with Gram's iodine solution.

3.2.5.3: Assessment for heavy metal tolerance

Based on the imputed functional analysis, most of the pathways were mapped for xenobiotic degradation at the sub-system levels. Therefore, such properties of the strains could be harness for environmental applications. Here, representative strains were evaluated for their heavy metal tolerance properties against some toxic heavy metals. Initially, heavy metal tolerance tests were performed against the chromium (Cr^{6+}) and arsenic (As^{3+}) using determination of Maximum Tolerance Limit (MTL). The stock solution of both the heavy metals (potassium dichromate, sodium arsenate) was prepared in distil water and filter sterilized. Different concentrations (chromium: 50 µg/mL–1000 µg/mL and arsenic: 1 mg/mL–350 mg/mL) were added separately to individual tubes of 10 ml LB broth. The tubes were inoculated with 50 µL microbial cultures (1 Optical Density (OD) at 620 nm) and incubated at 37°C for 24 hours. Further, MTL was determined by measuring the OD at 620 nm using a spectrophotometer (Systronics, India).

Based on the chromium and arsenic tolerance results, strains were short listed for further screening against other heavy metals. In total, 27 different bacterial strains were selected and screened for cadmium (II), mercury (II) and zinc (II) tolerance. Screening was done by growing each strain of the bacterial cultures in different concentrations of metal ions and monitoring their growth to determine the MTL for each strain. The stock solution of the heavy metals (Cadmium chloride, Mercury chloride and Zinc sulphate) was prepared and

filter sterilized. Different concentrations (cadmium: 50µg/mL– 500µg/mL, mercury: 1µg/mL - 10µg/mL and zinc: 50µg/mL- 500µg/mL) were added separately to individual tubes of 10 ml LB broth. The tubes were inoculated with 50 µL microbial cultures (1 Optical Density (OD) at 620 nm) and incubated at 37°C for 24 hours. Further, MTL was determined as mentioned previously for chromium and arsenic metals.

3.2.5.4: Assessment for antibiotic resistance

Antibiotic resistance was known among the microorganisms inhabiting the aquatic environments including rivers, sewage, and ocean water (Schwartz etal, 2003). Most of the Indian hot springs may also provide an ideal setting for the acquisition and dissemination of antibiotic resistance, because they are frequently impacted by anthropogenic activities. In addition, there also exist a correlation in between heavy metal and antibiotic resistance. Therefore, in this section, antimicrobial susceptibility testing (AST) of heavy metal tolerating bacterial strains were performed. The strains which were showing high heavy metal tolerance were evaluated for their antibiotic resistance to correlate the antibiotic-heavy metal resistance. Bacterial inoculums were prepared by growing the bacterial cells in NB medium at 37ºC for 24 hours. The 24 hours old grown culture was then spread evenly on MH agar plate using a sterile cotton swab. Antimicrobial susceptibility testing was conducted with the help of Icosa discs (Himedia) of twenty different antibiotics. These discs were placed on the culture spread plates using a sterile forceps and the plates were incubated at 37ºC for 24 hours. Resistance patterns were checked by measuring the diameter of the zone of inhibition around each of the antibiotic discs. The zone diameters of each drug were interpreted using CLSI guidelines (Clinical and Laboratory Standard Institute).

3.2.6: Genomic analysis of strains

3.2.6.1: Genome sequencing, assembly and annotations

Genome sequencing of three different strains was done using Illumina MiSeq M02845 sequencer was carried out using 2 × 300 bp chemistry. Raw reads were checked for the quality of paired-end reads using the FastQC tool and good quality reads (Phred score >Q30) and selected for assembly. Genome assembly was performed using SPAdes (Bankevich et al. 2012) and annotations were carried out using PATRIC (Wattam et al. 2017) and RAST toolkit (RASTtk) (Brettin et al. 2015) and ANI calculator (Han et al. 2016). Based on the annotation statistics and a comparison to other genomes in PATRIC, details of the analysis, including genes of interest (speciality genes), a functional categorization (subsystems) and phylogenetic analysis was carried out. Further, based on the ANI calculator, the genome comparisons of *Geobacillus* species (G86) was performed using RAST and BRIG tools. Raw reads and assembled contigs generated in the study were deposited in the gene bank, SRA (NCBI) under the accession numbers, SRX1704329, SAMN10107875 and SAMN10107876.

3.2.7: Studies of heavy metal tolerant strain *Achromobacter* sp.

Based on the MTL values, the strain (*Achromobacter sp*. WP24) showed the tolerance against most of the heavy metal. Therefore, this strain was further selected for growth curve analysis and other parameter studies. Growth under normal media conditions and in different concentrations of heavy metals was studied for this strain.

3.2.7.1: Growth pattern under normal conditions

Growth curve of *Achromobacter sp*. WP24 strain was determined by monitoring the growth of the culture at equal time intervals under defined parameters. The strain was grown in NB medium for 24 hours and OD was adjusted to 1 at 620 nm. This overnight grown culture was inoculated (1%) to a 100 ml sterile NB medium in 250 ml flasks along with NB as control. After every 3 hours of interval of incubation time till 24 hours and then at 48 hours, growth

was monitored by measuring the OD at 620 nm using NB as black in the spectrometric analysis.

3.2.7.2: Growth patterns under individual heavy metal

Growth curve analysis of *Achromobacter sp*. WP24 strain was performed in presence of different concentrations of heavy metals. Concentrations were selected based on the MTL values of each heavy metal as determined previously. Working solutions of all five metals were added separately in the tubes containing NB media to make the final volume of 10 ml. Initially optimum concentrations such as 200 µg/ml for chromium, 300 µg/ml for cadmium, 6 mg/ml for Arsenic, 3 µg/ml for mercury and 600 µg/ml for zinc was performed. Finally, different levels of concentrations such as sub-lethal concentrations, different MTL concentrations (1/2, 2/3, 1/4 of MTL values) of the respective heavy metal were used for the experiments. Cultures were added as mentioned previously and incubated in shaker incubator at 37ºC at 160 rpm for 48 hours along with the controls (only nutrient media). Growth was monitored as mentioned in previous section.

3.2.7.3: Growth patterns under mixed/pooled heavy metals

The *Achromobacter sp*. WP24 strain was grown in pooled concentrations of different heavy metals (concentrations were selected based on MTL values). Different levels of MTL concentrations (1/2, 2/3, 1/4 of MTL values) of the respective heavy metal were used for pooling experiments.

3.2.7.4: Effect of mixed/pooled heavy metals on cell morphology

To evaluate the effect of heavy metal stress condition on the cell morphology of the *Achromobacter sp*. WP24, scanning electron microscopy (SEM) studies were performed. The overnight grown culture was inoculated into a 100 ml NB media in 250 ml flasks. Flasks were incubated at 37ºC in shaker incubator at 150 rpm for 48 hours along with the control flasks. The cells were harvested by centrifugation, washed two-three times and then

resuspended in sterile saline solution. Both the control and heavy metal-stressed cells were prepared for SEM analysis. The incubated cultures were centrifuged for 5-10 min at 6000 rpm and cell pellet was collected, resuspended in sterile distilled water. Samples preparation was performed using glutaraldehyde and ethanol in an appropriate concentrations. A loopful of suspension was kept on the silicon wafer and dried under vacuum for 4-5 h and observed for SEM images using a JEOL JSM-6360A microscope.

3.2.7.5: Chromium (VI) reduction assay

The *Achromobacter* sp. (WP24) strain found to tolerate high level of chromium, here, the objective is to evaluate the reduction potential of *Achromobacter* sp. (WP24) for chromium (VI) using a diphenyl carbazide assay (Camargo et al, 2003). Culture was grown in 250 ml flasks containing 100 ml nutrient broth with different concentration of Cr (VI) (50 µg - 300µg) by inoculating with 1% overnight grown culture (OD_{620}: 1). Flasks including controls (without culture) were incubated at 37 ºC shaking at 150 rpm for 24-48 h. Aliquots of 1 ml form the samples were taken out at 24 and 48 hours of incubation, Cr (VI) concentration and extracellular chromium reductase enzyme activity. For enzyme activity 0.2 ml cell free extract was added to 1.2 ml of 1mM $K_2Cr_2O_7$, 0.6ml of 100mM Phosphate buffer pH 7 and kept for 30 minutes for incubation at 30ºC. Tubes were centrifuged, supernatant was collected and added with 0.2 ml of 1, 5- diphenylcarbazide (DPC). The reaction mixture was kept at room temperature at 5 minutes and absorbance was measured at 544 nm.

3.3: Results and discussions

3.3.1: Cultivation using solid media and enrichment methods

Using all the cultivation strategies, a total of 454 isolates were isolated. Among these 454 isolates, 290 isolates were obtained using multiple culture media with the remaining from enrichment techniques (temperature stress: 46, nutrient stress: 55; chemical stress: 63;

phenol, 44 + PAH compounds, 19). In general, use of multiple media for isolation found to be effective for isolation of diverse bacteria. After morphological investigations, it was observed that the bacteria with different colony morphologies were captured on very low nutrient agar plates. Culture media with high nutrient content has favoured the growth of fast-growing bacteria, ultimately suppressing the growth of other slow-growing bacteria. All the isolated cultures were checked for their morphologies and finally characterized using molecular techniques.

3.3.2: Molecular characterization of isolated strains

3.3.2.1: Genomic DNA, PCR amplification and purification

Genomic DNA isolation of all the isolated strains was performed. The isolated DNA was checked for its integrity on 0.8% agarose gel electrophoresis along with 1 Kb DNA ladder and visualization using Gel Documentation (Protein simple). A representative gel image of the agarose gel electrophoresis of genomic DNA, PCR amplification and purification of few samples is shown in figure 3.1.

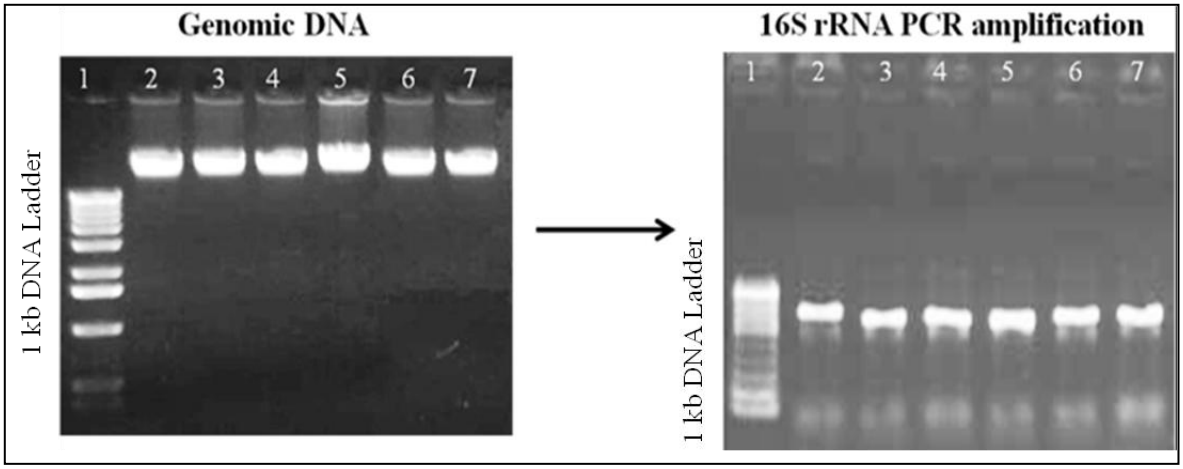


Fig. 3.1: Representative gel images of the genomic DNA and PCR amplified of the 16S rRNA gene (1.5 kb)

The concentration and purity of extracted DNA was analyzed using spectrophotometer (Nanodrop LTE) and appropriated quantities were used for PCR amplification of 16s rRNA

gene. Furthermore, amplified products were purified; quality and quantity assessment was performed and used for sequencing.

3.3.2.2: Sequencing analysis and nucleotide accession numbers

The purified products were subjected to cycle sequencing followed by purification and analyzed for sequencing using capillary sequencer (Applied Biosystems, USA). The raw sequences data obtained in the .abi format from the analyser edited using chromas software. The edited sequences were then checked for sequence similarity to the sequences from genebank database using nucleotide BLAST (BLASTn). The 16S rRNA sequence similarity was also performed using Eztaxon database having validly published names. The 16S rRNA gene sequencing and BLAST analysis at NCBI as well as EzTaxon database mapped all the isolates into 57 unique bacterial genera. All the representative sequences were deposited in NCBI database. The detailed analysis of representative strains with accession number is shown in (Table 3.1).

Table 3.1: Representative bacterial isolates, closest match in database and percent similarity based on 16S rRNA sequencing analysis

Strain name	Closest match in EzTaxon	Percent Similarity	Accession number
U17	*Acinetobacter junii* CIP 64.5(T) (APPX01000010)	99.61% (1305/1310)	MF101709
U23	*Staphylococcus gallinarum* ATCC 35539(T) (D83366)	100.0% (1404/1404)	MF101714
U24	*Limnobacter thiooxidans* CS-K2(T) (AJ289885)	98.13% (1370/1396)	KT345630
U252	*Citrobacter murliniae* CDC 2970-59(T) (AF025369)	99.02% (1308/1321)	KT345637
U270	*Ramlibacter tataouinensis* TTB310(T)(CP000245)	98.54% (1353/1373)	KT345639
U274	*Phreatobacter oligotrophus* PI_21(T) (HE616165)	99.53% (1269/1275)	KT345640
U275	*Acidovorax delafieldii* ATCC 17505(T) (AF078764)	99.38% (1451/1460)	MF101692
U277	*Bacillus subtilis subsp.* KCTC 13429(T)(AMXN01000021)	99.86 (1450/1452)	KT345642

U31	*Azospirillum zeae* N7(T) (DQ682470)	98.40% (1294/1315)	KT345631
U340	*Ferrovibrio xuzhouensis* LM-6(T) (KM978211)	97.52% (1296/1329)	MF101693
U342	*Phenylobacterium muchangponense* A8(T) (HM047736)	98.30% (1333/1355)	MF101711
U348	*Rhodobacter blasticus* ATCC 33485(T) (D16429)	96.57% (1054/1108)	KT345646
U374	*Methyloversatilis universalis* FAM5(T) (AFHG01000027)	99.73%(752/754)	KT345647
U382	*Caulobacter daechungensis* H-E3-2(T)(JX861096)	99.26% (1338/1348)	KT345648
U42	*Novosphingobium subterraneum* IFO 16086(T)(AB025014)	98.01% (1330/1357)	KT345633
U47	*Cupriavidus pauculus* LMG 3413(T)(AF085226)	100.0% (1269/1269)	MF101710
U49	*Caldimonas taiwanensis* On1(T)(AY845052)	99.51% (748/750)	KT345634
U58	*Barrientosiimonas humi* 39(T)(JF346171)	99.93% (1438/1439)	KT345635
U67	*Kocuria palustris* DSM 11925(T) (Y16263)	100.00% (998/998)	KT345636
UAC10	*Enterobacter hormaechei* ATCC 49162(T) (AFHR01000079)	99.5% (1416/1423)	MF101702
UAC108	*Aquabacter spiritensis* DSM 9035(T)(FR733686)	98.32% (1234/1255)	MF101701
UAC146	*Oleomonas sagaranensis* HD-1(T)(D45202)	100.00% (641/641)	KT345656
UAC151	*Xanthobacter autotrophicus* 7c(T) (X94201)	98.44% (1141/1159)	KT345657
UAC255	*Rhodococcus cercidiphylli* YIM 65003(T) (EU32554)	99.30% (1411/1421)	MF101691
UAC16	*Gordonia terrae* NBRC 100016(T) (BAFD01000032)	99.78% (1423/1426)	KT345650
UAC17	*Microbacterium invictum* DC-200(T)(AM949677)	98.79% (1393/1410)	KT345651
UAC23D	*Methyloterrigena soli* M48(T) (KP876580)	97.22% (1363/1404)	MF101685
UAC253	*Bradyrhizobium daqingense* CGMCC1.10947(T) (jgi.1041378)	100.00 % (246/246)	MF101708
UAC257	*Labrenzia suaedae* DSM 22153(T) (jgi.1107719)	97.43% (1363/1403)	KT345658
UAC259	*Corynebacterium coyleae* DSM 44184(T) (X96497)	100.00% (706/706)	KT345659

UAC265	*Brevundimonas viscosa* CGMCC 1.10683(T) (jgi.1076140)	99.85% (687/688)	KT345660
UAC275	*Rhizobium alvei* TNR-22(T) (HE649224)	97.27% (1352/1390)	MF101684
UAC46	*Pseudomonas alcaligenes* LMG 1224(T) (Z76653)	99.66% (1467/1472)	MF101712
UAC26	*Nocardioides iriomotensis* IR27-S3(T) (AB544079)	97.57% (1327/1360)	KT345653
UAC15	*Hydrogenophaga bisanensis* K102(T)(EF532793)	99.92% (1318/1319)	MF101716
UAC198	*Porphyrobacter neustonensis* DSM 9434(T) (AB033327)	99.30% (1151/1159)	MF101717
UAC72	*Cronobacter pulveris* 601/05(T)(DQ273684)	99.44% (713/717)	KT345654
WP24	*Achromobacter aegrifaciens* LMG 26852(T) (HF586507)	98.10% (673/686)	KT345674
SA34	*Bhargavaea ginsengi* ge14(T)(EF371375)	100.00% (1422/1422)	KT345672
SA64	*Exiguobacterium aurantiacum* DSM 6208(T) (JNIQ01000001)	100.00% (1213/1213)	KT345673
SA9	*Macrococcus caseolyticus* ATCC 13548 (T) (Y15711)	98.98% (1456/1469)	MF101694
EU14	*Lysinibacillus fusiformis* NBRC 15717(T) (AB271743)	100% (517/517)	MF101715
EM34	*Paenibacillus thiaminolyticus* NBRC 15656 (T) (AB073197)	99.82% (576/577)	MF101703
GH105	*Deinococcus arenae* SA1(T) (KF790633)	99.78% (889/891)	MF101700
GH118	*Aeromicrobium ponti* HSW-1(T) (AM778683)	98.85% (1379/1395)	MF101688
GH119	*Cellulosimicrobium funkei* ATCC BAA-886(T)(AY501364)	99.31% (734/739)	MF101704
GH12	*Arthrobacter globiformis* NBRC 12137(T) (BAEG01000072	98.60% (1334/1353)	MF101713
GH125	*Ochrobactrum intermedium* LMG 3301(T) (ACQA01000003)	99.93% (1406/1407)	MF101696
GH39	*Streptomyces gancidicus* NBRC 15412(T) (AB184660)	99.28% (1382/1392)	MF142758
GH71	*Actinomadura apis* IM17-1(T) (AB557596)	99.68% (622/624)	MF101706
GH81	*Dietzia schimae* YIM 65001(T) (EU375845)	98.64% (1380/1399)	MF101695

GH99	*Micromonospora aurantiaca* ATCC 27029(T) (CP002162)	99.65% (1432/1437)	MF101683
GHP2	*Janibacter indicus* 0704P10-1(T) (HM222655)	99.78% (1333/1336)	MF101680
GHP2B	*Brevibacterium pityocampae* DSM 21720(T) (EU484189)	99.56% (1357/1363)	MF101689
GHP3	*Rothia dentocariosa* ATCC 17931(T)(CP002280)	99.92% (1313/1314)	MF101707
GHP77	*Cellulomonas carbonis* T26(T) (HQ702749)	99.54% (1309/1315)	MF101705
GP2	*Micrococcus terreus* CGMCC 1.7054(T) (jgi.1058018)	99.52% (1437/1444)	MF101699

3.3.3: Phylogenetics and taxonomic diversity

3.3.3.1: Phylogenetics

Phylogenetic analysis revealed that all strains showed around 97-100% identity with sequences present in curated databases as shown in (Table 3.1). Here, we found an almost equal number of genera from both Gram-positive (27 genera) and Gram-negative (30 genera) bacteria. Among Gram-negative bacteria, 16S rRNA sequences of 3 strains showed 100% similarity to the type strains present in the database, 14 strains shared >99% similarity, 8 strains shared >98% similarity whereas 4 strains shared >97% similarity and one strain shared >96% with cultured type strains present in the database (EzTaxon). In case of Gram-positive bacteria, strains belonging to *Firmicutes* shared 99-100% similarity, whereas 13 Actinobacterial strains shared >99%, 5 strains shared >98% and 1 strain shared >97% similarity with the cultured type strains present in the database. The phylogenetic relationship of the representative isolates of both Gram positive and Gram negative groups are shown (Fig. 3.2A and 3.2B).

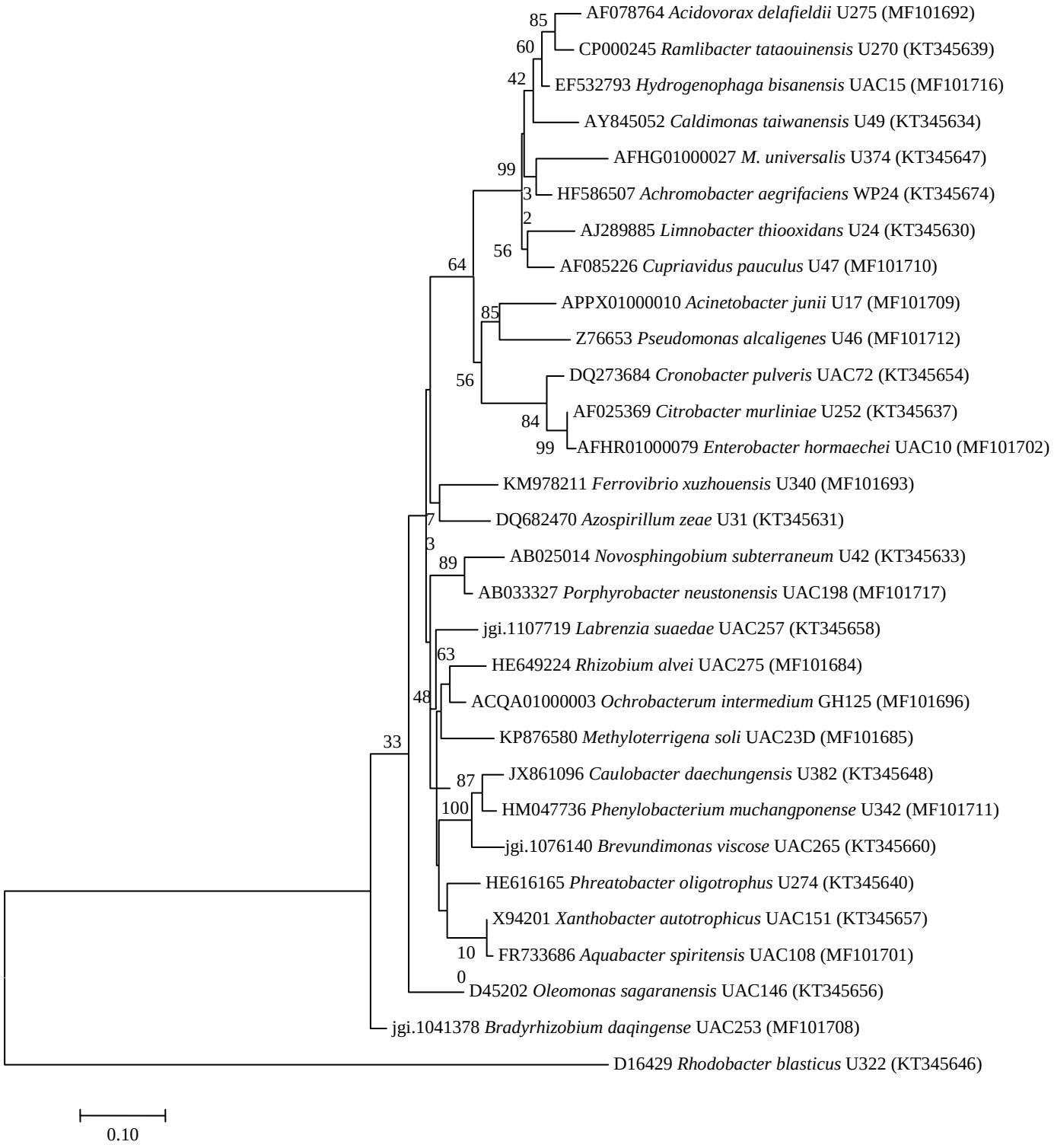


Fig 3.2A: The Phylogenetic tree of the representative Gram negative bacterial genera, constructed using the Neighbour-Joining method with bootstrap test (500 replicates) using MEGA7 software

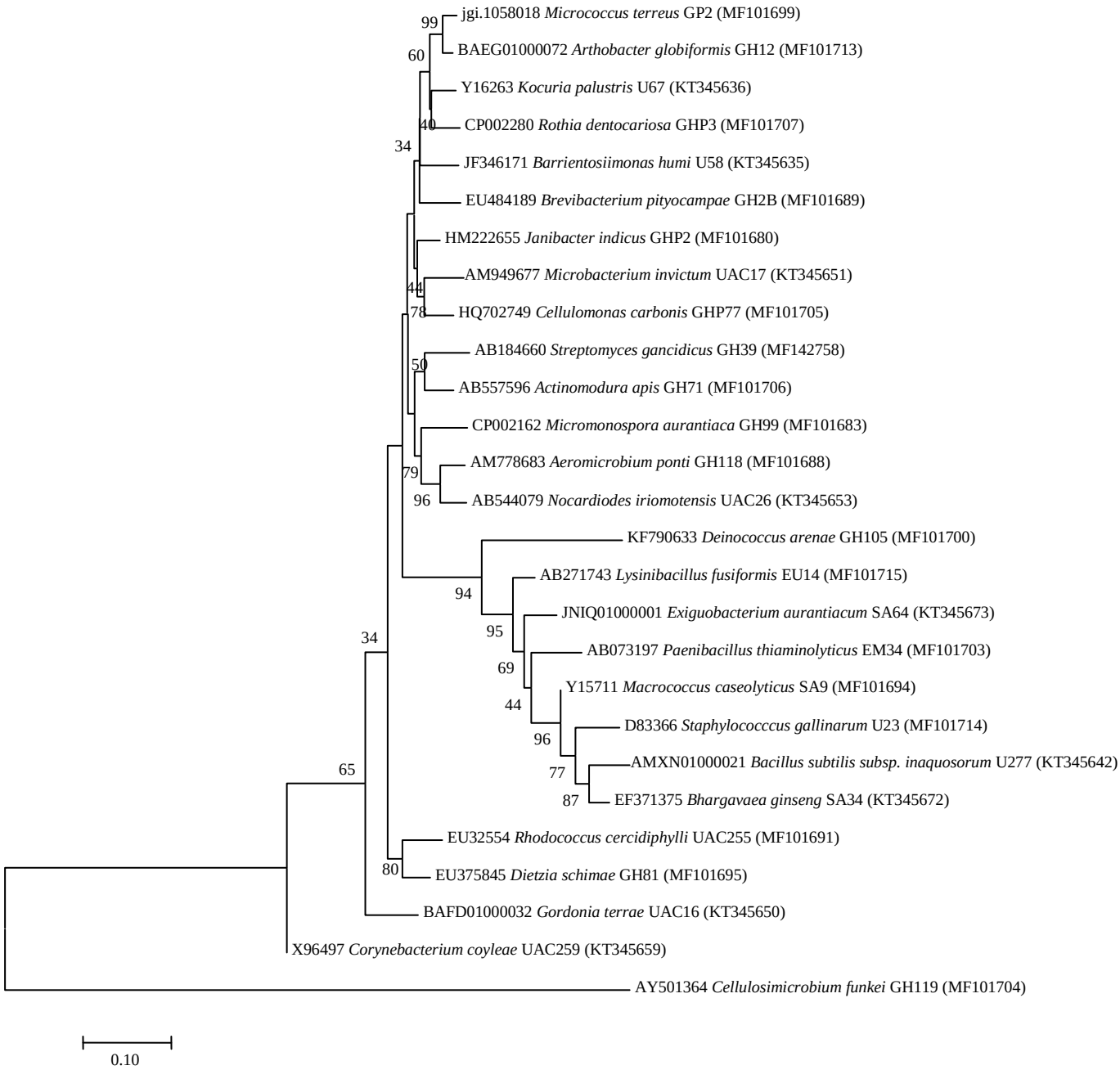


Fig 3.2B: Phylogenetic tree of the representative Gram positive bacterial genera, constructed using the Neighbour-Joining method with bootstrap test (500 replicates) using MEGA7 software.

3.3.3.2: Taxonomic diversity

The taxonomic position of all the 57 bacterial genera showed that 56 genera (one genus was from an unassigned order) belong to 17 different taxonomic orders under 6 different classes in the four bacterial phyla (Fig. 3.3 and Table 3.2).

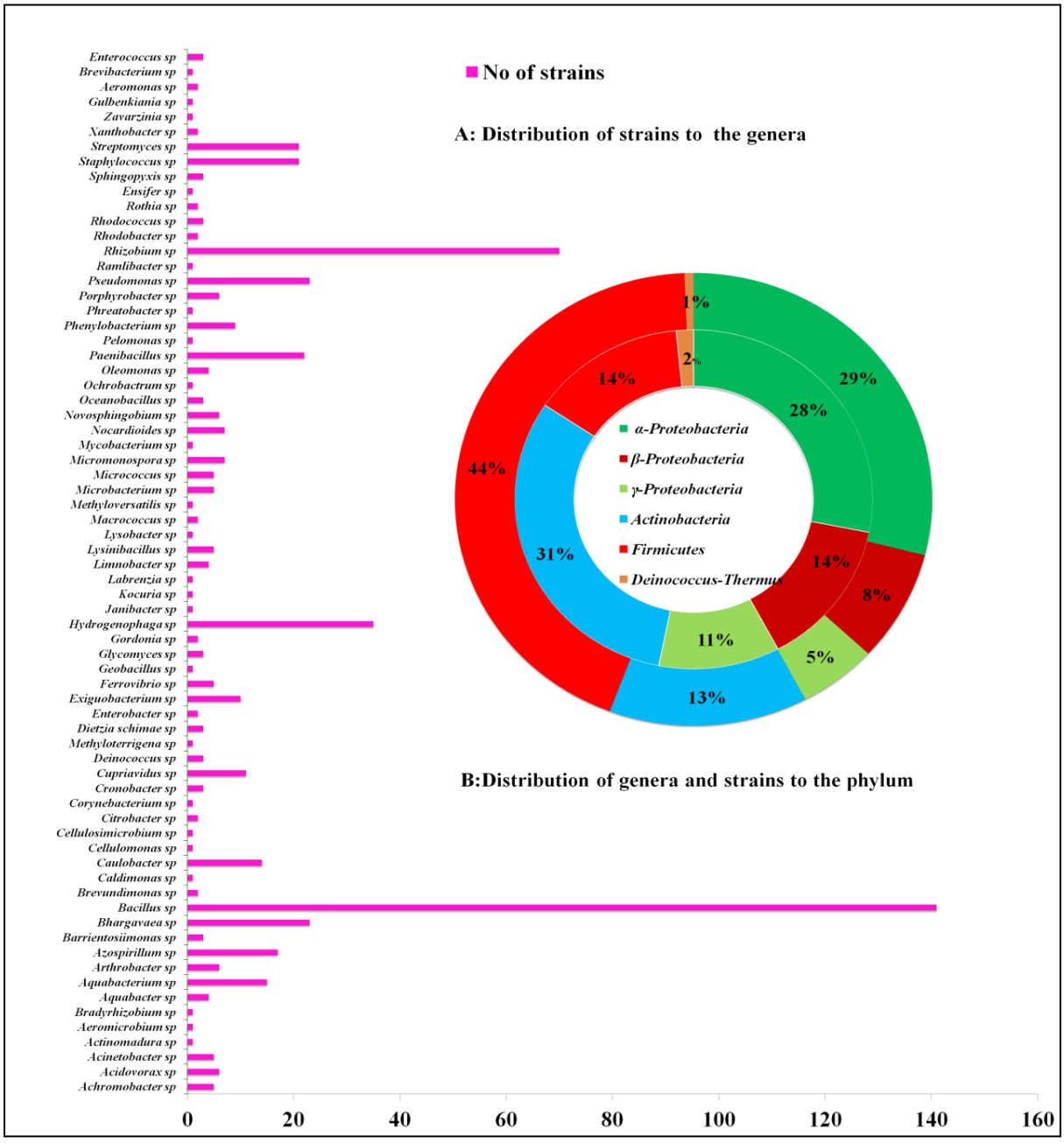


Fig. 3.3: Distribution of number of strains to each genus and phylum, **A:** showing number of strains obtained for each genus; **B:** percentage distribution of genus and strains to each phylum.

The relative abundance was highest for *Bacillales*, *Rhizobiales*, *Burkholderiales*, *Rhodospirillales*, *Caulobacterales*, *Micrococcales*, and *Streptomycetales* (Table 3.2)

Table 3.2 Relative abundance of the isolates with respect to the phylum, class and order at taxonomic levels

Phylum	**Class**	**Order**	**No of strains**	**Relative abundance (%)**
Proteobacteria	α-Proteobacteria	*Rhizobiales*	82	17.62
		Rhodospirillales	26	5.7
		Caulobacterales	25	5.5
		Rhodobacterales	3	0.66
		Sphingomonadales	6	1.32
		Unassigned	1	0.22
	β-Proteobacteria	*Burkholderiales*	37	8.14
		Rhodocyclales	1	0.22
	γ-Proteobacteria	*Pseudomonadales*	16	3.52
		Enterobacterales	7	1.54
Actinobacteria	Actinobacteria	*Actinomycetales*	9	1.98
		Micrococcales	20	4.40
		Corynebacteriales	6	1.32
		Micromonosporales	5	1.10
		Propionibacteriales	4	0.88
		Streptomycetales	10	2.20
Firmicutes	Bacilli	*Bacillales*	197	42.95
Deinococcus-Thermus	Deinococci	*Deinococcales*	3	0.66

3.3.3.3: Composition of dominant and rare bacteria

The phylum level distribution showed that *Firmicutes* are more dominant (197 isolates, 7 genera); constituting a majority of the *Bacillus* spp. The second most dominant group was *Proteobacteria* with great taxonomic varieties (200 isolates, 30 genera); *Rhizobium*, *Azospirillum*, *Caulobacter*, *Pseudomonas*, *Cupriavidus*, *Hydrogenophaga*, *Phenylobacterium* comprised the maximum number of isolates along with some rare genera such as *Ramlibacter*, *Phretobacter*, *Methyloversatilis*, *Rhodobacter*, *Caldimonas*, *Methaloterigenea*. While in the case of *Actinobacteria*, the proportion of isolates was found to be more evenly distributed among genera (54 isolates, 24 genera) among which *Streptomyces*, *Arthrobacter*, *Micromonospora*, *Micrococcus*, *Microbacterium* and *Nocardioides* were found with a relatively higher number of isolates. Few genera with the least number of isolates were also obtained such as *Actinomodura*, *Cellulomonas*, *Cellulisimicrobium*, *Corynebacterium*, *Barrentisimonas* and *Dietzia*. Lastly, isolates belonging to phylum *Deinococcus-Thermus* were found in a low number (3 isolates) and within only one genus. Important dominant genera of each phylum as well as overall rare bacterial genera are shown in the figure 3.4 and 3.5 respectively.

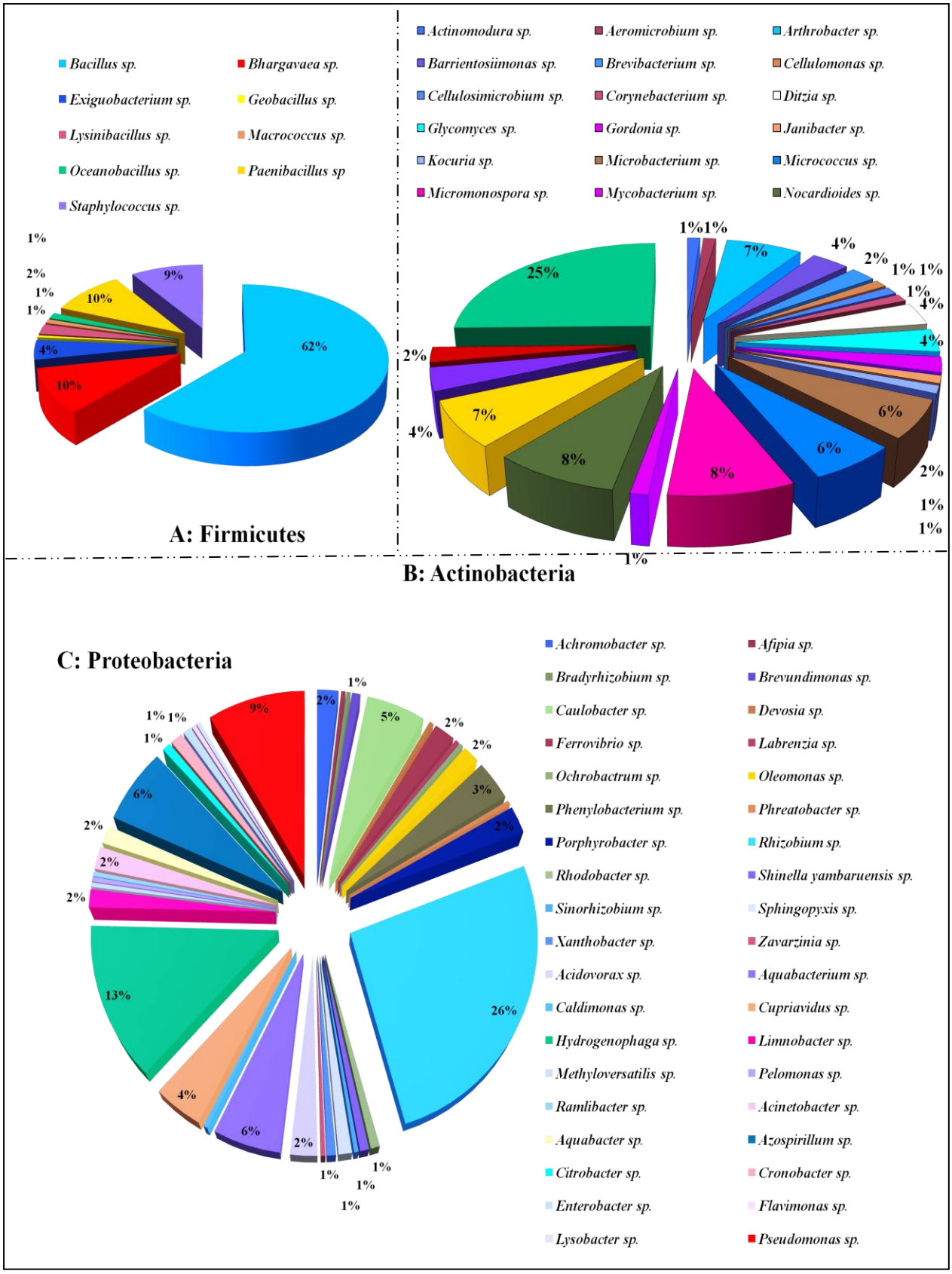


Fig 3.4: Diversity of dominant bacterial genera among four different bacterial phlya

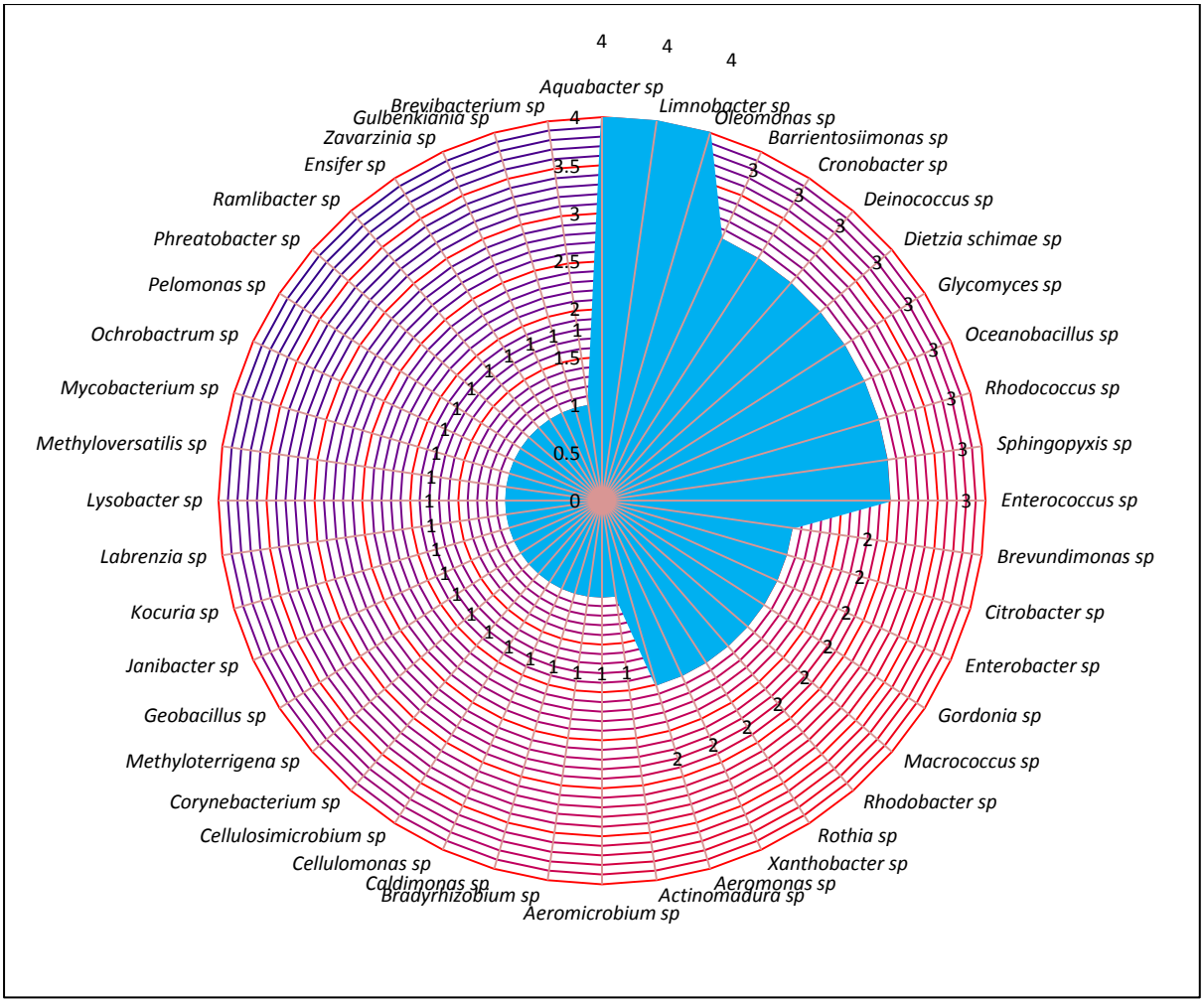


Fig. 3.5: Distribution of the rare bacteria genera cultivated using various cultivation methods and strategies

3.3.4: Comparing culture-based diversity with metagenomic communities

3.3.4.1: Within same (Unkeshwar) hot spring

Taxonomic analysis of the isolated bacterial strains has revealed the presence of 57 different bacterial genera. These genera were mapped with metagenome data (chapter 2), the result revealed that in total 42 bacterial genera were matched with the metagenomic analysis. However, other 15 genera do not any match with the metagenome data. The overall comparison is shown in the figure 3.6. These results indicate that the culture-dependent and culture- independent analysis could capture the different portion of microbial communities.

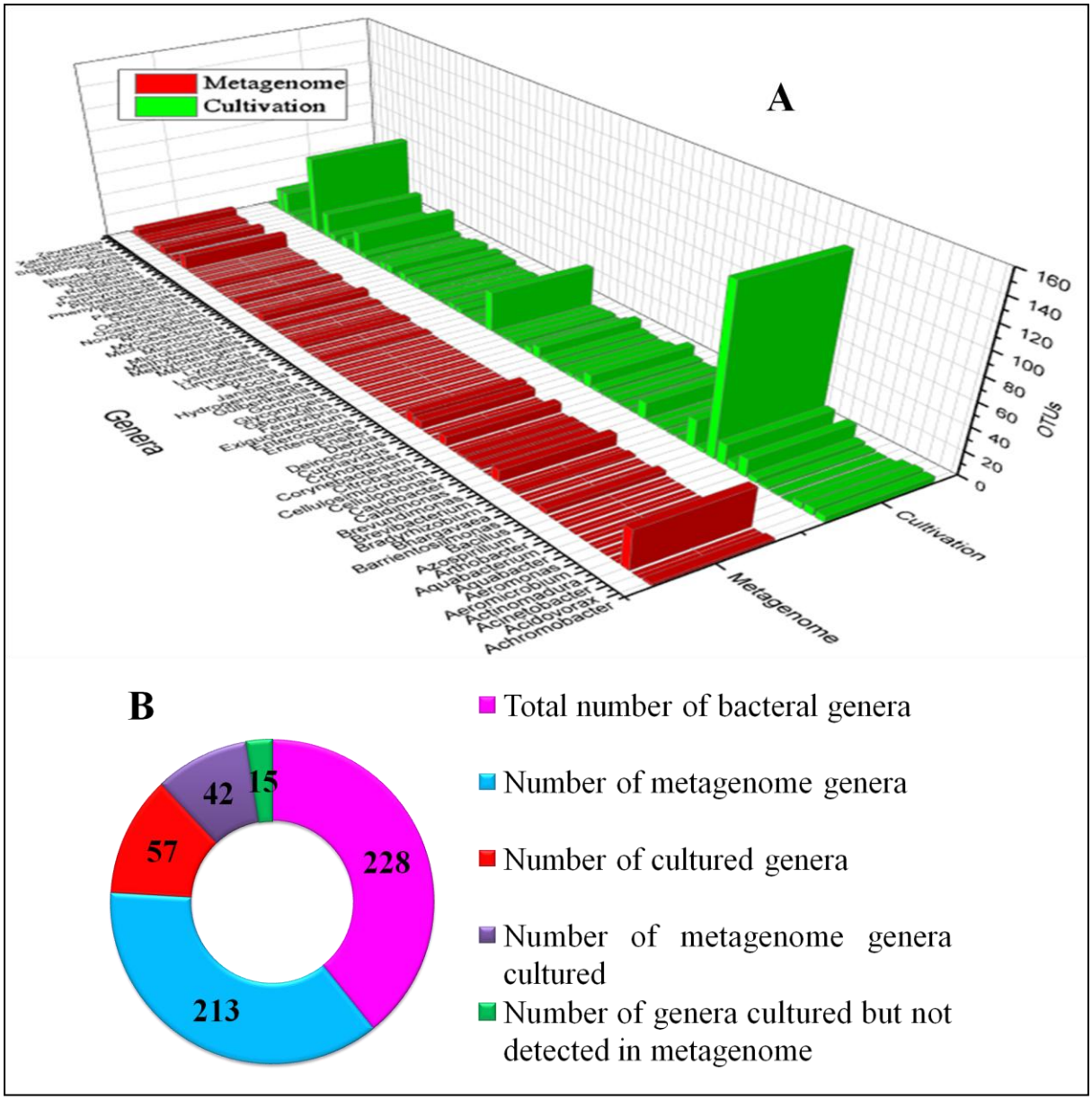


Fig. 3.6: Comparative analysis of the cultured isolates with metagenomic community

3.3.4.2: With other Indian hot springs

A comparative analysis of the culture based diversity (genus level) performed in this study with the microbial diversities of other Indian hot springs was performed. Among all the bacterial genera obtained, only 20 different genera were found to be isolated in the previous studies of hot springs. Whereas, more than thirty genera were detected in the culture-independent analysis of other hot springs. While most other genera reported for their isolation for the first time in this study (Table 3.3).

Table 3.3: List of bacterial genera, first time isolated from Unkeshwar hot spring

Strain name	Closest match in EzTaxon	References	Accession numbers
GHP3	*Rothia dentocariosa ATCC 17931(T)(CP002280)*	99.92% (1313/1314)	MF101707
GHP77	*Cellulomonas carbonis T26(T) (HQ702749)*	99.54% (1309/1315)	MF101705
U274	*Phreatobacter oligotrophus PI_21(T) (HE616165)*	99.53% (1269/1275)	KT345640
UAC72	*Cronobacter pulveris 601/05(T)(DQ273684)*	99.44% (713/717)	KT345654
SA9	*Macrococcus caseolyticus ATCC 13548 (T) (Y15711)*	98.98% (1456/1469)	MF101694
GH125	*Ochrobactrum intermedium LMG 3301(T)*	99.93% (1406/1407)	MF101696
UAC23D	*Methyloterrigena soli M48(T) (KP876580)*	97.22% (1363/1404)	MF101685
UAC253	*Bradyrhizobium daqingense CGMCC 1.10947(T)*	100.00 % (246/246)	MF101708
UAC257	*Labrenzia suaedae DSM 22153(T) (jgi.1107719)*	97.43% (1363/1403)	KT345658
UAC259	*Corynebacterium coyleae DSM 44184(T)(X96497)*	100.00% (706/706)	KT345659
U58	*Barrientosiimonas humi 39(T)(JF346171)*	99.93% (1438/1439)	KT345635
UAC108	*Aquabacter spiritensis DSM 9035(T)(FR733686)*	98.32% (1234/1255)	MF101701
UAC146	*Oleomonas sagaranensis' HD-1(T)(D45202)*	100.00% (641/641)	KT345656
UAC151	*Xanthobacter autotrophicus 7c(T) (X94201)*	98.44% (1141/1159)	KT345657

However, in the present study those genera that matched with previously cultivated hot spring isolates were mostly found to belong to the *Firmicutes,* and other members of *Actinobacteria* and some *Proteobacteria*. Interestingly, with the help of diverse culture media and conditions around 37 bacterial genera were cultivated for the first time from hot springs of India (Table 3.4). Most of them found to belongs to *Proteobacteria* (22 genera) and *Actinobacteria* (14 genera) and only one was found from *Firmicutes*. However, some of these genera were found to be present in other hot springs but were reported by metagenomics studies. For instance, 13 genera; *Ramlibacter*, *Acidovorax*, *Caulobacter*, *Novoshingobium*, *Gordonia*,

Nocardioides, *Hydrogenophaga*, *Porphyrobacter*, *Achromobacter*, *Arthobacter*, *Dietzia*, *Streptomyces*, and *Brevibacterium* were previously identified from different Indian hot springs using omics-based techniques.

Table 3.4: List of bacterial genera isolated in present study and matched with previous metagenomics analysis of hot springs

Strain name	**Closest match in EzTaxon**	**References**
U24	*Limnobacter thiooxidans* CS-K2(T) (AJ289885)	Amin et al. 2017
U270	*Ramlibacter tataouinensis* TTB310(T)(CP000245)	Saxena et al. 2017
U275	*Acidovorax delafieldii* ATCC 17505(T) (AF078764)	Mehetre et al. 2015a; Saxena et al. 2017
U31	*Azospirillum zeae* N7(T)(DQ682470)	Lavrinenko et al. 2010
U340	*Ferrovibrio xuzhouensis* LM-6(T) (KM978211)	Amin et al. 2017
U342	*Phenylobacterium muchangponense* A8(T)(HM047736)	Amin et al. 2017
U374	*Methyloversatilis universalis* FAM5(T)(AFHG01000027)	Amin et al. 2017
U382	*Caulobacter daechungensis* H-E3-2(T)(JX861096)	Mehetre et al. 2015a
U42	*Novosphingobium subterraneum* IFO 16086(T)	Mehetre et al. 2015a; Sharma et al. 2017
UAC16	*Gordonia terrae* NBRC 100016(T) (BAFD01000032)	Sharma et al. 2017
UAC275	*Rhizobium alvei* TNR-22(T) (HE649224)	Amin et al. 2017
UAC26	*Nocardioides iriomotensis* IR27-S3(T) (AB544079)	Sharma et al. 2017
UAC15	*Hydrogenophaga bisanensis* K102(T)(EF532793)	Sharma etal. 2014
UAC198	*Porphyrobacter neustonensis* DSM 9434(T)(AB033327)	Sangwan et al. 2015; Sharma et al. 2017
WP24	*Achromobacter aegrifaciens* LMG 26852(T) (HF586507)	Mehetre et al. 2015a; Sangwan et al. 2015
GH118	*Aeromicrobium ponti* HSW-1(T) (AM778683)	Amin et al. 2017
GH12	*Arthrobacter globiformis* NBRC 12137(T)	Bhatia et al. 2015a; Panda et al. 2016

GH39	*Streptomyces gancidicus* NBRC 15412(T) (AB184660)	Panda et al. 2016
GH71	*Actinomadura apis* IM17-1(T) (AB557596)	Liu et al, 2016
GH81	*Dietzia schimae* YIM 65001(T) (EU375845)	Sharma et al. 2017
GH99	*Micromonospora aurantiaca* ATCC 27029(T)	Amin et al. 2017
GHP2	*Janibacter indicus* 0704P10-1(T) (HM222655)	Amin et al. 2017
GHP2B	*Brevibacterium pityocampae* DSM 21720(T)	Sharma et al. 2017

3.3.5: Metaboli-genetic based functional analysis

3.3.5.1 Imputed functional analysis

Sequences of the isolated strains were analysis for imputed functional analysis, which has revealed the presence of 6235 molecules. Among them, 3364 molecules were having the assigned KEGG identity. Our own in-house scripts identified 315 pathways from the KEGG database. Subsystem level classification of the pathways showed that metabolism being the most abundant followed by environmental information processing and genetic information processing (Fig. 3.7). Further level categorization of metabolism, showed that carbohydrate metabolism was more dominant, very closely followed by amino acid metabolism. Some of the KEGG subsystems were also mapped to the pathways for degradation of xenobiotic compounds and biosynthesis of secondary metabolites, which indicates the strong potential of these microorganisms in biotechnological applications. Also, some KEGG subsystems were found associated with the metabolism of sulphur and methane.

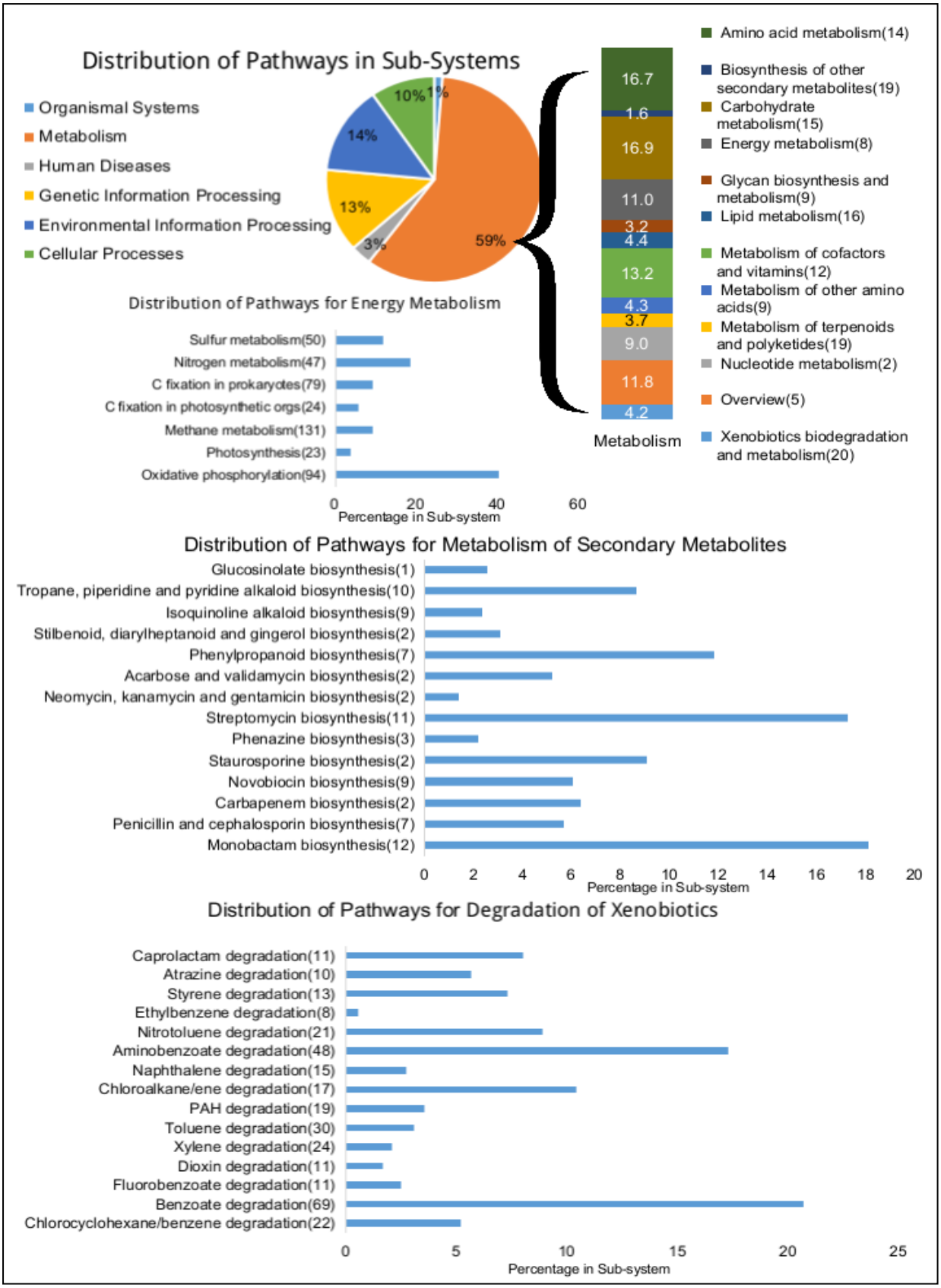


Fig. 3.7: Imputed functional analysis with the relative abundance of assigned KEGG identity to the pathways at the subsystem levels

3.3.5.2: Assessment for industrially important enzymes

Screening for hydrolytic enzyme production showed that around 33 genera were found positive for production of at least one enzyme. Interestingly, among these 33 positive genera, 18 genera were from a set of 37 genera were previously not explored. Among these 18 genera, 11 were positive for cellulase, 5 for xylanase, 7 for amylase and 8 for protease. Plate based screening and overall positive strains for production of each enzyme is shown in the figure 3.8A and B.

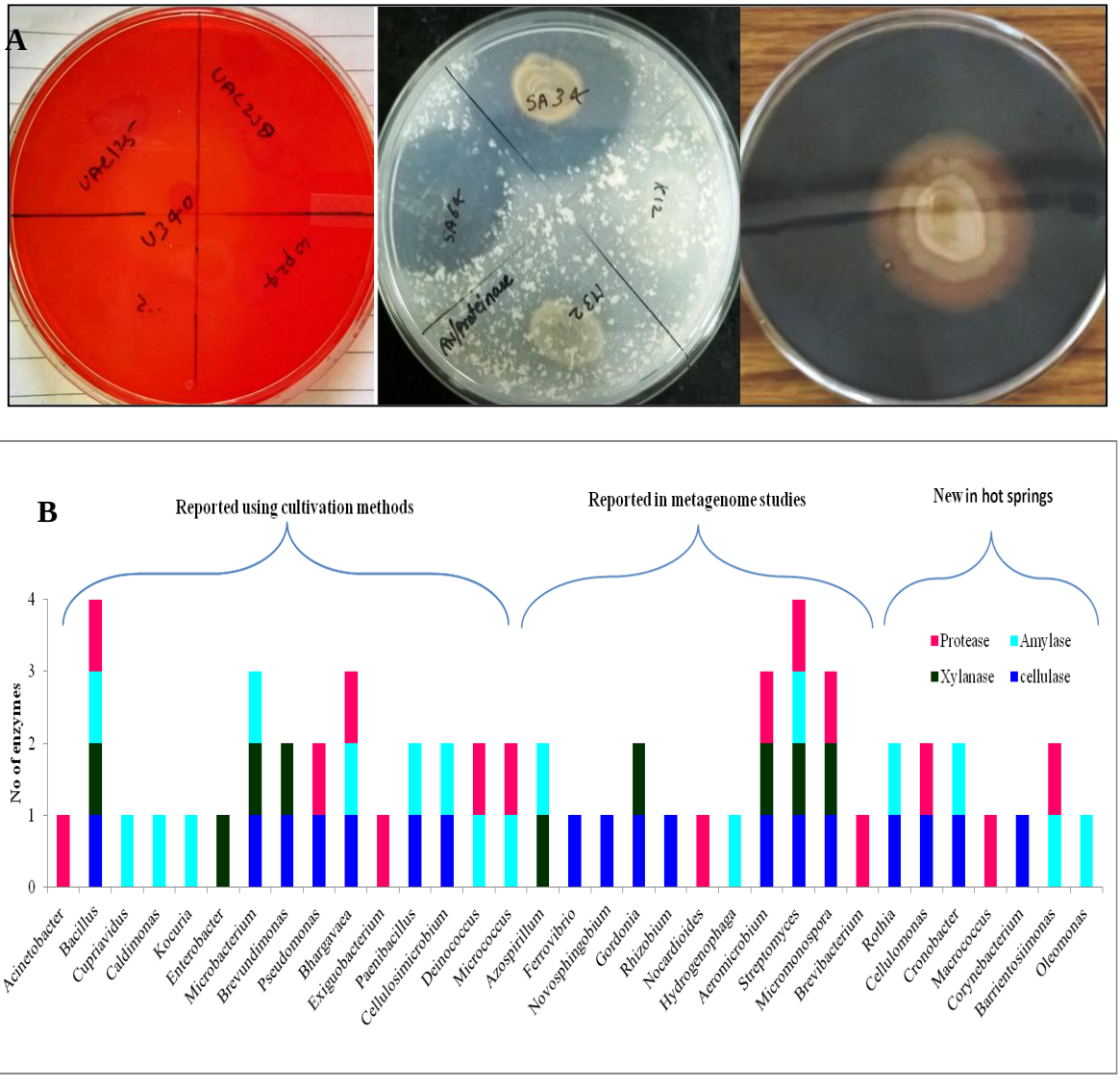
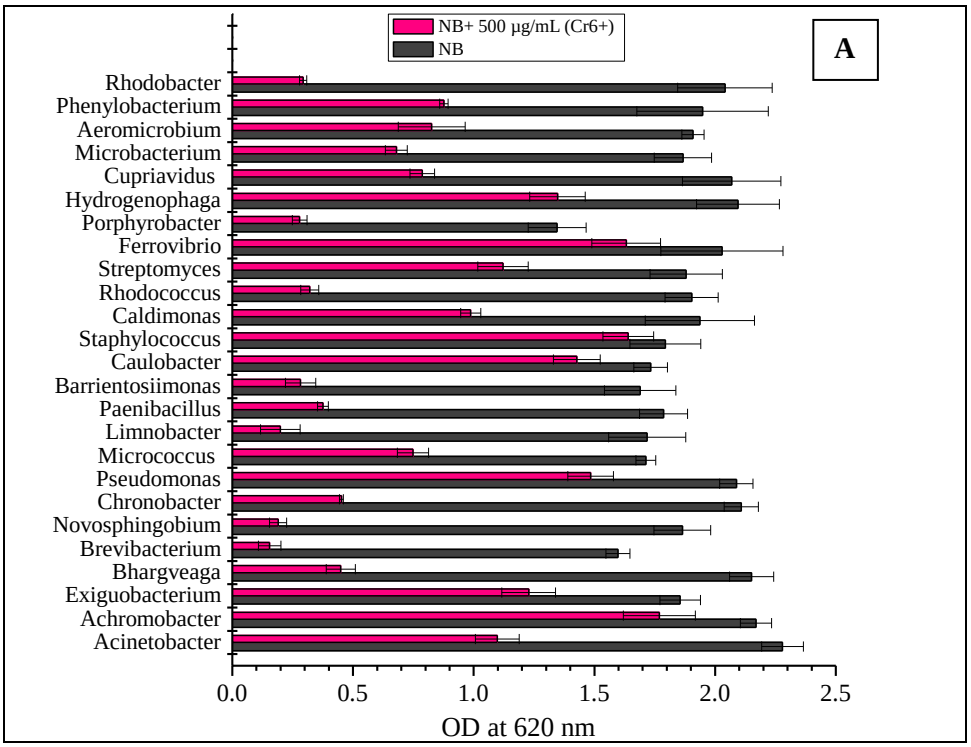


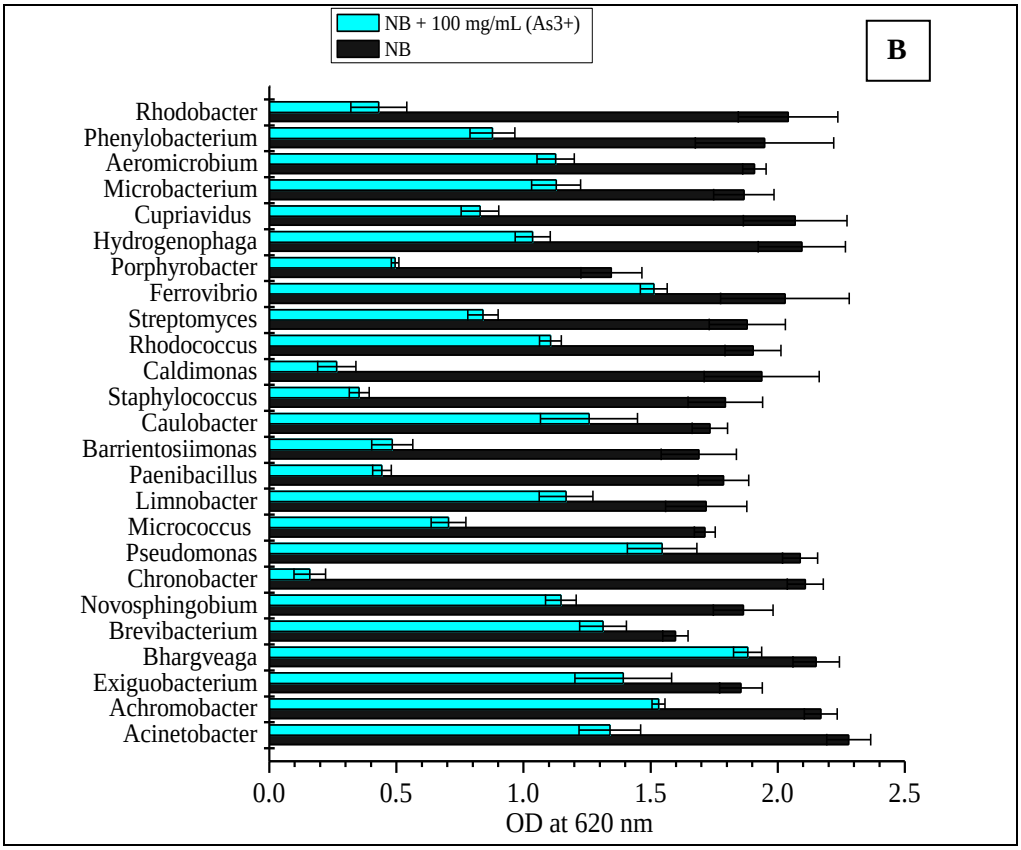
Fig. 3.8: Positive bacterial strains producing important hydrolytic enzymes; cellulase, xylanase, amylase and protease production

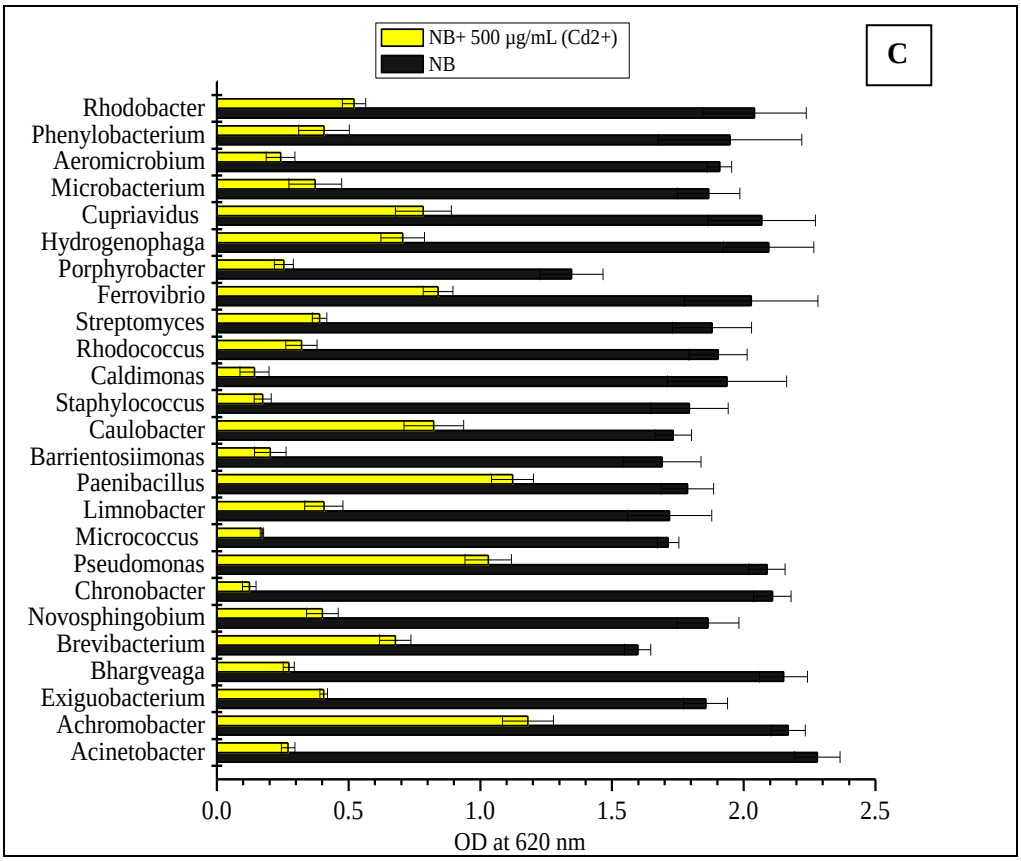
Overall results showed that *Bacillus* (U277) and *Streptomyces* (GH39) were found to produce all four enzymes, while other strains such as *Bhargavaea* (SA34), *Paenibacillus* (EM34), *Microbacterium* (UAC17), *Aeromicrobium* (GH118) and *Micromonospora* (GH99) were found to produce more than one enzyme. The enzymes cellulase and xylanase were mostly produced by actinobacterial strains, whereas *Bacillus* and related genera were found to produce amylases and proteases.

3.3.5.3: Assessment for heavy metal tolerance

Heavy metal tests of all the representative genera performed against the chromium and arsenic have revealed that most of the genera showed the high levels of tolerance. Around 21 genera were found to tolerate >500 µg/ml chromium and 25 were able to tolerate >100 mg/ml arsenic. From these, 25 genera which were selected for further analysis based on their tolerance to both of these heavy metals. The selected 25 genera were tested for minimum tolerance limits (MTL) determination against five different heavy metals including chromium and arsenic. MTL was determined based on the growth estimation in presence of heavy metal along with controls (without heavy metals) as shown in the figure 3.9A-E




NB + 100 mg/mL (As3+)
NB
B
Rhodobacter
Phenylobacterium
Aeromicrobium
Microbacterium
Cupriavidus
Hydrogenophaga
Porphyrobacter
Ferrovibrio
Streptomyces
Rhodococcus
Caldimonas
Staphylococcus
Caulobacter
Barrientosiimonas
Paenibacillus
Limnobacter
Micrococcus
Pseudomonas
Chronobacter
Novosphingobium
Brevibacterium
Bhargveaga
Exiguobacterium
Achromobacter
Acinetobacter
0.0
0.5
1.0
1.5
2.0
2.5
OD at 620 nm


NB+ 500 µg/mL (Cd2+)
NB
C
Rhodobacter
Phenylobacterium
Aeromicrobium
Microbacterium
Cupriavidus
Hydrogenophaga
Porphyrobacter
Ferrovibrio
Streptomyces
Rhodococcus
Caldimonas
Staphylococcus
Caulobacter
Barrientosiimonas
Paenibacillus
Limnobacter
Micrococcus
Pseudomonas
Chronobacter
Novosphingobium
Brevibacterium
Bhargveaga
Exiguobacterium
Achromobacter
Acinetobacter
0.0
0.5
1.0
1.5
2.0
2.5
OD at 620 nm

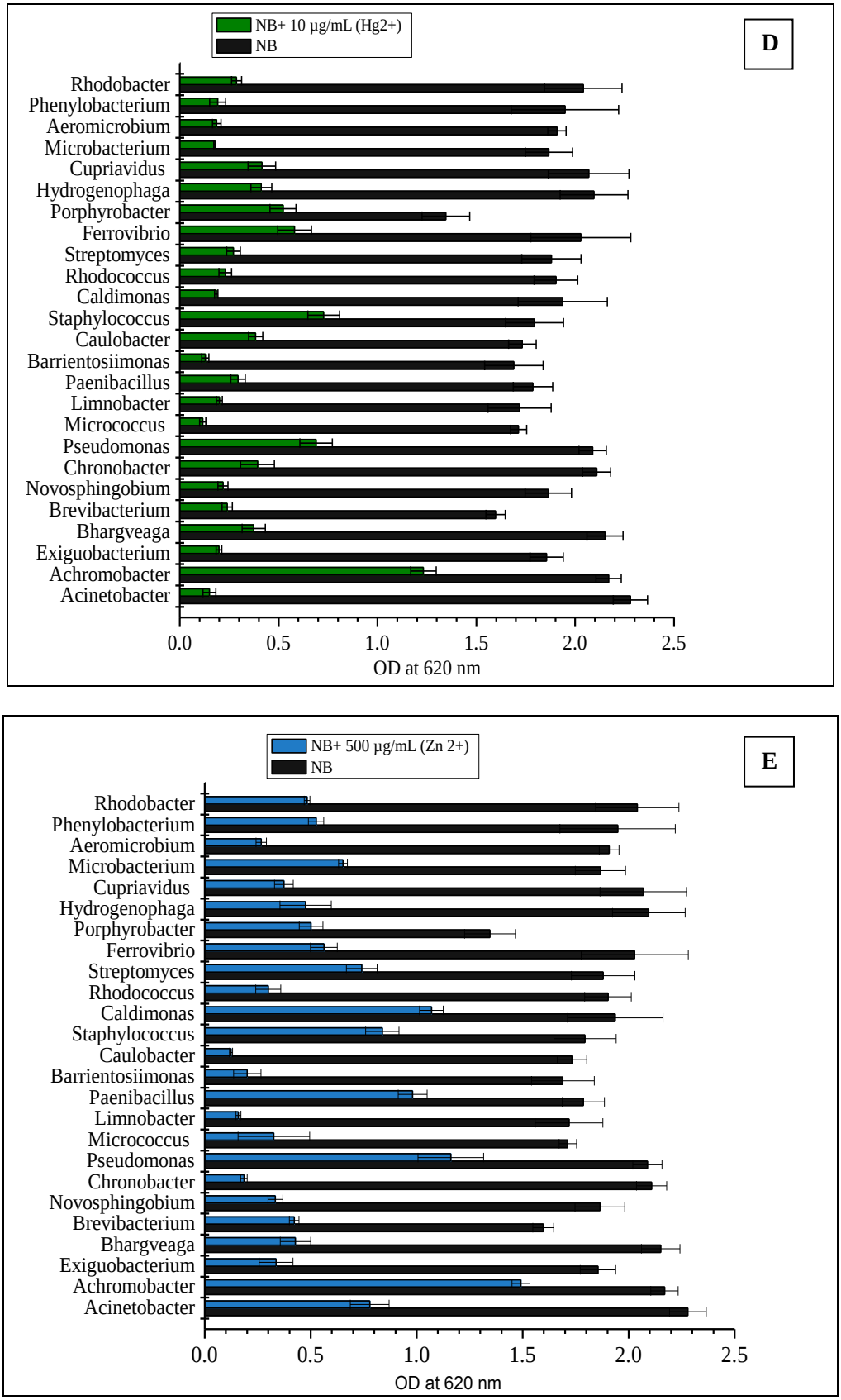


Fig. 3.9: Growth of selected bacterial genera in presence of different concentrations of heavy metals (A: chromium, B: arsenic, C: cadmium, D: mercury, E: zinc)

3.3.5.3.1: Maximum tolerance limit (MTL) determination

In case of chromium tolerance, *Hydrogenophaga, Ferrovibrio, Staphylococcus, Caluobacter, Pseudomonas, Achromobacter* was found to be highest concentration tolerating strains. In case of Arsenic tolerance, *Microbacterium, Hydrogenophaga, Ferrovibrio, Caulobacter, Pseudomonas, Bhargveaga, Exiguobacterium, Achromobacter* and *Acinetobacter* were found among the highest tolerating strains. While in case of other metals, *Cupriavidus, Ferrovibrio, Caulobacter, Paenibacillus, Pseudomonas, Achromobacter* for Cadmium; *Staphylococcus, Pseudomonas* and *Achromobacter* for Mercury and *Caldomonas, Paenibacillus, Pseudomonas* and *Achromobacter* for zinc were found to be highest tolerating strains as shown in table 3.5. Based on the heavy metal tolerance against all the tested heavy metals, *Achromobacter* (WP24) strain was found be multi-mental stress tolerance. It has shown the MTL of 800 µg/mL, >80 mg/mL, 700 µg/mL, 8 µg/mL, >800 µg/mL against the chromium, arsenic, cadmium, mercury and zinc respectively.

Table 3.5 MTL values of selected bacterial genera against different heavy metals

Strain Name	**Cr^{6+} µg/ml**	**As^{3+} mg/ml**	**Cd^{2+} µg/ml**	**Hg^{2+} µg/ml**	**Zn^{2+} µg/ml**
Caulobacter (U382)	650	>70	400	3	300
Aquabacterium sp. K8	600	>60	300	4	300
Pseudomonas (UAC46)	800	>80	300	7	700
Exiguobacterium (SA64)	700	>85	200	5	400
Brevibacterium (GHP2B)	500	>50	300	3	500
Bhargveaga (SA34)	500	>90	200	6	600
Rhodococcus (UAC255)	300	>80	100	3	100
Novospingobium (U42)	350	>80	500	4	500
Phenylobacterium (UAC116)	400	>80	400	5	200
Chronobacter (UAC72)	500	30	200	6	500
Caldomonas (U49)	100	40	300	5	700
Streptomyces (GH39)	700	>80	200	3	300

Acinetobacter (U17)	300	80	500	4	200
Paenibacillus (EM34)	500	50	600	4	>800
Achromobacter (WP24)	800	>80	700	8	>800
Barrientosiimonas (U58)	450	60	400	4	300
Staphylococcus (U23)	700	40	400	8	200
Cupriavidus (U47)	500	30	600	2	200
Ferrovibrio (U340)	700	>80	800	6	>700
Porphyrobacter (UAC227)	50	20	200	4	200
Hydrogenophaga (UAC15)	700	>80	500	6	400
Microbacterium (U17)	400	>80	500	4	300
Micrococcus (EU15)	700	>80	300	3	600
Rhodobacter (U255)	300	50	100	2	100
Limnobacter (U366)	200	30	50	4	500

3.3.5.4: Assessment for antibiotic resistance

Heavy metals tolerating bacterial genera were examined for their antibiotic resistance to assess the association of metal tolerance with multiple antibiotic resistances. Based on the diameter of the zone of inhibition around the disc (Fig. 3.10), results were interpreted according to the CLSI guidelines. Here, most of the isolates were found to be sensitive to the antibiotics indicating their nature of inhabiting natural environmental niches. Antibiotic and metal resistance phenotypes were associated with nearly all the genera (Table 3.6).

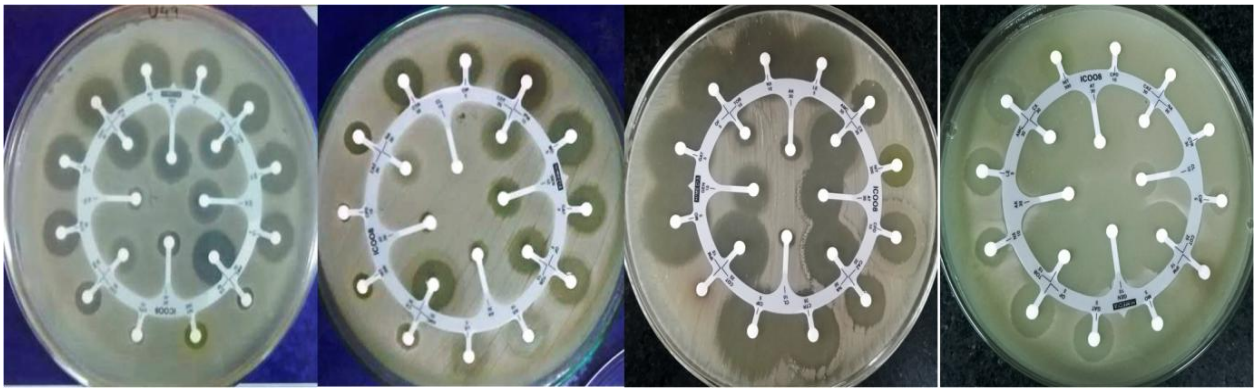


Fig. 3.10: Antibiotic sensitivity testing showing the zone of inhibition

However, those genera showing high levels of metal tolerance were also found to be highly antibiotic resistance. A positive correlation between tolerance to high levels of heavy metals and multiple antibiotic resistances were noted among genera of *Achromobacter*, *Ferrovibrio*, *Pseudomonas*, *Staphylococcus*, *Chronobacter*, *Hydrogenophaga* and *Micrococcus* strains. These genera were among the highest tolerating high levels of heavy metal concentrations. These results suggest that simultaneous selection phenomena occurred in hot spring water which showed the link between heavy metal and antibiotic tolerance.

Table 3.6: Antibiotic sensitivity testing of the heavy metal tolerating bacterial genera *

Antibiotics(mcg)/ Genera	LE (5)	AMC (30)	NIT (300)	CX (30)	CPD (10)	CAZ (30)	NA (30)	CTR	CL (10)	CIP (5)	IMP	COT (25)	MO (5)	GEN (10)	GAT (5)	TOB (10)	OF (5)	NX (10	AK (30)	AT (30)
Acinetobacter	S	R	R	S	S	R	R	S	R	S	S	R	S	R	R	R	S	S	S	S
Achromobacter	S	R	R	R	R	I	R	R	R	R	R	S	S	R	S	S	R	R	R	R
Exiguobacterium	S	S	I	S	S	S	R	S	R	R	R	R	S	R	S	S	S	S	S	R
Bhargveaga	S	R	R	S	S	R	R	S	R	S	R	S	S	I	S	S	S	S	S	I
Brevibacterium	S	R	R	S	R	S	R	S	R	S	I	S	S	I	S	S	S	S	S	S
Novosphingobium	R	R	R	R	S	R	R	S	S	S	I	R	R	R	R	R	S	I	I	S
Chronobacter	S	R	R	R	R	R	S	S	R	S	R	S	S	S	S	R	S	S	R	R
Pseudomonas	S	R	R	R	R	S	R	R	S	R	R	R	S	S	S	S	R	I	S	I
Micrococcus	S	S	R	S	R	R	S	S	S	S	S	S	S	S	S	S	S	R	R	R
Limnobacter	S	R	I	R	S	S	R	S	R	S	R	S	S	S	S	S	I	S	R	R
Paenibacillus	S	S	S	S	S	S	S	S	R	S	R	S	S	S	S	R	S	S	R	S
Barrientosiimonas	S	R	R	I	I	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R
Caulobacter	S	S	S	S	R	S	R	I	S	R	R	S	S	S	S	S	S	R	I	R
Staphylococcus	R	R	S	R	R	R	S	S		S	S	R	R	R	S	S	R	R	S	R
Caldimonas	S	R	R	S	R	R	R	R	R	S	S	S	S	S	S	S	S	S	R	R
Rhodococcus	S	R	R	R	S	S	S	S	R	S	R	R	S	R	R	S	S	S	S	S
Streptomyces	S	R	S	R	S	S	R	S	R	S	R	S	R	S	S	R	S	S	R	S
Ferrovibrio	R	R	R	R	R	R	R	S	S	S	R	R	R	R	R	R	S	R	R	R
Porphyrobacter	S	R	R	R	I	R	S	S	S	S	I	S	S	S	R	R	S	S	R	R
Hydrogenophaga	S	I	R	I	S	R	S	S	S	S	R	S	S	S	R	R	S	S	R	S
Cupriavidus	S	I	R	R	S	R	R	S	S	S	S	S	S	S	S	R	S	R	R	R
Microbacterium	S	R	R	R	S	R	S	S	S	S	I	R	I	R	S	R	S	S	R	R
Aquabacterium	S	R	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	R
Phenylobacterium	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
Rhodobacter	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Note: R: Resistance, S: Sensitive, I: Intermediate, (Full name of the antibiotics are provided in the appendix IV).

3.3.6: Genomic analysis of strains

Genome based functional annotation was performed for three strains of *Bacillaceae* family, which included a *Geobacillus* sp. (G86) strain and two other *Bacillus* sp. (U2, U277) strains selected form bioprospecting experiments. These three strains were selected based on temperature dependent growth profile. The detail of the distribution of the genome annotations of the two *Bacillus* sp. strains is shown in the figure 3.11.

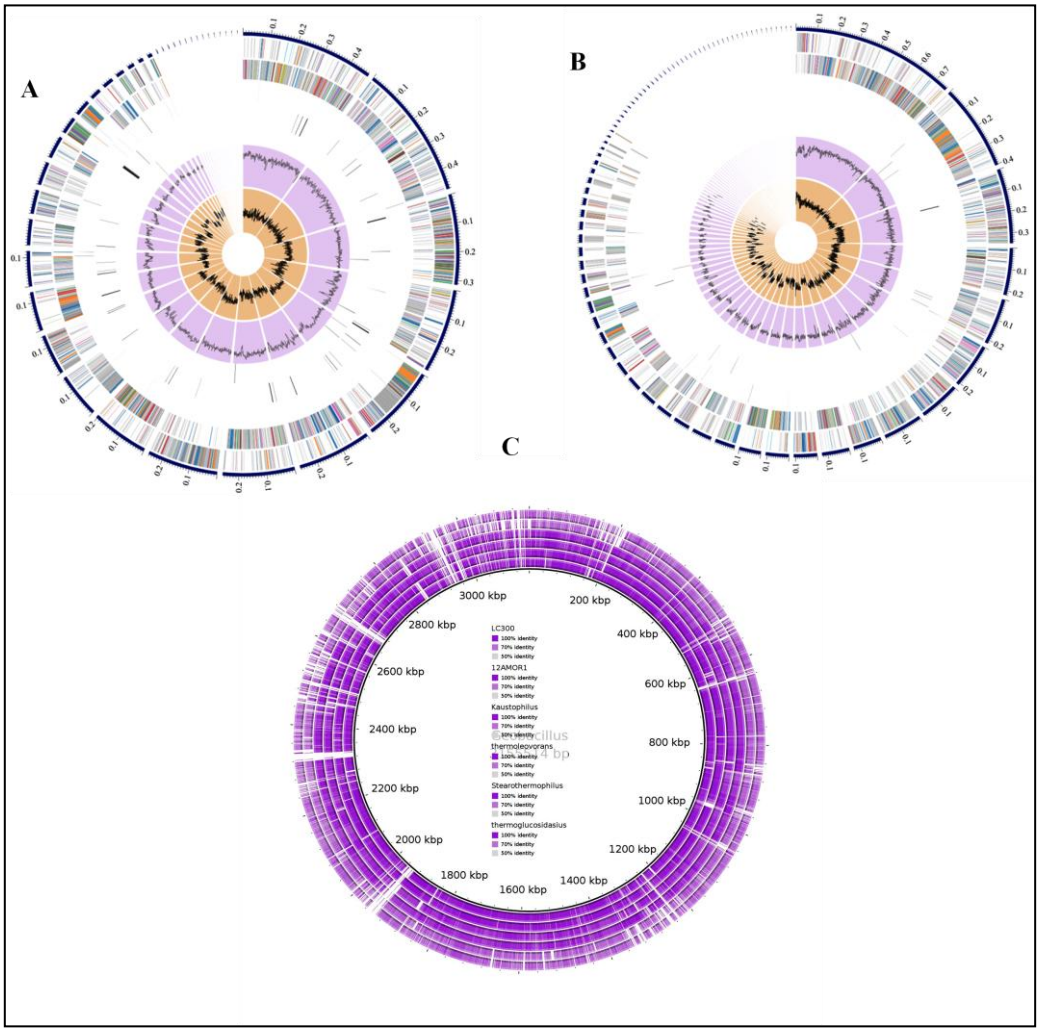


Fig. 3.11: A circular graphical display of the distribution of the genome annotations of U2 (A) and U277 (B), from outer to inner rings, the contigs, CDS on the forward and reverse strand, RNA genes, CDS to known antimicrobial resistance genes, CDS with to known virulence factors, GC content and GC skew. A whole genome comparison of the *Geobacillus* strain (G86) with reference strains using BRIC (C).

The *Bacillus* sp. strain (U2), showed 82 contigs, genome length of 4,412,281 bp, and an average G+C content of 45.75%. This genome has 4,947 protein-coding sequences (CDS), 53 transfer RNA (tRNA) genes, and 4 ribosomal RNA (rRNA) genes. The annotation included 1,209 hypothetical proteins and 3,738 proteins with functional assignments. The proteins with functional assignments included 1,079 proteins with enzyme commission (EC) numbers, 902 with gene ontology (GO) assignments and 787 proteins that were mapped to KEGG pathways. In the case of U277, 40 contigs and estimated genome length of 3,988,468 bp, and an average G+C content of 43.84% were observed. This genome has 4,164 protein-coding sequences (CDS), 52 transfer RNA (tRNA) genes, and 5 ribosomal RNA (rRNA) genes. The annotation included 650 hypothetical proteins and 3,514 proteins with functional assignments. The proteins with functional assignments included 1,050 proteins with EC numbers, 873 with GO assignments, and 773 proteins that were mapped to KEGG pathways. While in the case of *Geobacillus*, 357 contigs, an estimated genome size, 3155514 bp and a total of 3618 protein coding sequences (CDS) were detected. In the ANI calculator, the *Geobacillus* strain (G86) showed 90.18% similarity with *G. kaustophillus*, 78.21% with *G. stearothermophilus* and 83.13% with *G. thermodenitrificans*. Based ANI calculator, *Geoabcillus* strains probably found to be novel species. Therefore, genome sequence of this strain was further analysed for prediction of possible genes and proteins using GeneMark 3.0. The results revealed prediction of 3399 genes involved in various subsystem level functions (Fig. 3.12A). Further, these predicted possible genes were used for prediction using RAST, which has resulted in identification of 2244 proteins out of 3682 genes, and among which around 1360 were annotated for subsystem functions (Fig. 3.12B)

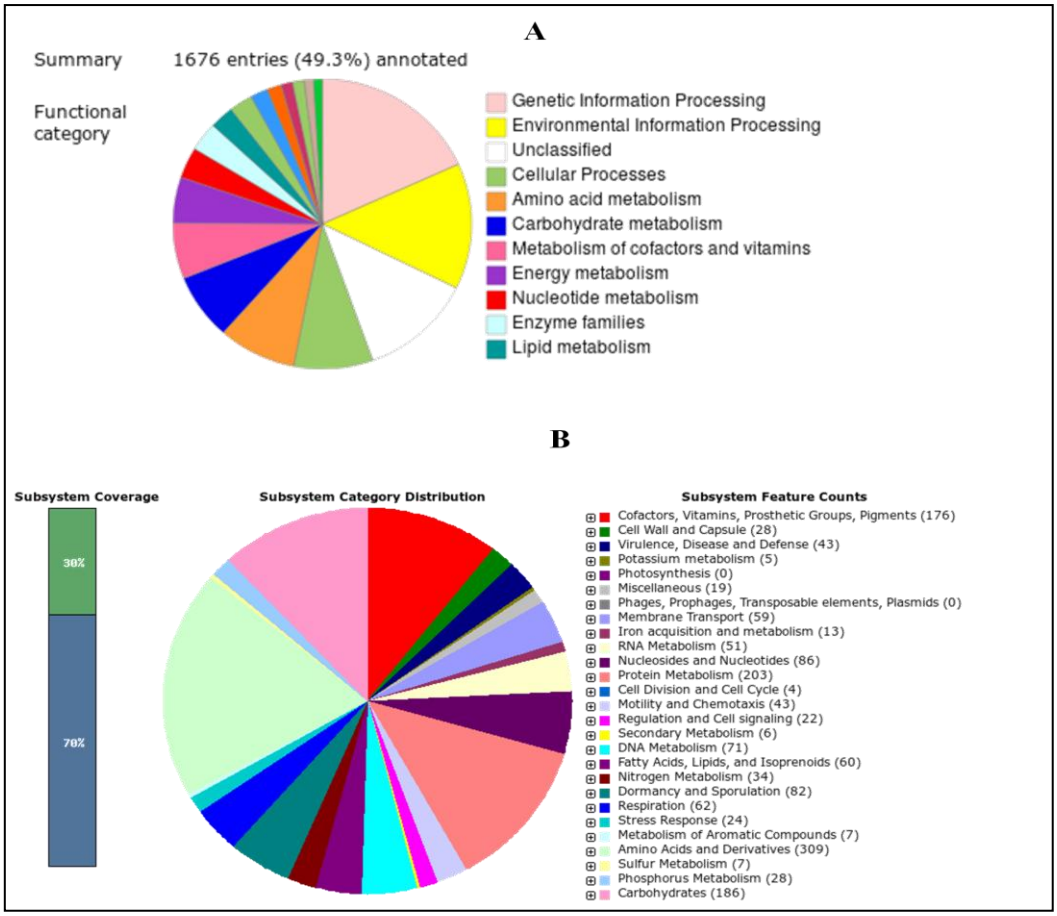


Fig. 3.12: Gene prediction of *Geobacillus* sp. (G86) (A) and functional annotations (B)

3.3.7: Studies of heavy metal tolerant strain *Achromobacter* sp. (WP24)

3.3.7.1: Growth pattern under individual/mixed heavy metal stresses

Growth curve studies of *Achromobacter* sp. (WP24) strain was performed under normal conditions *i.e.* in nutrient media (Fig. 3.13A) and under heavy metal stress. Under normal condition this strain grows within 24 hours and has a log phase between 3 to 12 hours. However, under heavy metal stress this log phase got extended. In case of optimum concentration of heavy metals, *i.e.* Cr^{6+}, the exponential growth was observed between 18 to 24 hours, in presence of As^{3+}, it was 15 to 24 hours. For Cd^{2+} the exponential growth was between 15 to 24 hours, for Hg^{2+} and, the exponential growth phase was 6 to 12 and 9 to 18 hours respectively (Fig. 3.13B).

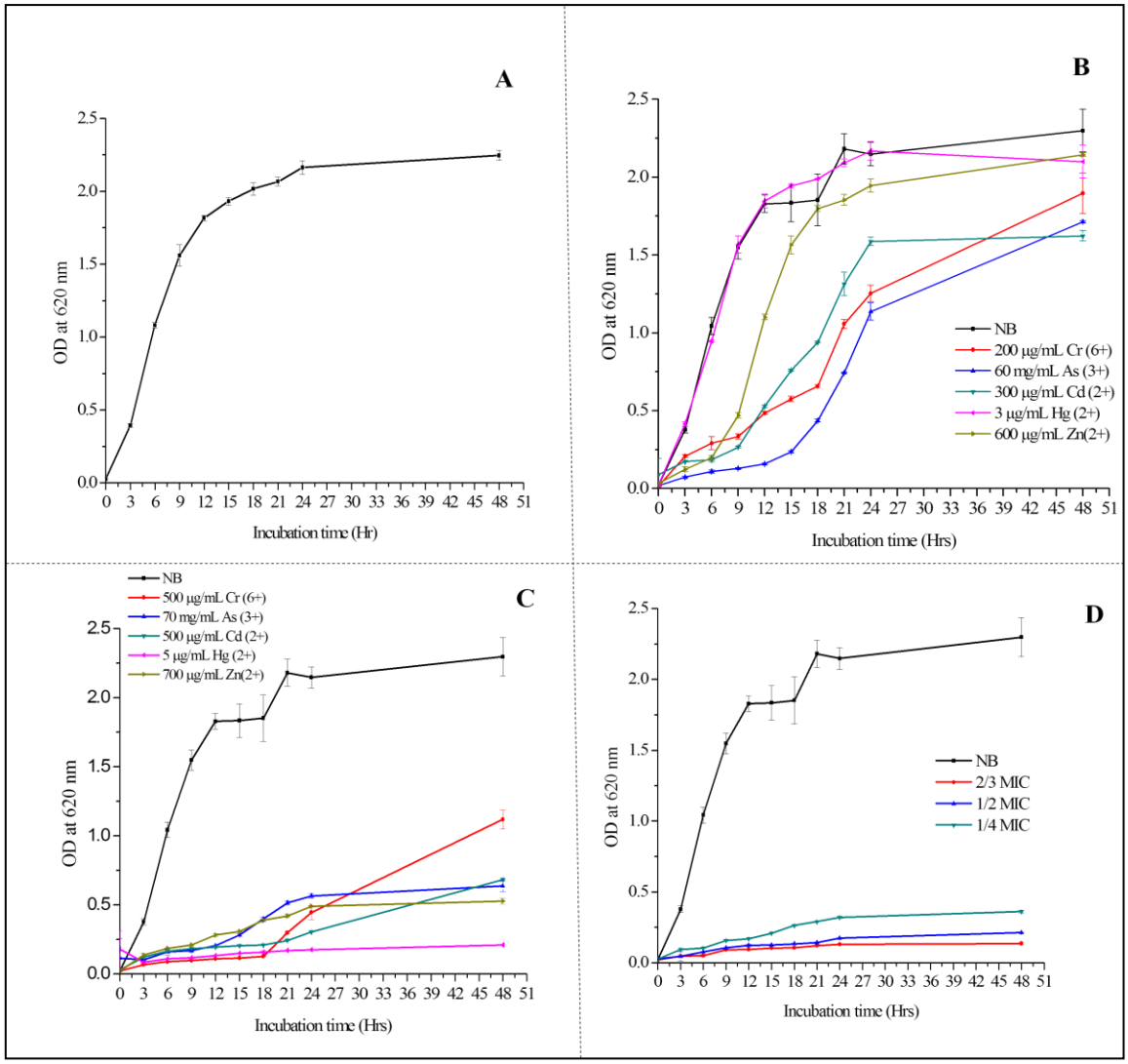


Fig. 3.13: Growth curve analysis of the *Achromobacter* sp. (WP24) strain in NB (A), heavy metal stress conditions (B-D)

In case higher concentrations of heavy metals (less than MTL values), log phase has extended by far and was achieved in between 18 to 48 hours and 21 to 48 for chromium and Cadmium respectively. However for other metals no significant growth was observed (Fig. 3.13C). In the pooled heavy metal experiment, growth was totally hampered and only observed for one forth of MTL values (Fig. 3.13D) of all the heavy metals which indicate that the organism could survive under multi-metal stress conditions but relatively at lesser concentrations than the individual metal stress.

3.3.7.2: Effect mixed/pooled heavy metals on cell morphology

Morphological changes of *Achromobacter* sp. (WP24) strain under stress conditions of heavy metals such as Cr^{6+}, As^{3+}, Cd^{2+}, Hg^{2+} and Zn^{2+} were observed. A large number of SEM images were obtained and analyzed and representative images showing peculiar morphological features in response to the presence of different metals were shown in the figure 3.14.

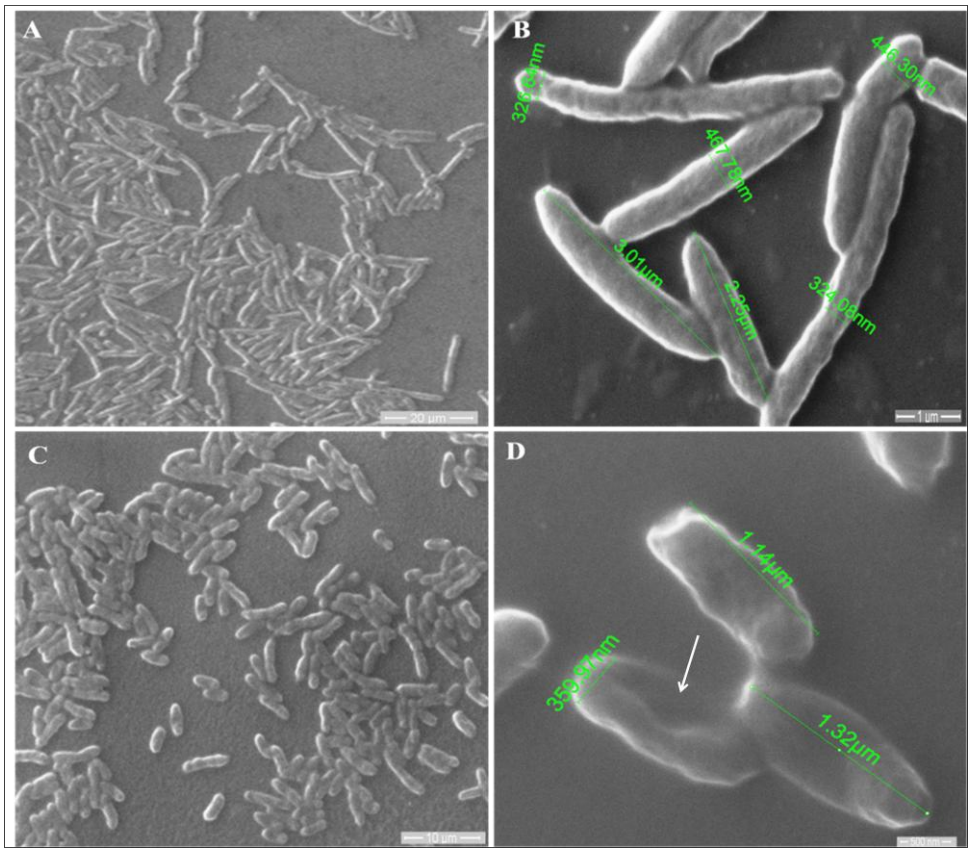


Fig.3.14: Representative scanning electron micrograph of *Achromobacter* sp. (WP24) strain NB media without metals (A and B), in the presence of pooled heavy metals (C and D).

Growth in NB (without heavy metals) (Fig. 3.14A, B) shows that cells are elongated (3.4- 3.7 nm in length) and tapering at the ends. However, some variation in the surface growth was observed in the presence of different metals. Under such stress cells has become more wrinkled (Fig. 3.14C, D) with reduced cell length. Thus, in the presence of different heavy

metals concentrations, morphological changes in the *Achromobacter* sp. (WP24) strain were observed.

3.3.7.3: Chromium (VI) reduction assay

Achrombacter sp. (WP24) strain found to reduced 60-66% of chromium (VI) at lower concentrations *i.e.* 50 - 100 µg/mL. While at higher concentrations reduction is low as shown in table 3.7. Although, this strain was found to tolerate high concentrations of various heavy metals including chromium, but its reduction rates are lower, possibly indicating that other mechanism are used by this strain for survival under heavy metal stress conditions. Additionally, this strain was also found to be resistance to maximum antibiotics, which also infer preferentially it uses other mechanisms for removal of heavy metals.

Table 3.7: Percentage of reduction of chromium (VI) by *Achromobacter* sp. (WP24) strain

Sr No.	Concentration (µg/ml)	% reduction after 24 hr	% reduction after 48 hr
1	50	33 %	66 %
2	100	30 %	60 %
3	200	8 %	17 %
4	300	6.6 %	16 %

3.4: General discussions

Exploring the culturable diversity from hot springs is an important aspect for the understanding of microbial ecology and also holds a great interest for bioprospecting. In this chapter, we have analysed the microbial diversity using multiple cultivation techniques and used cultured isolates for various bioprospecting activities based on imputed functions. Analysis of culture based diversity revealed that Unkeshwar hot spring host diverse bacterial species, probably due to its moderate level of temperature. It is well noted that, the

temperature of hot spring has a greater impact on microbial compositions in hot springs. Generally, with an increase in temperature, microbial diversity decreases (Wang et al. 2013; Sharp et al. 2014; Badhai et al. 2015) and moderate temperature range hot springs could support high microbial diversities. Here, a great variety of bacterial genera were isolated, most of them found to be moderately thermophilic to mesophilic in nature, and such bacterial populations were frequently observed in the terrestrial hot springs having a temperature of less than 75°C (Lau et al. 2009; Wang et al. 2013). Furthermore, other physicochemical factors (analysed in chapter 2) such as total organic carbon, sulphur and magnesium which were found with relatively higher concentrations may also have contributed to the presence of diverse microbial flora. For example, total organic carbon and magnesium may have supported the growth of hydrolytic enzyme producing bacteria. The organic content can support the growth of heterotrophic bacteria in the hot springs as revealed in some of the previous studies (Chan et al. 2015; Saxena et al. 2017). The enzymatic activities of some of the microorganism may require magnesium ions, which therefore may affect the compositions of such enzyme-producing microbes in hot springs (Grivalský et al. 2016). However, the sulphur generally present in the form of sulphate or sulphite in hot spring environments have a reported greater impact on microbial composition in such environments (Purcell et al. 2007). The genus; *Limnobacter* a thiosulfate-oxidizing heterotrophic bacterium was also found to be present in this hot spring. Such correlations among physicochemical factors and microbial diversity could help in the understanding of the microbial ecology of these environments. Though the culture-based studies could not possibly explain such correlations in a better ways, such information could aid for better amendment of media compositions for isolations of different bacterial groups from such hot environments. With culturing, media composition and laboratory conditions are the most important factors for isolations of microbial groups from environmental samples. In addition, most of the bacterial

genera were recovered using low nutrient media compositions such as diluted and R2A agars. Such low nutrient media were often found to be useful for isolations of rare bacteria genera from environmental samples (Vartoukian et al. 2010).

Further we have compared the general occurrence of these genera in hot spring environments based on some previous studies performed using both culture-dependent and independent methods. Such comparisons revealed some interesting attributes of microbial diversities of hot spring environments. Most of the genera were found to be common inhabitants of hot spring environments as many of them were detected by previous metagenomic analysis of such environments but still were not cultivated. Among all the bacterial genera obtained, only 20 different genera were found to have been previously isolated from hot springs. Some were only detected by culture-independent analysis, while others were reported for the first time in this study from hot spring environments. However, in the present study those genera that matched with previously cultivated hot spring isolates were mostly found to belong to the *Firmicutes,* and other members of *Actinobacteria* and some *Proteobacteria*. Among them, *Firmicutes* mostly were found to be previously cultivated from high-temperature hot springs. For example, genera such as *Bacillus*, *Paenibacillus* (Singh et al. 2010 Pandey et al. 2015 Sahay et al. 2017); *Lysinibacillus*, *Exiguobacterium, Bhargavaea* and *Staphylococcus* (Kumar et al. 2014a, b) have been isolated from Himalayan geothermal hot springs with the temperature range of 90-95 ºC. The predominant occurrence of *Firmicutes* and some other actinobacterial members such as *Microbacterium, Kocuria, Micrococcus* and *Cellulosimicrobium* in such high-temperature hot springs reveals that these groups of bacteria possess high-stress tolerance properties (Cerritos et al. 2011; Kumar et al. 2014a; Sharma et al. 2014). Among the members of *Proteobacteria, Acinetobacter* and *Pseudomonas* are the ubiquitous genera and they were also found to be present in hot springs environments. However, the genus; *Caldimonas* is a thermophilic

bacterium and still now isolated only from hot springs and represented only by two valid species (Takeda et al. 2002; Chen et al. 2005). However, some other thermophilic species were also reported from hot springs having the moderate temperature range (Rakshak et al. 2013). The genus *Caldimonas* particularly has attracted more attention as members of *Caldimonas* are thermophilic and have various metabolic properties; for example, one of the species reported by Takeda et al. (2002) has the poly (3-hydroxybutyrate) (PHB) degradation as well as manganese oxidation abilities, while other species are reported to produce amylase enzyme (Chen et al. 2005). Another genus, *Rhodobacter,* is rarely found to be associated with hot springs environments. However, they can obtain energy through photosynthesis and therefore some species of this genus are used as model organisms to study bacterial photosynthesis (Imam et al. 2013).

Interestingly, with the help of diverse culture media and conditions around 37 bacterial genera were cultivated for the first time from hot springs of India. Most of them found to belongs to *Proteobacteria* (22 genera) and *Actinobacteria* (14 genera) and only one was found from *Firmicutes*. However, some of these genera were found to be present in other hot springs but were reported by metagenomics studies. For instance, 13 genera; *Ramlibacter*, *Acidovorax*, *Caulobacter*, *Novoshingobium*, *Gordonia*, *Nocardioides*, *Hydrogenophaga*, *Porphyrobacter*, *Achromobacter*, *Arthobacter*, *Dietzia*, *Streptomyces*, and *Brevibacterium* were previously identified from different Indian hot springs using omics-based techniques. Interestingly, members of the group *Actinobacteria* such as *Gordonia*, *Nocardioides*, *Arthobacter*, *Dietzia*, *Streptomyces* and *Brevibacterium* were reported in the metagenomics analysis of high temperature (90-95ºC) hot springs (Sharma et al. 2017). The temperature tolerance range of these genera seems to be wide, as they were isolated in this moderate level temperature hot spring which possibly could reveal that the moderate temperature and dilute nutrient media might be the necessary conditions for their isolation from hot spring

environments. Among the group members of *Proteobacteria*, genera such as *Hydrogenophaga*, *Porphyrobacter*, *Achromobacter* and *Ramlibacter* were found to be present in hot springs of wide ranges of temperature (Sangwan et al. 2015; Badhai et al. 2015; Panda et al. 2016). Among them, *Porphyrobacter* has been reported from various habitats but mostly from freshwater and hot spring environments. The members of this genus are known to contain bacteriochlorophyll and some species were found to have toxic environmental pollutant degradation potentials (Hiraishi et al. 2002). Another genus, *Ramlibacter*, was also detected in higher abundance in hot springs present in central India (Saxena et al. 2017). Presently the genus is represented by four valid species and most of them were isolated from sub-desert soil or forest soil. The species isolated from desert environments were found to form a cyst and reported as a chemoautotrophic bacterium (Heulin et al. 2003). However, their dominant occurrence in hot spring habitats revealed in recent studies by Saxena et al. (2017) and their isolation from the present hot spring indicate that they may have some ecological role in such hot environments.

Although out of 37 bacterial genera, only 13 were found to be present in other Indian hot springs which was reveled through metagenomics studies. Surprisingly, another 10 genera were matched with the microbial composition at the genus level of recently studied hot springs of Pakistan ranging in temperature from 60 to 95°C (Amin et al. 2017). Interestingly, these hot springs of Pakistan were also mapped in the Himalayan geothermal belt, in which other Indian hot springs were located; however, we could not find these 10 genera in these Indian hot springs. The possible reason for such a difference in abundance of microbial communities of hot springs may be due to the physicochemical factors, as some of the sites of these Pakistan hot springs were reported with high bicarbonates, which was also, evidenced the Unkeshwar hot springs through the previous lithological studies (Varun et al. 2012). However, such results also suggest that the sampling size could be one of the reasons

for such differences, for instance, during the analysis of Pakistan hot springs the authors used around nine different samples from three different sites for metagenomics analysis.

Among those genera that were matched with Pakistan hot springs were members of *Proteobacteria* and *Actinobacteria*. Some of the genera such as *Limnobacter*, *Phenylobacterium*, *Methyloversatilis*, and *Rhizobium* of *Proteobacteria* were found to be interesting, as they possibly play important role in biogeochemical cycling in hot springs environments. For example, still now only two species of *Limnobacter* were reported and both are heterotrophic bacteria with the ability to oxidize thiosulfate to sulphate, one was isolated from the littoral zone of a freshwater lake and another from volcanic deposits (Lu et al. 2011). Another genus; *Methyloversatilis* is a facultative methylotrophic bacterium and can use methanol or methylamine as the source of carbon and energy (Kalyuzhnaya et al. 2006). Most of the strains of *Phenylobacterium* were isolated from soil and they can grow optimally only on artificial chemical compounds like chloridazon and antipyrin. However, one thermophilic species was isolated from the subsurface thermal aquifer and grows best with yeast extract as the sole carbon and energy source (Kanso and Patel 2004; Eberspächer and Lingens 2006). Among the actinobacterial genera; *Aeromicrobium*, *Micromonospora*, and *Janibacter*, the species of *Janibacter* were isolated from various extreme habitats including hydrothermal sediments of the ocean (Zhang et al. 2009) to the upper atmosphere (Shivaji et al. 2009). Overall, out of 57 genera, only 20 genera were found to be previously isolated from other hot springs and the remaining 37 genera were isolated for the first time from Indian hot spring environments among which 13 were detected in the previously performed metagenomics studies of other Indian hot springs. However, around 10 different genera were detected in recently studied hot springs of Pakistan, several factors such as such as temperature, sampling size; geographical locations may explain their absence in the previous culture-based and metagenome analysis of Indian hot springs.

The overall analysis, to the best of our knowledge, revealed that around 14 bacterial genera were reported for the first time from hot springs environments. Although some of them may not be endemic to the hot springs environments, as they might have migrated from other sources to the hot spring. However, their fraction was found to be very low compared to the overall number of bacterial genera isolated. Interestingly, other genera were found to be rare genera and therefore could be natural bacterial inhabitants of such environments, but were missed in previous studies of both culture-based and metagenomics approaches. Several factors might account for their absence in previous studies. One possible reason is that they may represent a minor fraction of microbial communities in such environments and still were reported with a very low number of strains. For example, *Methyloterrigena*, which is a newly isolated genus, has only one species. The genus *Barrientosiimonas* is also found to be represented by only one species and was isolated from Barrientos Island in the Antarctic (Lee et al. 2013). An increase in the number of species and strains of such rare genera could help to assign precise taxonomy to such species which might be possible through the use of culturomics techniques. It was also observed that some other genera were taxonomic reshuffled genera with no proper presence of type species which could also limit their identification and isolations of closely associated species of such taxa. For instance, *Aquabacter*, *Labrenzia* and *Phreatobacter* and *Oleomonas* are taxonomically reshuffled genera and most of them were found to be represented by few numbers of species. However, most of them were reported from various types of environments and with different metabolic abilities. The genera *Methyloterrigena* and *Aquabacter* are members of the *Hyphomicrobiaceae* family and *Methyloterrigena* can metabolize C1 (Methanol) compounds (Kim et al. 2016). Another genus, *Phreatobacter*, has not yet been assigned to any family, and species of this genus were reported to be oligotrophic in nature, which makes isolations from environmental samples difficult. So far, only one species has been isolated from

ultrapure water samples (Tóth 2014). *Oleomonas* species, isolated from the oil fields environment, were reported for their abilities to grow on aromatic compounds such as toluene, benzene and phenol, and aliphatic hydrocarbons (Kanamori et al. 2002). Another genus, *Cellulomonas* has the ability to degrade cellulose and was isolated from alkaline environments (Jones et al. 2005).

During comparative analysis, it was also observed that both the culture-dependent and independent methods capture different portions of microbial communities from the hot springs environments. The occurrence of such differences was also found in previous studies from other types of environments (Stefani et al. 2015). Different factors can account for such differences in the microbial analysis since proper physicochemical sources and nutrients are needed for the growth of a microorganism; these factors possibly facilitated the cultivation of some organisms (Pham and Kim 2012). While in the case of culture-independent studies, bias analyses of predominance give rise to different microbial portions (Poretsky et al. 2015). However, as observed in the present study, most of the bacterial genera detected in culture-independent studies could be cultivated with the help of diversifications of culture conditions. In addition, such comparative analysis may also be helpful for ecological and bioprospecting studies of hot springs environments. In addition, it also makes reorganization of other factors such as sampling size, physicochemical factors important while analyzing microbial diversities or culturing them from such robust environments. Therefore, microbial investigation of such environments should follow in a coordinated way using both methods, where data obtained from advanced metagenomics approaches possibly could help to design diverse cultivation strategies using high throughput in order gain access to previously hidden metabolic diversity. In recent years, such efforts are accomplished through the use of 'culturomics', a high-throughput approach that multiplies culture conditions in order to increase the culture population of bacterial species (Stewart 2012; Lagier et al. 2015). Use of

such techniques apparently has greater advantages, as it allows access to rare population present in the ecosystem, also could provide a large number of strains for newly identified bacterial species for the greater elucidation of metabolism and viability of microbial species (Greub 2012). Furthermore, this facet of microbial ecological studies will change using culturomics as these techniques allow studying the interaction between microbial species of a particular habitat, which helps to increase our knowledge about microbial ecosystem dynamics (Greub 2012; Gibbons and Gilbert 2015). More importantly, these techniques could help to design appropriate culture conditions for newly isolated bacteria which further can be found useful with some variation of parameters for isolation of previously uncultured species. Presently, such an approach is being used for the description of human-associated micro-flora, and 1000 human-associated bacterial species including 247 new species have been isolated and described in a short time (Lagier et al. 2016; Abdallah et al. 2017). In the coming years such techniques will possibly be used for other types of environments, as different types of these techniques have been employed for marine and aquatic environments (Gao et al. 2013). Furthermore, use of culturomics in the future will possibly add a large number of bacterial species to the databases which can also pose a challenge inevitably for present bacterial taxonomic approaches (Rosselló-Móra et al. 2017; Abdallah et al. 2017). However, use of such complementary approaches of both culturomics and metagenomics in coordination and with in-depth investigations will possibly change the face of microbial ecology studies, which is the need of future microbial research (Ma et al. 2014; Gutleben et al. 2017; Poddar and Das 2017).

Here, we tried a combination of both of such methods for functional analysis; tools generally used for predictive functional analysis of microbial communities were instead used for cultured isolates to assess their functional potentials. Such assessment reveals an abundance of genes for metabolic pathways, among which a number of pathways were

mapped to the production of secondary metabolites, and xenobiotic compound degradations which could further help to harness the metabolic potential via bioprospecting. For example, the pathways mapped for secondary metabolites synthesis such as polyketides indicates that possibly some of the isolates could have such metabolite production abilities. In accordance, the genera having such biosynthetic potential can have applications in the production of antibiotics or other bioactive compounds. For instance, genera such as *Actinomodura*, *Micromonospora*, *Streptomyces* obtained from this hot spring were also isolated previously from hot springs with the presence of such biosynthetic genes (Liu et al. 2016).

The hot spring microbes are usually sought in bioprospecting for hydrolytic enzymes due to their thermo-stability and various studies have reported such enzymes from these environments, still, further research effort is necessary to find suitable enzymes which will meet the industrial demands (Niehaus et al. 1999; Obeng et al. 2017). From the present study, those were previously not reported for such applications from hot springs were also found to produce enzymes. It is also observed that group members of *Firmicutes* and *Actinobacteria* have greater abilities for enzyme production than members of the *Proteobacteria.* This could be informative as recently more attention was given to members of thermophilic *Actinobacteria* for production of such enzymes (Shivlata and Satyanarayana 2015; Liu et al. 2016). Among the genera which seem to be previously underexplored from hot springs for enzymes productions such as *Azospirillum*, *Ferrovibrio*, *Novosphingobium*, *Gordonia*, *Rhizobium*, *Nocardioides*, *Hydrogenophaga*, *Aeromicrobium*, *Micromonospora*, *Brevibacterium* were found positive for at least one enzyme. Although, further biochemical and thermo-stability of enzymes needs to be evaluated to have a possible new microbial source for industrial applications. However, other genera such as *Cellulomonas*, *Streptomyces* were reported for cellulase production (Goswami et al. 2016; Liang et al. 2014) and *Bacillus*, *Exiguobacterium* and *Bhargavaea* for amylase and protease production, among them

Exiguobacterium was found to be an important member for such enzyme productions (Kasana and Pandey 2017).

In the predictive functional analysis, pathways were also mapped for degradation of xenobiotic compounds which could reveal the potential use of the isolated strains for environmental applications. In general, microbial species from such environments have the ability to survive under various stress conditions including heavy metal stresses. Recently, microbes with the ability to oxidize arsenate were reported from Jinqing hot spring (Yang et al. 2017). From the present hot spring, around 25 bacterial genera were found to tolerate higher concentrations of both the heavy metals employed in the study. However, here we found more such genera for heavy metal tolerance from *Proteobacteria* along with some actinobacterial members. This may be due to differences in the mechanisms used by Gram-positive and Gram-negative bacterial strains for heavy metal stress tolerance. The Gram-negative bacteria can show tolerance to heavy metal tolerance via either by oxidation-reduction of heavy metal or by efflux mechanisms (Silver and Misra 1984). Gram-negative bacterial genera such as *Achromobacter*, *Ferrovibrio*, *Caulobacter*, *Hydrogenophaga*, *Pseudomonas*, *Cupriavidus*, and *Chronobacter* were found among the highest heavy metal tolerating strains. In case of Gram-positive strain, *Staphylococcus*, *Microbacterium*, *Bhargveaga*, *Paenibacillus* and *Exiguobacterium* were also found to tolerate and were able to survive under heavy metal stress conditions. Interestingly, the highest heavy metal tolerating strains were also found to resistance to different antibiotics. Genera such as *Achromobacter*, *Ferrovibrio*, *Caulobacter*, *Hydrogenophaga*, *Pseudomonas*, *Cupriavidus* and *Chronobacter* were antimicrobial resistance (AMR). Such result indicates the possibility of co-selection among antibiotic and heavy metal resistance. It is of great concern about heavy metals selecting indirectly for antibiotic resistance by co-selection (Koditschek and Guyre, 1974). This is an indirect selection process occurs through various mechanisms such as mechanisms

those are coupled physiologically (cross-resistance) and genetically (co-resistance). Cross-resistance describes mechanisms that provide tolerance to more than one antimicrobial agent such as antibiotics and heavy metals. As an example, several multidrug efflux pumps are known to mediate decreased susceptibility toward antibiotics and heavy metals by rapid extrusion of the toxins out of the cell (Seiler and Berendonk, 2012). Although antibiotic resistance has become a major threat to human health worldwide, this phenomenon has been largely overlooked in studies in environmental settings such as hot springs influenced by anthropogenic pressure needs more attention. Though, most of the bacterial genera have shown tolerance to such tolerance properties, but they were not explored previously from hot spring environments. It becomes necessary to identify their potentials, which further requires more focused studies regarding their taxonomy, biochemistry and genetics and pathogenicity to identify and fully exploit these microorganisms. To evaluate such properties of potentials strains, some of preliminary studies were performed over the potent strains. A strain of *Achromobacter* species has shown significant tolerance to most of the heavy metals used in the study. This strain was studied for its growth properties, cell morphologies and metal reduction potentials. Growth studies have shown that the strain could tolerate different heavy metals individually and to lesser extent pool of heavy metals. Although the strain has performed well for tolerance of heavy metals, but not found very significant for reduction of chromium metal. However, such property of multi-metal tolerance possibly indicates that the strain might be using multidrug efflux pumps to rapidly extrude the heavy metals and other toxic compounds from the cells. These mechanisms are also known to mediate decreased susceptibility toward antibiotics, which may cause AMR in the environmental microbes (Baker-Austin et al. 2006; Seiler and Berendonk, 2012).

3.5: Conclusion and future prospects

The present study reports the isolation and characterization of diverse members of bacterial phylum using microbial cultivation techniques from Unkeshwar hot spring of India. Microorganisms were isolated using various cultivation strategies, which has made possible the isolation of previously underexplored microbes form Indian hot springs. Therefore, this study shares an interesting aspect in basic microbiology that the use of multiple isolation techniques and cultivation strategies could be useful for isolating the diverse range of microbial taxa including some rare bacterial genera from hot spring environments and probably from other extremophilic niches too. Additionally, it also sheds light on the factors which could be considered while analysing microbial diversities form such environments. Factors such as sampling size, understanding of physicochemical parameters of the microbial habitats were found to be important for culturing the microbes from such hostile environments. Using culture-based approaches most of dominant as well as rare bacteria were isolated from this hot spring. Metabolic potential of observed diversity revealed that many bacterial genera has various properties such as production of industrially important enzymes, tolerance of heavy metal stresses. Additionally, most of the bacterial genera showing resistance to heavy metal were also found resistance to most of the antibiotics. This is of great concern, as most of the Indian hot springs were known to have anthropogenic load due to the belief of holy place. Therefore, this study also suggest for the conservation of such pristine natural environments as a natural sources for possible future use for biotechnological potentials. In addition, the study also promotes the use of larger scale microbial cultivation studies for isolating the unexplored species missed previously, which can be further exploited in both basic and applied microbiology. In future, there is need to use high throughput culturomics techniques for deeper understanding and to get an increased access to the bacterial communities in hot spring environments.

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CHAPTER 4

Biodegradation potential and biosurfactant production

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic priority pollutants. They are of great concerns due to their toxic, mutagenic, carcinogenic and immunotoxic properties which may adversely affect human and environmental health. Therefore, studies focusing their removal or detoxification using environment-friendly approaches such as bioremediation have attracted much research attention. However, bioremediation processes are mostly affected by various environmental conditions, such as temperature, nutrients, chemical property of the PAHs, which in turn needs the implementations of the naturally occurring microorganisms, which are well adapted to such conditions. In the present study, thermo-tolerant microorganisms isolated under PAH enriched conditions from hot spring environments were evaluated for biodegradation of PAHs and biosurfactant production. Based on various isolation and screening techniques, a few of the selected strains belonging to *Bacillus* group were used for degradation of PAHs. Various strategies were also designed to find out the influence of different factors such as mixed compounds, degradation in crude oil, temperatures and nature and number of degrading strains, total microbial community and amendment with biochar to maximize the degradation rates. The results revealed that degradation depends on the nature and chemical type of compounds. Use of mixed culture at higher temperature found more effective for degradation of low molecular weight (LMW) PAH compounds. In contrast, amendment with biochar and degradation by the whole community; enhanced degradation of High molecular weight (HMW) was observed. Moreover, PAHs degrading strains were also producing biosurfactants, which were analyzed by both molecular and analytical tools. The study infers that hot springs microbes has well adaptation properties and therefore could be the source for the development of an effective bioremediation strategy.

Key Words: Biodegradation, PAHs, Mixed culture, Crude oil, Biochar, Biosurfactants

4.1: Introduction

Over the past few decades, immense industrial growth and development have resulted in overuse of oil and petroleum products leading to severe environmental pollution. These activities have threatened the natural habitat and ecosystem, releasing huge toxic wastes in the environment. Such improperly discharged wastes containing toxic compounds deteriorate the quality of terrestrial and aquatic resources (Hu et al. 2004). Among several such toxic compounds, polycyclic aromatic hydrocarbons (PAHs) are the major contributor to environmental pollution, owing to their physicochemical and recalcitrant nature (Martorell et al. 2010). PAHs belong to the diverse class of non-polar, aromatic compounds which are compactly arranged fused benzene ring, and are thermodynamically stable. They are ubiquitous, non-polar organic compounds comprised of two or more than two benzene ring in various structural configurations and exist as colourless, white, or pale yellow-green solids having a faint, pleasant odour (Ghosal et al. 2017). These compounds are generated during the incomplete combustion of organic materials like coal, tar, oil and petrol and industrial processes as well as from some natural processes such as volcanic activities or geothermal reactions in the coal deposits (Abdel-Shafy and Mansour, 2016). However, they are present in relatively high concentrations in products of fossil fuels and during exploration activities, an accidental release may result in the high loading of these compounds in the environments (Varjani et al. 2017). The PAHs are of great concern due to their various toxic, mutagenic, carcinogenic, immunotoxic and teratogenic properties which may adversely affect human and other living organism health. These compounds could interfere with the cellular membrane and enzyme system functioning and few among them could act as carcinogenic and mutagenic agents (Yu et al. 2002). Some are potent immune-suppressants and affects immune system development, humoral immunity and affects host resistance (Armstrong et al. 2004). Therefore PAHs have been listed as priority pollutants of critical environmental and public

health concerns and needs immediate attention (Davie-Martin et al. 2017). With the increased awareness of their adverse effect, studies focusing their removal or detoxification from the environment using environment-friendly approaches of bioremediation have attracted much research attention.

Among the several remediation technologies developed for removal of the environmental contaminants, bioremediation is safer and also a cost-effective solution to the environmental problems. It is an emerging green technology with the upper hand of cost, an eco-friendly mode which is also a sustainable solution for the treatment of recalcitrant, toxic pollutants (Bamforth et al. 2005). In general, bioremediation processes involve either addition of exogenous potential microbial strains to the contaminated sites or boosting the growth of the indigenous microbial community through biostimulation (Sarkar et al. 2017). Microbial systems have the quick adaptability to extreme environment and their diverse genetic machinery enforces them to survive and proliferate on toxic pollutants, degrading them to less or non-toxic products (Khan et al. 2001). They are being tremendously exploited for remediation of recalcitrant compounds including hydrocarbons, pesticides, heavy metals, and dyes released from different industrial by-products (Seo et al. 2009; Arun et al. 2011). In the microbial mediated remediation processes, to a more extent, bacterial and fungal strains mostly used either as a potential individual strain or in microbial consortia. Taxonomically diverse ranges of microbial species have a great potential for degradation of the different class of toxic PAH compounds. A great variety of genera of gram-positive and -negative bacteria, fungi and to some extent algae were characterized for their abilities to degrade PAHs. A number of effective hydrocarbon degraders were reported; particularly for the degradation of low molecular weight (LMW) PAHs due to their less complex nature (Kanaly and Harayama, 2000, Reda, 2009). The high molecular weight (HMW) PAHs compounds, being highly recalcitrant, only a few microbial species were reported with abilities to degrade

or detoxify these compounds. Among the several HMW PAHs, pyrene is a relatively persistent compound and is often used as a model substrate in studies pertaining to high molecular weight PAH degradation (Kanaly and Harayama, 2000; Gupta et al. 2017).

Though bioremediation processes thought to be effective tools for remediation of the contaminated sites, these processes are often affected by several environmental factors. Environmental factors could enhance or may retard the rates of biodegradation processes. For instance, increasing temperature leads to a decreased viscosity, higher solubility and faster diffusion of hydrophobic contaminants to the cell which could enhance the biodegradation rates (Perfumo et al. 2007). However, the metabolic activities of mesophilic microorganisms slow down at higher temperatures implying that both the bioavailability of compound and microbial metabolisms were being controlled by temperatures (Zhang et al. 2012; Margesin and Schinner, 2001). In the contaminated environments, pollutants always exist as mixtures of compounds. Therefore the effect of the toxicity of a chemical mixture on different microbial species is much more complex, which could influence the degradations processes. Microbial community members able to degrade pollutant compounds are majorly inhibited by chemical mixtures, which greatly contribute to the resilience and resistance of pollutant in the environments (Ramakrishnan et al. 2011). In addition, various other environmental conditions directly or indirectly influence the microbial degradation processes. In such cases, microbial strains which are generally more metabolically active and stress tolerating could be more promising for degradation studies. Thermophilic and thermo-tolerance microbial strains were receiving a great deal of attention from last few years in bioremediation processes. Such strains have high metabolic rates and also possess more stable enzymes which can withstand the physical and chemical denaturing conditions of hydrocarbon contaminated sites and also possess thermo-stable enzymes (Mnif et al., 2011; Wang et al., 2006). This is an important property required when exogenous microbial strains were used for bioremediations. As, after

the addition of such strain either in solid or liquid phase, next degradation process majorly depends on the survival of these organisms in contaminated environments (Das and Mukherjee, 2007). In all cases, the temperature is one of the important factors in biodegradation processes, therefore thermophilic or thermo-tolerant strains could be a potential solution for biodegradation of PAH compounds. In a recent study, it was reported that the microorganisms prevailing in hot springs possess the hydrocarbon degradation potentials, which could be exploited for PAHs compound degradation studies (Saxena et al. 2017). Nevertheless, still, only a few thermophilic species were exploited in bioremediation of PAHs, which therefore implies further isolation and exploitation of novel and potentially useful microbial strains from various natural as well as extreme habitats. The aim of the present study was to evaluate the aromatic hydrocarbon degradation potentials of microorganisms isolated from the hot springs under enriched conditions. Biodegradation was studied using different strategies and temperature conditions, mixed cultures, additions of enhancement factors to make an effective bioremediation tool. Additionally, biosurfactants production abilities and their products were evaluated, which is also the part of degradation processes.

4.2: Material and Methods

4.2.1: Chemicals

All the chemicals used in this study were of analytical grade. All the PAH compounds were procured from sigma-Aldrich (USA) with a purity >98%. The surfactants, surfactin, Triton X-100 was purchased from Merck (USA), while other chemicals, culture media and solvents such as Acetone, Ethyl acetate, Dichloromethane, Chloroform, n-Hexane, Hexadecane Acetonitrile, Sodium sulphate, and other culture media components were purchased from

either HiMedia or Sigma-Aldirich (USA). Crude oil was kindly obtained from the ONGC (Bombay high, Mumbai).

4.2.2: Screening of bacterial strains (non-enriched) for pyrene degradation

4.2.2.1: Preliminary screening based on solid agar method

Selected bacterial strains were screened using a solid agar plate based method as described by Kiyohara et al. (1982) with some minor modifications. Bacterial strains were grown in nutrient broth for an overnight to obtain the sufficient cell suspension for inoculations. A mineral salt medium (MSM) as described by Patowary et al. (2016); composition (gm/lit): KCl (0.1), $NaNO_3$ (4.0), K_2HPO_4 (1.0), KH_2PO_4 (0.5), $CaCl_2$ (0.01), $MgSO_4 \cdot 7H_2O$ (0.5), $FeSO_4 \cdot 7H_2O$ (0.01), Yeast extract (0.1), and 10 ml of trace element solution containing (g l^{-1}): $CuSO_4 \cdot 5H_2O$ (0.5), H_3BO_3 (0.26), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.06), $MnSO_4 \cdot 7H_2O$ (0.5) and $ZnSO_4 \cdot 7H_2O$(0.7 and pH was adjusted to 7.0 ± 0.2 by agar plates were prepared; dried in a laminar hood for 48 hours. After a sufficient dryness of the plates, a 100 mg/L pyrene solution prepared in acetone spread over the agar surface of the plates and kept in the laminar hood still the development of a white layer of pyrene. These plates were streaked with loopful of overnight bacterial cultures as a single spot over the agar surface; plates were wrapped with aluminium foil and incubated at 37ºC up to 7 days. After every 24 h of intervals; plates were observed for growth and the clear zone around the colonies; positive bacterial strains were subcultured.

4.2.2.2: Growth monitoring and selection of strains

Using solid agar plate method, few of the selected bacterial strains were further evaluated based on growth analysis in liquid medium containing pyrene to confirm their PAHs (pyrene) utilization efficiencies. In the 250 ml conical flasks, 100 ml of MSM (Potawary et al. 2016) was prepared and amended with different concentrations of pyrene (100 mg/L - 500 mg/L) by adding the appropriate volume of pyrene from the stock solution prepared in acetone. All the

flasks were kept at 37ºC shaking (150 rpm) to evaporate the acetone. Meanwhile, bacterial cultures were grown in nutrient broth; OD_{620nm} (Optical density at 620 nm) was measured and adjusted to 1 for each culture using dilutions for inoculations. To the pyrene-containing flasks, cell pellet obtained from each strain inoculums, 5% (v/v) was added and incubated at 37°C with a 150 rpm in a shaker incubator for 18 days. Along with the tests cultures, a set of MSM containing flasks without pyrene was also maintained as a control. Growth was estimated by measuring the OD_{620nm} after an interval of every 3 days. Based on the growth profiling, highest growing strains were selected for biodegradation studies.

4.2.3: Enrichment based isolation and screening for PAHs degraders

Since the initial screening was performed using randomly selected strains, and through the screens, only a few of the strains were found to be putative PAH degrader. To obtain the diverse variety of cultures with high degradation potential for PAHs, enrichments of samples were performed. Enrichments were performed at different temperature *i.e.* 37ºC and 50ºC and using mixtures of PAH compounds.

4.2.3.1: Enrichment at 37ºC and 50ºC

The enrichment was performed for the isolation of diverse and thermo-tolerance bacterial species. Therefore, two different temperatures *i.e.* 37ºC and 50ºC were selected for enrichment. In the conical flasks containing 100 ml of MSM, a mixture of PAH compounds, namely anthracene, fluorene, phenanthrene and pyrene solution (100 mg/L) was added as the sole source of carbon and energy. Flaks were kept in a fume hood under shaking for the evaporations of the solvent. After complete removal of solvent, these flasks were inoculated with a 5 ml of the water sample and different sets were incubated at 37°C and 50ºC with shaking at 150 rpm under dark conditions. After every 7 days of intervals, an aliquot of the enriched sample was transferred to fresh medium and new flasks were incubated under the same conditions. Such subsequent transfers were performed until 21 days of incubations.

After every subsequent transfer, enriched samples were used for plating and isolation of bacteria.

4.2.3.2: Isolation of bacteria from enriched cultures

Isolation of bacteria from the enriched broths was performed using two different isolation strategies mentioned as follows

4.2.3.2.1: Isolation using standard spread plate method

For isolation of bacteria, different dilutions of the enriched samples were prepared. From each dilution, appropriates quantities were used for spreading on Bushnell-Haas (BH) agar plates preloaded with a mixture of PAH compounds. All the plates were incubated at respective temperatures of enriched broth (37ºC and 50ºC) under dark conditions. After every 24 h of incubation, plates were observed for bacterial colonies; isolated colonies were subcultured on the same agar plates under the same conditions and obtained as pure cultures for storage.

4.2.3.2.2: Isolation using filter membrane based method

In this method, under aseptic conditions, individuals, as well as mixtures of PAH compounds (anthracene, fluorene, phenanthrene and pyrene) were spiked on the sterile 0.22 µm polytetrafluoroethylene (PTFE) filter membrane (HiMedia, India) surfaces as acetone solution and allowed to evaporate the solvent completely. The membrane was placed in a sterilized filtration assembly (Merck Millipore, USA) and different dilutions of enriched broths were filtered through the filter membrane using a vacuum. The membrane was removed from the filtration assembly and placed in the 25 mm Petri dish containing sterile cotton linters pre-moist with liquid BH medium. Plates were incubated at respective temperatures of enriched broth, observed for the appearance of bacterial colonies after every 24 h of incubation. Subsequently, individual colonies were subcultured on agar plates and stored for further studies.

4.2.3.3: Identification and taxonomic analysis

The isolated cultures were subjected to genomic DNA isolation using standard phenol/chloroform/isoamyl alcohol (25:24:1) method as described earlier in the previous chapter (chapter 3). The isolated genomic DNA was checked for its integrity using 1% agarose gel and visualized under gel doc system (Protein simple); quantified using Spectrophotometer (Nanodrop 1000 UV) and used for the amplification of the 16S rRNA gene using universal bacterial primers 16F27(5′CCAGAGTTTGATCMTGGCTCAG3′) and 16R1525 (5′TTCTGCAGTCTAGAAGGGGTGWTCCAGCC3′). PCR amplification, purification, and sequencing were performed as described earlier (chapter 3, section 3.4). The sequences were edited using CHROMASLITE (version 1.5) and analyzed by Basic Local Alignment Search Tool (BLAST) with the closest cultured sequences retrieved from the National Center for Biotechnology Information (NCBI) database (Altschul et al. 1990) and EzTaxon server (Chun et al. 2007). The phylogenetic tree was constructed by a neighbor-joining method using Molecular Evolutionary Genetic Analysis (MEGA) 6 software (Tamura et al., 2013).

4.2.3.4: Screening and selection of strains for PAH utilization

All the bacterial strains obtained from enrichments were screened for selection of more efficient strains for PAH degradation. Screening was performed as described previously in the section 4.2.2.

4.2.4: Selection of suitable media for biodegradation studies

Various mineral salt medium as composed by different researchers varies with the composition of mineral salt solutions. Some of the commercially available mineral salt media are also used for degradation studies at lab scale. Here, to understand the effect of mineral salt composition on growth under PAH stresses conditions. We have chosen four different

MS media, including commercially available BH medium to be used for growth analysis. These four media are as follows,

MSM1, composition (gm/lit): K_2HPO_4; 7.0 gm, KH_2PO_4; 2.0 gm, $C_6H_5Na_3O_7$; 0.5gm, $NH_4)_2SO_4$; 1gm, $MgSO_4.7H_2O$; 0.1 gm (Kumari et al. 2013), **MSM2**, composition (gm/lit): KCl (0.1), $NaNO_3$ (4.0), K_2HPO_4 (1.0), KH_2PO_4 (0.5), $CaCl_2$ (0.01), $MgSO_4{\cdot}7H_2O$ (0.5), $FeSO_4{\cdot}7H_2O$ (0.01), Yeast extract (0.1) and 10 ml of trace element solution containing (g l^{-1}): $CuSO_4{\cdot}5H_2O$ (0.5), H_3BO_3 (0.26), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.06), $MnSO_4{\cdot}7H_2O$ (0.5) and $ZnSO_4{\cdot}7H_2O$(0.7 and pH was adjusted to 7.0 ± 0.2 (Patowary et al. 2016), **MSM3**, composition (g/L): KH_2PO_4 8.5 gm/lit, K_2HPO_4 21.75 gm/lit, Na_2HPO_4 33.4 gm/lit, NH_4Cl 5.0 gm/lit, $MgSO_4$ 22.5 gm/lit, $FeCl_3$ 0.25 gm/lit, $CaCl_2$ 36.4 gm/lit, Trace element 1 (ml), pH 7.2 ; Trace element composition: $CuSO_4.5H_2O$ 0.5 gm/lit, H_3BO_3 0.26 gm/lit, $(NH_4)_6MO_7O_{24}.4H_2O$ 0.06 gm/lit, $MnSO_4.7H_2O$ 0.5 gm/lit, $ZnSO_4.7H_2O$ 0.7 gm/lit (Sukhdhane et.al, 2016), **MSM4**, BH medium, composition (g/L): $MgSO_4.7H2O$ (0.2); $CaCl_2.4H_2O$ (0.02); KH_2PO_4 (1.0); K_2HPO_4 (1.0); NH_4NO_3 (1.0); $FeCl_3$ (0.05). For growth analysis, in separate conical flasks, 100 mL of each MS medium was prepared and augmented with 100 mg/L pyrene as mentioned earlier. Growth was monitored after every 3 days of incubation still 18 days.

4.2.5: Workflow for biodegradation studies

4.2.5.1: Chemicals and solvents

All the chemicals, solvents required for the degradation analysis were procured from either from HiMedia or Sigma. All the solvents were stored with appropriate safety in safe containers and used precisely.

4.2.5.2: Degradation using liquid medium

Degradation was performed at the lab scale in a liquid medium using shake flask conditions. In a set of conical flasks, 100 ml MSM amended with 100 mg/L of PAH compounds either as

an individual or in a mixture were added. Mother culture of selected strains was prepared as mentioned earlier, from which a 5% inoculums obtained in the form of cell pellet inoculated to the PAH containing flasks. These sets of flasks were covered with aluminium foil and incubated in a shaking incubator at the respective temperature of the selected strains *i.e.* at 37ºC and 50ºC with 150 rpm for 15 days. Another set of flask without an addition of cell cultures was also maintained as abiotic control. After the completion of the incubation time, residual PAH compounds from the test as well as control flasks were extracted three times with equal volume of ethyl acetate as a liquid-liquid extraction method. The organic phase obtained filtered through the anhydrous sodium sulphate to remove the water molecules if any. The filtrate was concentrated using a rotary vacuum evaporator (Heidolph, Germany) till dryness and then dissolved in an appropriate (usually Acetonitrile) organic solvent for analytical analysis.

4.2.5.3: Analytical analyses

The residual PAHs obtained from test and control samples were quantitatively analyzed by Ultra high-performance liquid chromatography (UHPLC) system (Thermo Fisher, scientific, USA) equipped with UV detectors. Separation of the compound was carried out using a reverse-phase C18 ODS2 4.6*250 mm column (Waters, USA), using 20 µl injection volumes of samples and Acetonitrile: water (80:20) as a mobile phase at a flow rate of 1 ml per minutes. The amount of degraded PAH compound was calculated as a difference in the percentage of degradation between the sampling day (15 days) and 0 days and with the subtraction of abiotic losses. The calculations were performed using the following mathematical formula. Degradation percentage = (control peak area- sample peak area)/ (control peak area) × 100. Final degradation percentage = (sample values - abiotic control values). The workflow of whole process used for biodegradation analysis is shown in figure 4.1.

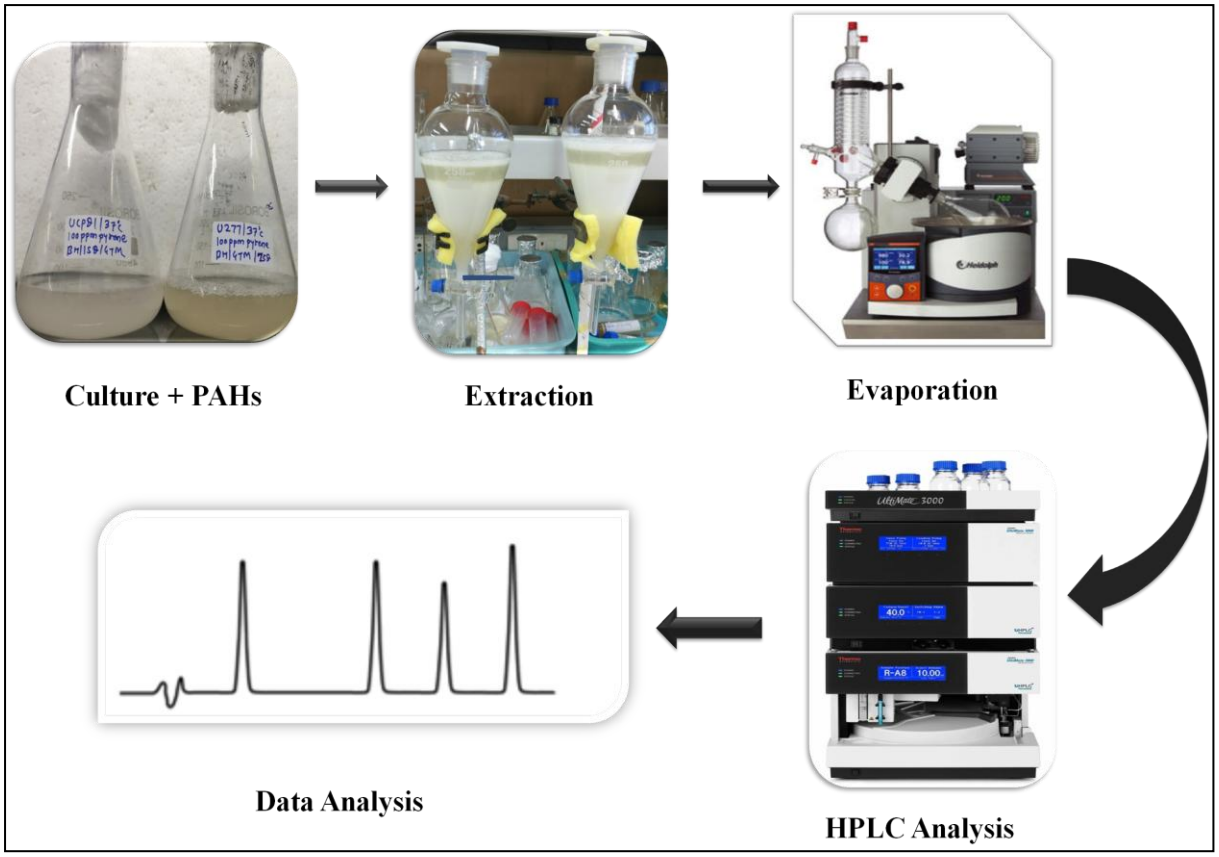


Fig. 4.1: Workflow for the degradation analysis of PAH compounds using different biodegradation strategies

4.2.6: Biodegradation strategies

4.2.6.1: Bacterial strains and incubation temperatures

In total, four bacterial strains were selected for biodegradation studies based their PAH utilization efficiencies. These four strains belong to two genera of the *Bacillaceae* family. These strains include three *Bacillus* sp (U277, GHP76, and UCPD1) and one *Aeribacillus* sp (UCPS2). All three *Bacillus* species were selected from 37ºC temperature set (one (U277) form direct screening); *Aeribacillus* sp was selected from the 50ºC temperature enrichment set.

4.2.6.2: Degradation of HMW PAHs using individual cultures

Biodegradation was performed using individual strains at respective temperatures of their growth. Three strains *i.e. Bacillus* sp, U277, GHP76, and UCPD1 were used for

biodegradation at 37ºC, while only one strain *Aeribacillus* sp UCPS2 at 50ºC. Biodegradation experiment was carried out as described in the workflow (Section 4.2.4). Here, however, the changes regarding the use of PAH compounds, inoculation cultures, incubation temperatures as per the strategy if made are described. In this first biodegradation strategy, degradation of High molecular weight compound (pyrene) was carried out using individual strain to find out the HMW PAHs degradation efficiencies of these strains.

4.2.6.3: Degradation of mixed PAHs using individual cultures

In this biodegradation strategy, degradation of mixed PAH compounds (anthracene, fluorene, phenanthrene and pyrene) *i.e*. containing both HMW and LMW PAHs was performed by individual strain. Biodegradation was performed at 37ºC using individual *Bacillus* sp. U277, GHP76, and UCPD1, while by *Aeribacillus* sp UCPS2 at 50ºC. An efficient method for separation and resolution of peak of each individual compounds form the mixture was developed and optimized using HPLC by manipulating solvent gradient program and flow rates.

4.2.6.4: Degradation of mixed PAHs using mixed cultures

In this biodegradation strategy, degradation of mixed compounds was performed using mixed culture of four strains. Individually overnight grown cultures of each strain were mixed in an appropriate manner, so as to obtain the mixed culture. For mixing, overnight grown cultures were adjusted to 1 OD_{620nm}. An equal volume from culture of each strain was mixed in a sterile fresh tube, OD_{620nm} was adjusted to one and used in the form of cell pellet as a 5% cell inoculums. Flasks were incubated at both the temperatures (37ºC and 50ºC) and harvested for analytical analyses as described in the previous section.

4.2.6.5: Degradation of mixed PAHs using total enriched community

In this experiment, degradation was performed using total enriched microbial community, so as to check the impact of the total microbial community on the degradation of mixed

compounds. Cell pellets obtained from the enriched broth cultures (during the enrichment procedures) was dissolved in sterile saline solution and adjusted to 1 OD_{620nm}. Finally, different inoculums % were prepared (0.5% - 3%) and used for degradation experiments. Incubation was performed at 37ºC and analytical analysis was performed as described earlier.

4.2.7: Strategies for enhancing degradation rates

Using different biodegradation strategies, degradation of mixed PAH compounds, as well as individual compounds, was performed. Here, the strategy in which highest degradation achieved was further used for the enhancement. Biodegradation using mixed culture at 50ºC has shown the highest degradation, therefore, this set of experiments was further used with amendments for enhancing the degradation rates.

4.2.7.1: Enhancement by biochar amendments

Various methods such as the development of microbial consortia, the addition of synthetic, natural biosurfactants as well as the addition of various substances such as biochar, humic acids which help in solubilising and making availability of PAHs molecules to the microorganisms. Here we have used biochar to enhance the rates of degradations.

4.2.7.1.1: Biochar preparation

Biochar was prepared using the agricultural waste product, *i.e.* corncob. In this method, corn cobs were collected from local places thoroughly washed with tap water and double distilled water and then dried in the oven at 50ºC for 2-3 days. Further, they were dried at 110ºC for a couple of days till to obtain a constant weight. After sufficient drying, Corncob was crushed with disintegrator and then using mortar and pistil to obtained fine powder. The obtained powder was then sieved (60 mesh) and used for pyrolysis. The slow pyrolysis experiments were carried out in a muffle furnace. Pyrolysis was performed with 25 gm of the sample at 500ºC temperature with ramp temperature of 10ºC/min for three hours. Biochar formed was collected and sterilized using dry autoclave at 121ºC for 20 minutes.

4.2.7.1.2: Degradation using biochar amended mixed cultures

Degradation mixed compounds by mixed culture at 50ºC was found higher as compared to other degradation strategies, therefore to further accelerate the degradation rates; addition of biochar to MSM containing PAHs was done. The biochar is known to accelerate the rate of degradation of PAH compounds by making the availability of PAH molecules to the degrading microbes. Here, 0.5% corncob biochar was added to liquid MSM amended with 100 mg/L of mixed PAH compounds. Flasks were inoculated with mixed culture (as described in section 4.2.6.3) and incubated at 50ºC for 15 days and harvested for analytical analysis.

4.2.8: Analysis of intermediate metabolites and pathway prediction

PAH compound degradation, especially HMW compounds occur through successive attack of enzymes involved in the metabolic pathway. During such degradation process, various intermediate metabolites are being formed, which may be toxic or nontoxic. Differences in the degradation pathways among various microbial groups found to occur, however, in most of the cases, well elucidation of metabolic pathways is still lacking in some microbial groups such as *Bacillus* group. In this study, we have analyzed the intermediate metabolites using GC-MS analysis of pyrene degradation in *Bacillus* species and attempt was made to predict the metabolic pathway of degradation.

4.2.8.1: Analysis using GC-MS

To analyze the intermediate metabolites of pyrene degradation, the culture of the *Bacillus* species, strain U277 was grown in 500 ml mineral salt medium containing 100mg/L pyrene as a sole source of carbon and energy. Cell pellet obtained from an overnight grown culture (OD_{620nm}: 1) was resuspended and added (5%) to the flasks containing 500 ml of MSM amended with pyrene. Based on the degradation profile, incubation was done at 37°C and 150 rpm for about 15 days. After completion of the incubation period, the broth was filtered

through glass wool and centrifuged at 10000 rpm for 10 minutes to remove cells and remaining pyrene crystals. The supernatant was collected in fresh flasks and extracted five times with an equal volume of ethyl acetate. Further, the extract was acidified to pH 2 with 6N HCl, and extracted again using ethyl acetate. Extracts were dried over anhydrous sodium sulphate and concentrated using and concentrated rotary vacuum evaporator (Heidolph, Germany) till the dryness and then dissolved in acetonitrile solvent. For initial detection and characterization of metabolites, aliquots of extracts were analyzed by gas chromatography (GC)-mass spectrometry (MS) along with the standard pyrene compound. The GC-MS analysis was carried out using Gas Chromatograph-Mass Spectrometer (GC/MS TQ8030, Shimadzu, Japan) equipped with an auto-injector. The Helium was used as the carrier gas with a flow rate of 1 ml per min, maintaining an injection temperature of 300°C. The column oven temperature was set at 60°C with a hold time of 5 min and was subsequently increased to 280°C with a ramp of 8°C min with the final hold of 37 min. The mass spectrometric data were acquired in electron ionization mode. The ion source temperature and interface temperature for MS was set at 230°C and 310°C, respectively.

4.2.8.2: Degradation pathway prediction

The chromatograms were analyzed with GC-MS solution software and metabolites were determined based on the molecular and fragment ions in the mass spectra and the chromatographic retention time (R_t) of authentic compounds using NIST11 library database. Further based on the literature analysis proposed few steps of the pathway were analysed.

4.2.9: PAHs degradation in crude oil

4.2.9.1: Crude oil and chemicals

Crude oil was obtained from Bharat Petroleum Refinery, Mumbai 400074, India and used throughout the study by their permission. Media and other chemicals were of purity grade from obtained from HiMedia and Sigma. Solvents such as acetonitrile, n-hexane,

dichloromethane and diethyl ether used are of high performance liquid chromatography (HPLC) grade.

4.2.9.2: Degradation studies

Degradation of PAHs in crude oil was performed using individual strain, mixed cultures and the total enriched microbial community at respective temperatures. For degradation, in the conical flasks, 100 ml MS medium was taken and added with 2% (v/v) crude oil as sole carbon and energy source. For inoculums developments, the selected bacterial cultures, mixed cultures, and enriched community were prepared as mentioned in earlier sections. Briefly, Cell pellet was obtained from the sets of all (individual, mixed and total community cultures) overnight grown cultures and resuspended in sterile saline and used as 5% inoculums (OD_{620nm}: 1) from each set were added to the flasks containing 100 ml of MSM amended with 2% crude oil. Inoculated flasks were incubated at respective temperatures, *i.e.* individual cultures (U277, GHP76, and UCPD1), mixed culture and the total enriched community at 37ºC and strain of UCPS2 and another set of mixed culture mixed at 50ºC at 150 rpm for 15 days. A set of flasks containing only MS media and crude oil (2%) was also maintained in the same conditions as abiotic control. After completion of the incubation time, growth of the cultures was determined by measuring the OD_{620nm}. Residual crude oil from the culture as well as control flasks was for extraction. Extraction was done using n-hexane: dichloromethane (1:1) solvent system as described previously (Patel et al. 2018). The solvent fraction was evaporated at room temperature, reconstituted in acetonitrile: methanol (1:1) and a suitable aliquot was analyzed on Ultra High-Performance Liquid Chromatography (UHPLC). An efficient method for separation and resolution of peak of each individual PAH compounds form the crude oil was developed and optimized using HPLC by manipulating solvent gradient program and flow rates.

4.2.10: Screening for biosurfactants

The bacterial strains used for biodegradation studies were also evaluated for their biosurfactant production ability. In general, bacterial species which could utilize efficiently aromatic hydrocarbons as a carbon source and energy source are known to produce surface active compounds for emulsifying the available hydrocarbon source. Here, we have screened the PAH-degrading bacterial strains for biosurfactant production.

4.2.10.1: Drop collapse and oil displacement methods

Screening for biosurfactant production was performed using drop collapse and oil displacement methods. Overnight grown cultures (OD_{620nm}: 1) of each strain was inoculated individually in the conical flasks containing 100 ml of nutrient broth. These flasks were incubated at the respective incubation temperature of individual strains at 150 rpm shaking for 48 h. The culture broth was harvested using centrifugation (10000 rpm for 20 minutes) at a temperature of 4ºC to collect the cell-free supernatant. Collected supernatant used for biosurfactant testing. In drop collapse method, a sample of 5 µl cell-free supernatant is added to the centre of a Petri dish containing oil (Olive oil) and observations are carried out for 1 min. If the drop of a sample collapses with the oil, it is an indication of the presence of BS in the culture broth (Bodour et al. 1998). To further confirm the BS production, another test, using oil displacement method was performed. In this experiment, oil displacement assay was carried out as described by Morikawa et al. (2000) with slight modifications. In a clean 25 mm Petri dish, appropriate quantity of distilled water was added. Over the surface of the water, around 50 µl crude oil was gently added to form a thin oil layer. Over this layer, using micropipettes, a 20 µl of cell-free supernatant was carefully placed without touching pipettes to the oil layer and observed for the displacement of the oil.

4.2.10.2: Surface tension (ST) reduction

Reduction in the surface tension was evaluated for the collected cell-free supernatant using the tensiometer (K11, Kruss, Germany). A sterile distil water was used as a control. Based on the reduction properties, positive strains were selected for further experiments.

4.2.11: Production and purification of biosurfactants

4.2.11.1: Process parameters for fermentation

During screening for BS, strains found to be positive were used for BS production in a 3 litre bioreactor. Inoculums were prepared by growing the cultures in 250 ml flasks containing 100 ml nutrient broth and growth was determined by estimating absorbance (OD_{620nm}). Inoculums of 5% were used to inoculate the 3L bioreactor (Eppendorf, New Brunswick Scientific, UK) containing 2L nutrient broth. Process parameters such temperature was set at 30ºC, pH 7 and incubation time of 48 h. The agitation system of the bioreactor contained two six-blade impellers on a single drive shaft connected to a motor whose speed was controlled by a fermentation control unit. Both pH and dissolved oxygen probes were also connected to a fermentation control unit and pH was monitored but not maintained. Dissolved oxygen (DO) conditions, was accomplished using an air flow rate ($1.5\ vvm^{-1}$) and a stirrer speed (250 rpm). During fermentation, temperature, dissolved oxygen and pH values were monitored and recorded prior to each sampling procedure. Sample of appropriate quantities was removed during the course of the fermentation at regular intervals in order to determine the BS.

4.2.11.2.: Extraction

After completion of the fermentation incubation time, broth was harvested and clarified by using centrifugation for 20 min at 8000 rpm at 4ºC temperature. The supernatant was collected in sterile flasks and acidified with 3N HCl till the pH becomes 2.0 and then kept for overnight at 4°C to obtain the precipitation. The precipitate was collected in 50 ml tubes and

dissolved in HCL and kept for shaking until it gets completely dissolved. The dissolved sample was then extracted with an equal volume of Chloroform: Methanol (2:1) solvent (Mnif et al. 2016). The obtained crude extract in the form of the organic phase was dehydrated by passing through the anhydrous sodium sulphate. Finally, the extract was concentrated in a rotary vacuum evaporator (Heidolph, Germany). Crude BS was freeze-dried using a vacuum freeze dryer, the weight of the dried crude BS was measured and yield (gm/L) was calculated.

4.2.11.3: Purification

Purification of BS was performed in a preparative mode by high-performance liquid column chromatography as described by Amanolahi (2017) with slight modifications. The crude BS was dissolved in 2 ml mixture of Acetonitrile: methanol (2:1) solvents. High-performance liquid chromatography (HPLC) was carried in a preparative mode. An aliquot of 40 µl of each crude BS solutions was injected automatically in a reversed-phase C18 column (15.0 × 4.6 mm; 5 µm particle size) held at 25°C. The mobile phase was held for 10 min before each run, and then the samples were eluted using a linear gradient mobile phase composed of acetonitrile (3.8 mM): trifluoroacetic acid (80:20, v/v) at a flow rate of 4 ml/min. The eluted components were detected using DAD at 205 nm ad 210 nm. Separation of compounds was monitored by analyzing peak in the chromatograms, based on which, fractions were collected and further analyzed using other parameter testing.

4.2.12: Characterization of biosurfactant

4.2.12.1: Using molecular tools

Many strains from genus *Bacillus* have the ability to produce biological surface active compounds belonged to lipopeptide (LPs) group. Therefore, as the BS producing strains belong to *Bacillus* group, we performed PCR using different primer sets for possible detection of lipopeptide genes. The PCR amplification of BS genes was done using primer

sets as described previously by various researchers such as Fengycin, Baccilomycin D (Ramarathnam et al. 2007), Iturin A (Tsuge et al. 2005) and Surfactin (Hsieh et al. 2004). For all sets of genes, PCR conditions were standarilized using sets of gradient PCR by modifications of conditions previously developed by Stankovic et al. (2012). The reaction was performed in 50 µl reaction mixture containing 10 nM (each) primer (Eurofins), 200 µM (each) deoxynucleoside triphosphate (dNTP) (Genei), 1 U of Taq polymerase (Genei) in the appropriate reaction buffer, and 100 ng of DNA extract as a template with following PCR conditions. Briefly, the initial denaturation for the fenD and bacillomycin D gene was at 94ºC for 3 min; 35 cycles for the fenD gene at 94°C for 1 min, at 56°C for 1 min, followed by a 72°C extension for 1 min for 45 s; 35 cycles for bacillomycin D were at 94°C for 1 min, at 56°C for 30 s, and extension at 72°C for 1 min. The final extension for both genes was at 72°C for 7 min. The initial denaturation for the iturin A and the sfp gene was at 94°C for 4 min; the final extension was at 72°C for 7 min. For the iturin A; at 94°C for 30 s, 56°C for 30 s, and a 72°C extension for 2 min, the 30s for 35 cycles and for the sfp gene 35 cycles with at 94°C for 30 s, 43°C for 30 s, and a 72°C extension for 1 min. The PCR products were then analysed using 1% agarose gel electrophoresis and visualised by gel documentation system (Protein simple). The PCR products were purified using Exo-rSAP (USB) and sequenced in ABI 3500xl genetic analyzer (Invitrogen/Life Technologies) and edited using CHROMASLITE (version 1.5). Edited sequencing data was analysed by blastN and blastX with closest cultured species sequences retrieved from the NCBI database (Altschul et al. 1990). The phylogenetic tree was constructed by the neighbour-joining method using MEGA 6 software (Tamura et al. 2013) to determine the percentage homology of each individual gene in the closely related reported strains of the database.

4.2.12.2: Using analytical tools

4.2.12.2.1: Thin layer chromatography

The purified BS was dissolved in chloroform-methanol (2:1) using the recommended concentration of 10 mg/ml as described by Smyth et al. (2010) and analyzed by thin layer chromatography (TLC). For analysis, 10 µl of each sample was spotted onto the TLC plates (Merk, USA) and allowed for drying. Meanwhile, a solvent mixture of chloroform: methanol: acetic acid: water (25:15: 4: 2 v/v/v/v) was prepared and filled in the TLC tank and closed for few minutes by a glass cover. The sample loaded TLC plate was placed in the chamber carefully without touching the samples spot to the solvent system directly. TLC chamber was closed using a glass lid and allowed to run with continuous monitoring till the solvent front moves for more than half of the plate. The plate was removed from the chamber and air dried in a fume hood for a few minutes and then spread with 1% ninhydrin reagent. For visualization of the spot, plate was kept in a hot air oven at 110ºC for about 10-15 minutes.

4.2.12. 2.2: FTIR analysis

FTIR is an effective analytical tool for primary identification of compounds, purified BS from UCPS2 strain along with standard compound was analysed. For analysis of functional groups of the biosurfactant molecule, the purified biosurfactant was analyzed in spectrum two Perkin Elmer instrument. The samples were analyzed on ATR (Attenuated total reflectance) mode considering in the range of 4500–500 cm^{-1} wave numbers with a resolution of 4 cm^{-1} for detection of functional groups and existing bond types.

4.2.12.2.3: HRMS analysis

The purified BS was dissolved in Acetonitrile (LC-MS grade) for identification using Accela™ ultra-high performance liquid chromatography (UHPLC) system (Thermo Fisher, Waltham, USA). Samples were run using C18 Hypersil Gold column (1.9 µm, 2.1 X150, Thermo) with a linear gradient of solvent A (acetonitrile with 0.1% of formic acid) against 2-

95% of B over 15 min solvent B (water with 0.1% of formic acid) at flow rate of 350 µl/min and 45°C. The molecular weight was identified by using Q-Exactive-Orbitrap mass spectrometer (Thermo Fisher) in the electrospray ionization-positive (ESI+) mode, mass scan range set from 100 to 1500 m/z and the data acquisition and processing were performed using the Thermo Scientific Xcalibur software (Version 3.0). The tandem mass spectrometry (data-dependent MS/MS) data were collected with collision energy between 30 and 40eV, data obtained was analyzed along with standard compound.

4.2.13: Activity assay: Emulsification Index (EI)

Emulsification activity of the produced biosurfactant was measured as described previously by Jagtap et al. (2009) with slight modifications. Purified BS was diluted and tested for emulsification of various oils and solvents. A three ml volume of various oils such as diesel oil, crude oil, olive oil, vacuum oil, engine oil as well as immiscible solvents such as n-Hexane, Hexadecane, Benzene, xylene were added separately in fresh 25 mm test tubes. To these tubes, an equal volume of purified BS (diluted) was added. The mixture was vigorously vortexed for about 2 minutes and allowed to stand for 24 h. The height of emulsion is measured by taking the layer formed in between aqueous and kerosene layer. EI is calculated by measurement of emulsion height using the formula:

Height of emulsified layer (mm)/ Total height of the liquid column (mm) x 100

4.3: Results and discussions

4.3.1: Screening bacterial strains

In total twenty-four bacterial strains were used for screening, among them few were grown solid agar plates preloaded with pyrene. Although not a clear zone around the colonies was observed, however, *Bacillus* strains (U2 and U277), *Staphylococcus* (U23), *Caldimonas* (U49) and *Achromobacter* (WP24) were found grow well on these plates. Therefore these strains were selected

to analyse their growth patterns in MSM medium containing pyrene to evaluate their pyrene utilization efficiencies. All the five strains grown in a liquid media having different concentrations of pyrene (100 mg/L - 500 mg/L), however, as concentration was increased, a decrease in growth was observed (Fig. 4.2). Among the Gram-positive strains, two *Bacillus* strains U2 and U277 has shown relatively higher growth in the all the concentrations of pyrene used in the study. Although both the strains were found to utilize pyrene efficiently as a carbon and energy source, strain U277 was more consistence in growth in all the concentrations of pyrene, therefore selected for further studies.

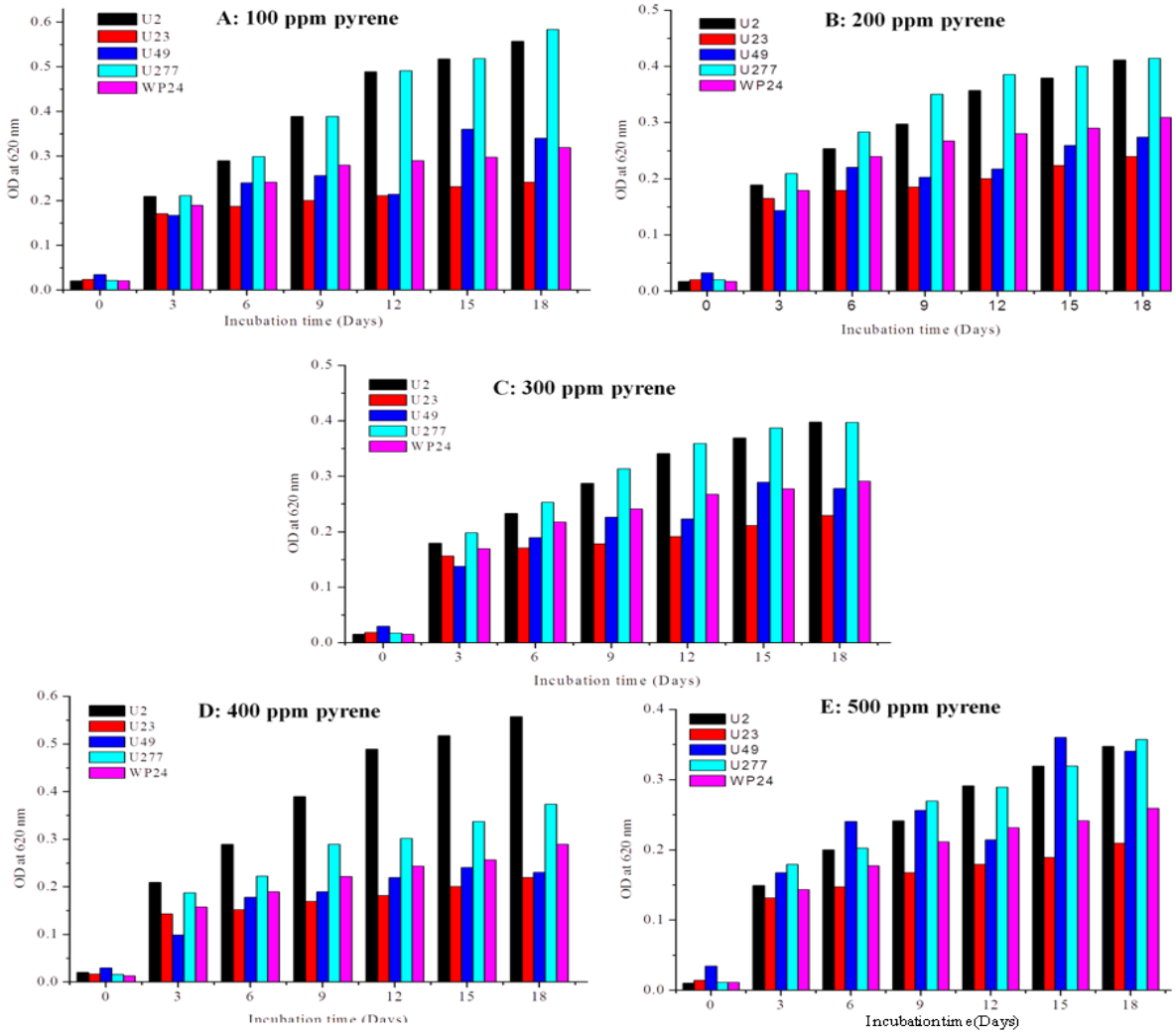


Fig.4.2: Growth analysis of selected bacterial strains in liquid medium containing different concentrations (100 mg/L-500 mg/L) of pyrene

4.3.2: Enrichment based isolation and screenings

Enrichment was performed as described in the methodology sections, the enriched broths were used for isolation of bacteria. Serial dilutions of enrichment broth were prepared and plated on BH (Bushnell-Haas) agar plates and also used for isolation using membrane filters. In both the methods, to enable the selection of clear zone forming colonies, a thin layer of mixtures of PAH compounds was coated plates as well as membranes.

4.3.2.1: Isolation of bacteria

Using standard spread plate isolation technique, different types of colonies were isolated. In this method, colonies were appeared on the plates after 48 h of incubation but without the zone of clearance. Further incubations of plates for some more time (5-7days) and careful observation, a zone of clearance was observed around the colonies as shown in the figure 4.3.

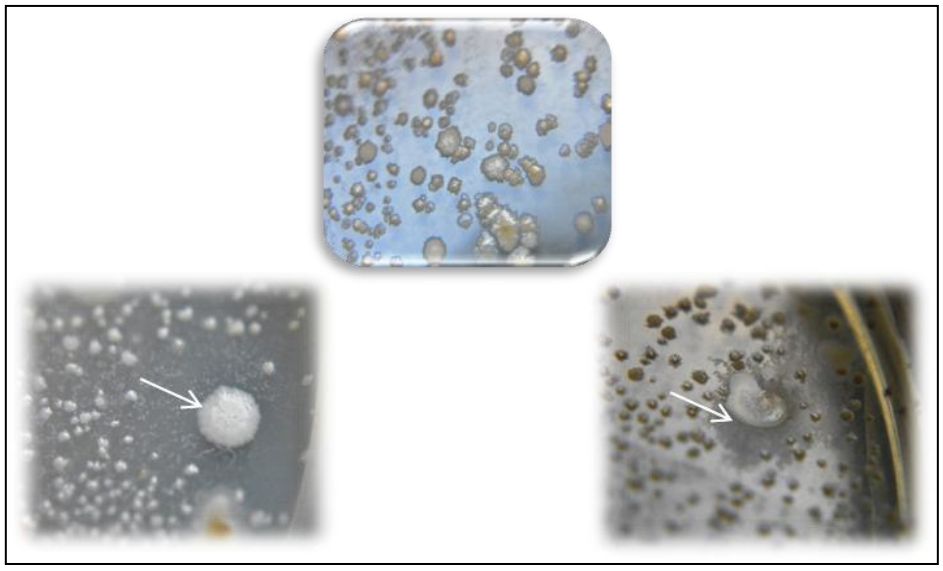


Fig. 4.3: Isolation of bacteria from the enriched broth on BH agar plates spiked with PAHs, arrow indicates the zone of clearance around the colonies.

From the plates, different types of colonies were isolated including those were not showing proper zone of clearance. In total, 55 bacterial isolates were isolated using this method, among them, 47 were from 37ºC and eight from 50ºC sets. Filter membrane method used for

isolation has also led to isolation of various bacterial strains, however, only fever isolates were obtained using this method. In total, 19 bacterial isolates (12 from 37ºC and 03 from 50ºC) were isolated. The reason for isolation of less numbers of colonies is due to difficulties in isolation of morphologically different individual colonies form the lawn of bacterial growth appeared on the membrane (Fig. 4.4). In this method, PAHs were directly associated with the bacteria on the membrane, also a mineral salt support was available embedded in the form of cotton linters below the membrane. Therefore, such PAH sorbed membrane method has given significantly different patterns of growth as compared to standard plate technique.

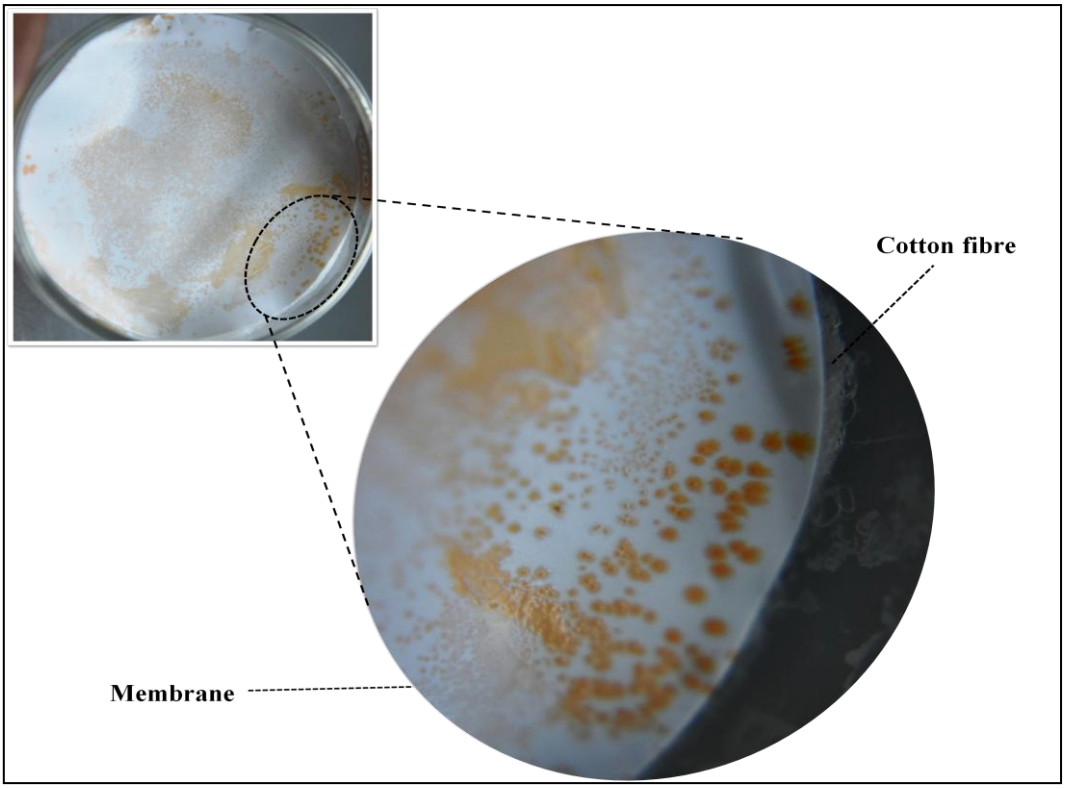


Fig.4.4: Isolation of bacteria using filter membrane technique, showing the numbers of colonies appeared on the membrane

Overall, using all sets of isolation a total of 74 bacterial isolates were obtained. Maximum numbers of bacterial isolates (63 isolates) were recovered from the enrichment done at 37ºC and remaining from 50ºC. All the bacterial strains were characterized using morphological

observation and gram staining methods. During morphological investigations, some of the isolates were observed with production of mucoid colonies indicating the production of extracellular substances (Fig. 4.5). Some of the bacterial colonies were also found to produce colour pigments.

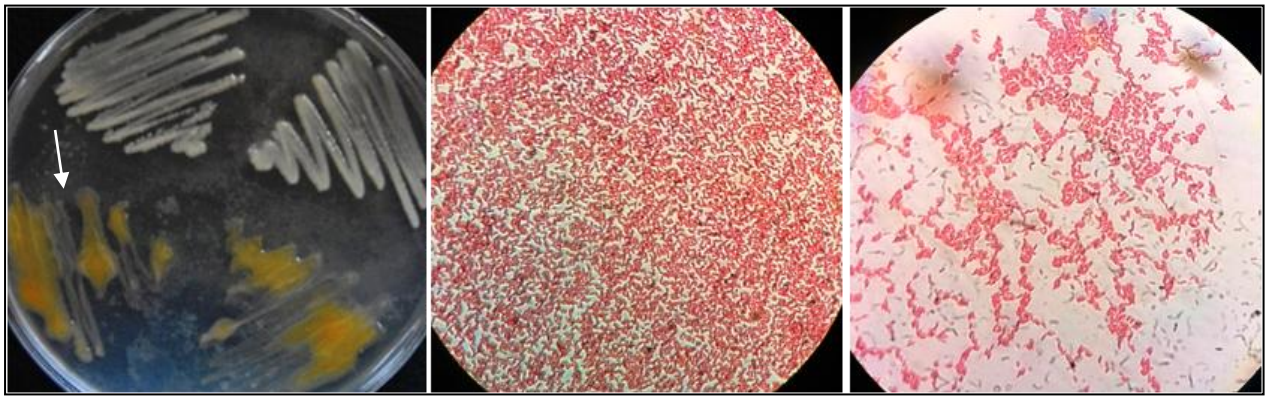


Fig. 4.5: A representative image of the isolated bacterial strains, showing production of extracellular substances and representative images of Gram staining.

4.3.2.2: Identification and taxonomic analysis

All the isolated strains were identified using 16S rRNA gene sequencing and phylogenetic analysis. The sequence similarity search results revealed that the isolates were found to belong to three bacterial phyla; *Firmicutes*, *Actinobacteria* and *Proteobacteria*. Most of the strains were found to belong to *Bacillus* (*Firmicutes*) followed by Actinobacterial genera such as *Janibacter*, *Cellulomonas*, *Arthobacter*, *Rothia*, *Dietzia*, *Micrococcus* and *Glycomyces* (Fig. 4.6). Among *Bacillus* group, different species were identified, which were found to be closely related to reported species previously used for degradation studies. The strains of *B. amyloliquefaciens* were reported for pyrene degradation (Nagger et al. 2010), *B. pumilus* (Patowary et al., 2016) and *B. subtilis* (Hunter et al. 2005) were reported for degradation and biosurfactant production. Therefore, the close association between the strains obtained in this study and those previously reported for degradation may reveal that these

genera possess such inheriting degradation potential, albeit they have been not studied from naturally occurring extreme environments.

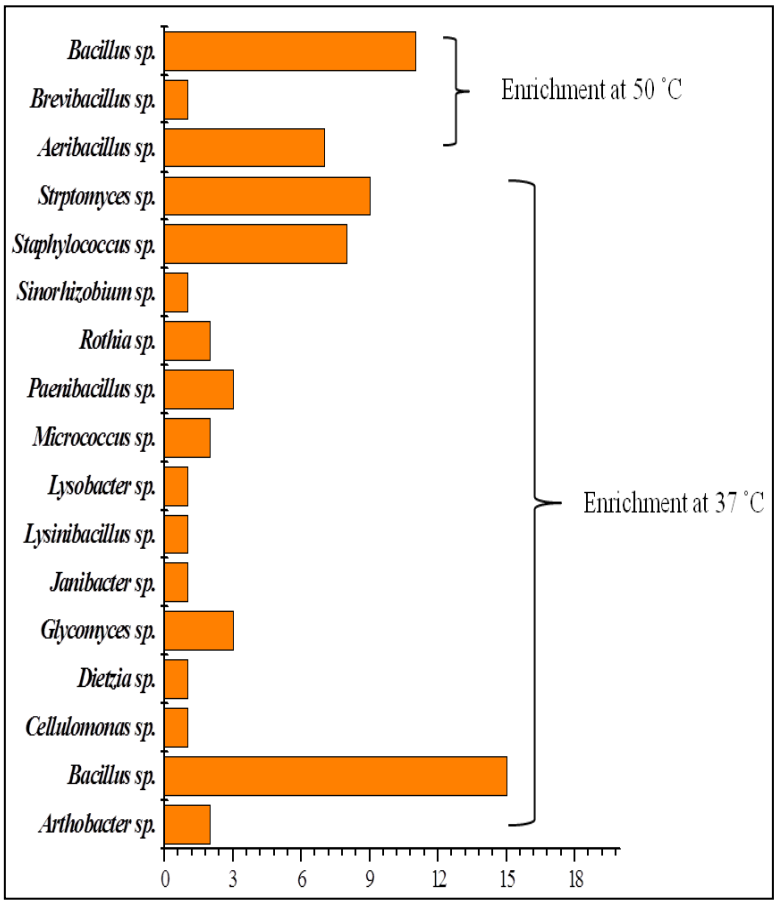


Fig. 4.6: Taxonomic grouping to the genus level of bacterial strains along with the numbers of strain of each genera isolated using enrichments

From the 50ºC enrichment sets, few of the thermophilic strains such as *Aeribacillus*, *Brevibacillus* and *Bacillus* were obtained. Among them, *Aeribacillus* strains were reported from different natural as well as contaminated thermal environments, however, only a few were reported for degradations of PAHs. Therefore, the numbers of different types of microbial strains obtained through the enrichment may represent a valuable resource for PAH degradation and bioremediations.

4.3.2.3: Screening and selection of strains

Strains for biodegradation studies were selected using various selection techniques as mentioned in the methods (Section 4.2.4). From the set of previously isolated strains used for direct screening, only one strain (U277) was selected. In case of enrichment done at 37ºC, four strains, those were showing clear zones around the colonies during isolation were selected. In total 5 bacterial strains, including U277 from the 37ºC set was selected for further evaluation based on growth analysis (Fig. 4.7).

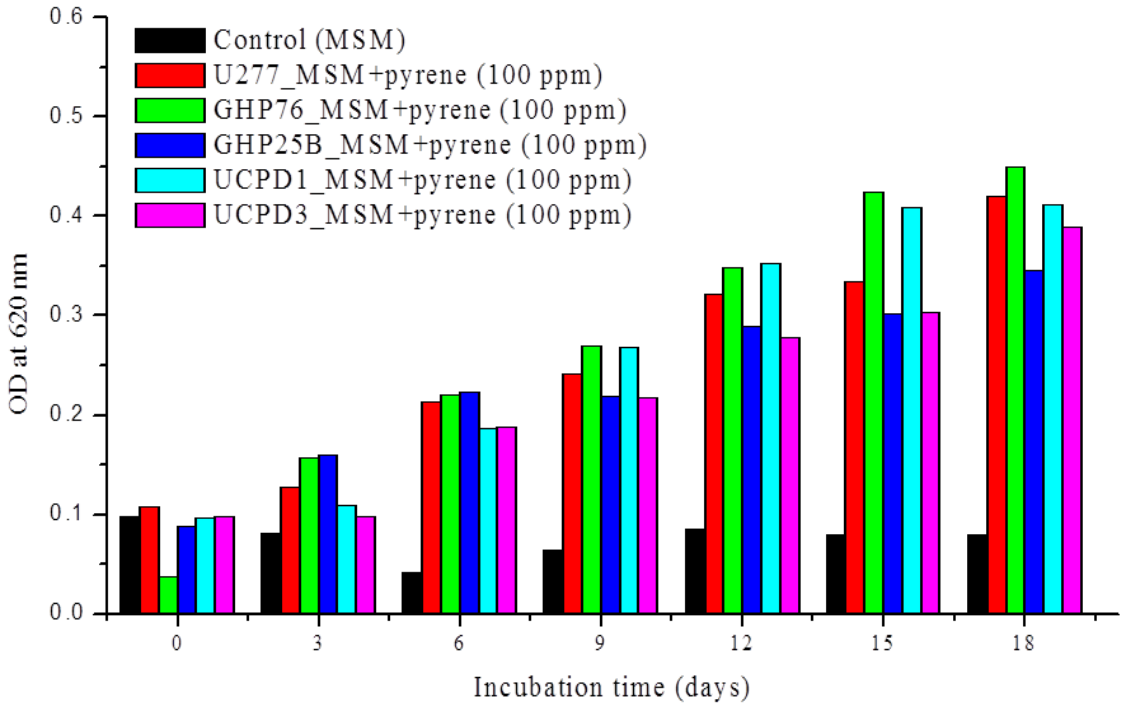


Fig. 4.7: Growth pattern analysis of selected strains from 37 ºC set

Based on growth pattern, finally three strains were selected form 37ºC set. From the 50ºC enrichment set one strain of *Aeribacillus* species (UCPS2) was selected based on its zone of clearance. In the final set, four strains belong to two genera of the *Bacillaceae* family; including three *Bacillus* sp (U277, GHP76, and UCPD1) and one *Aeribacillus* sp (UCPS2) were present.

4.3.3: Selection of media for biodegradation studies

Biodegradation studies were performed in liquid medium under shake flask conditions; therefore the composition of MSM could also affect the biodegradation process. To select a more effective medium for degradation studies, four different MSM (section 4.2.) were used for the growth analysis of selected strains. The growth analysis revealed that the composition of MSM could affect the growth of bacterial strains, which in turn affect the degradation process. Here, among four different MSM, a higher growth was observed in MSM2 and MSM4 (Fig. 4.8). MSM2 contents yeast extract in minor concentration, which may be acting as nutrient for growth, therefore higher growth does not necessarily reveals the higher degradation. However in case of MSM4 (BH medium, HiMedia), occurrence of higher growth could be correlated with the higher degradation rates as it contents only salt solutions but not any type of nutrient that will directly support the growth.

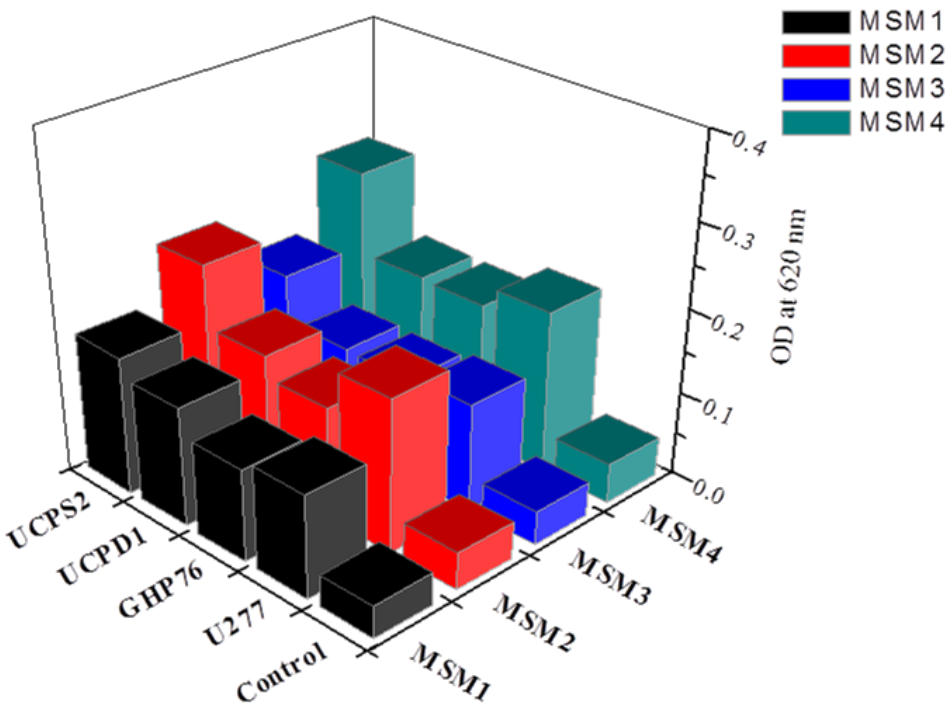


Fig. 4.8: Growth analysis of selected strain in different mineral salt media (MSM)

4.3.4: Biodegradation strategies

Biodegradation studies were performed at the laboratory scale using liquid medium shake flask conditions. Various strategies were designed for biodegradation of PAHs such as degradation by individual strains, mixed cultures at two different temperatures. Similarly, degradation of single compounds (HMW), mixed compounds and combinations of cultures and compounds were performed to evaluate their degradation efficiencies.

4.3.4.1: Degradation of PAHs (pyrene) using individual cultures

In the initial set of biodegradation studies, biodegradation of HMW PAH compound (pyrene) was studied using the individual strains at the respective temperatures of their growth. Degradation of pyrene (100 mg/L) was performed at 37ºC using three *Bacillus* sp (U277, GHP76, and UCPD) and at 50ºC by *Aeribacillus* sp strain UCPS2. A positive correlation between growth and degradation of pyrene among these strains was observed, indicating that all the selected strains could utilize pyrene as a source carbon and obtained energy for their growth (Fig. 4.9). However, at 50ºC, both growth and degradation found higher as compared to the 37ºC temperature. At 50ºC, the strain UCPS2 was able to degrade 79.47 ± 1.38% of pyrene within 15 days. At 37ºC, three *Bacillus* sp strains, U277, GHP76, and UCPD1 have shown 72.79 ± 0.33%, 56.39 ± 3.45% and 63.68 ±1.76% degradation of pyrene respectively within 15 days. It was also noted that, among these three strains, U277 was found more efficient than the other strains. A comparatively less degradation at 37ºC than the 50ºC may be because of temperature effect, or it may be due to the inherent property of the PAH degrading strains.

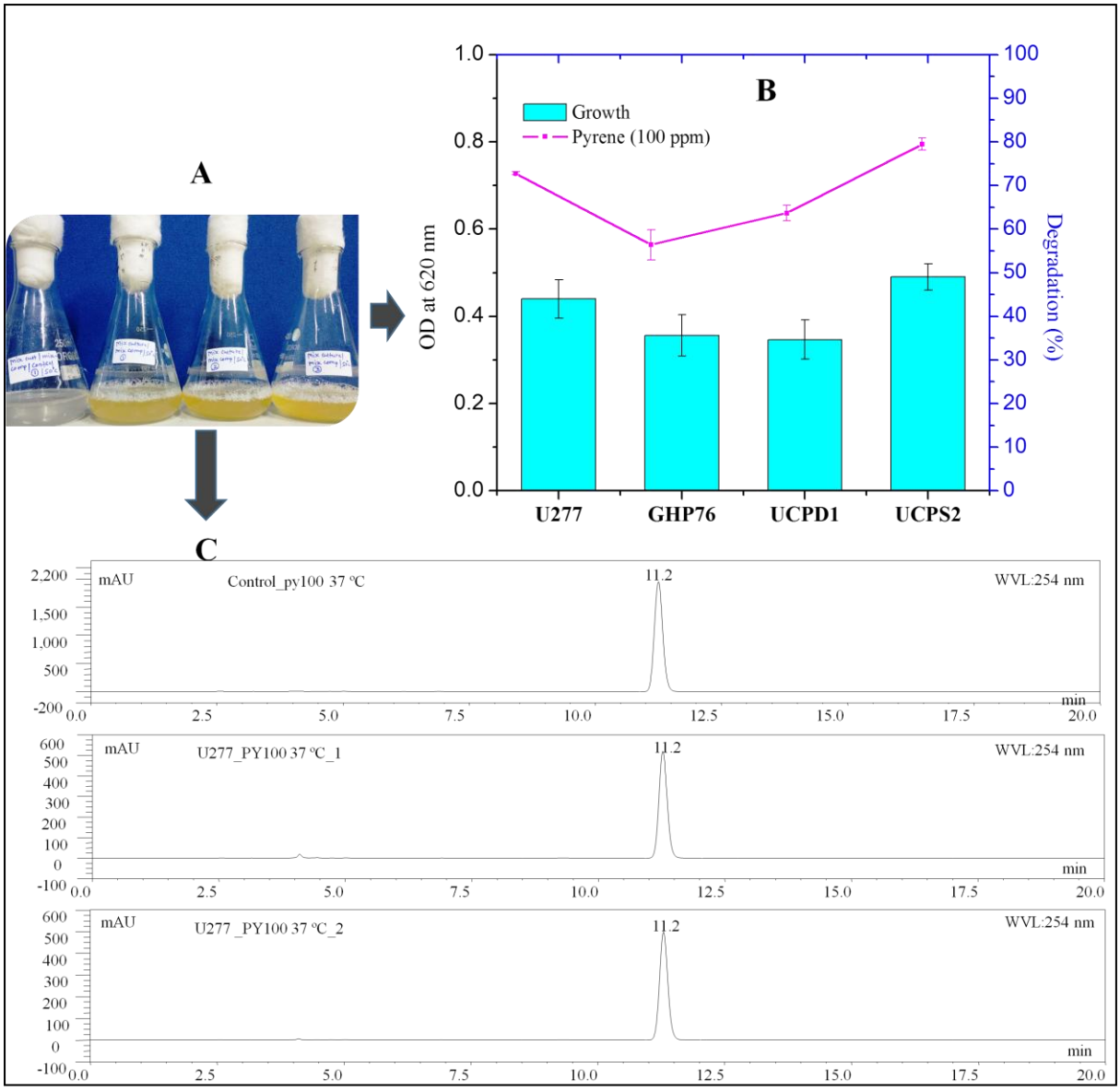


Fig. 4.9: Degradation of pyrene (HMW) using individual strains at 37ºC (U277, GHP76 and UCPD1) and 50ºC (UCPS2). **A:** Flasks showing the growth of cultures, **B:** Graph showing the growth and degradation of pyrene, **C:** Representative HPLC chromatogram of degradation analysis.

4.3.4.2: Degradation of mixed PAHs using individual and mixed cultures at 37ºC

In this experiment, a mixture of PAH compounds (anthracene, fluorene, phenanthrene and pyrene: final concentration 100 mg/L) was used for degradation using the individual as well as mixed culture at two different temperatures. In the first set of experiments, standard of mixture of four compounds was analysed for knowing their *Rt* and separation profile using HPLC. All the four compounds used for analysis found to separate well under standardized conditions as shown in figure 4.10.

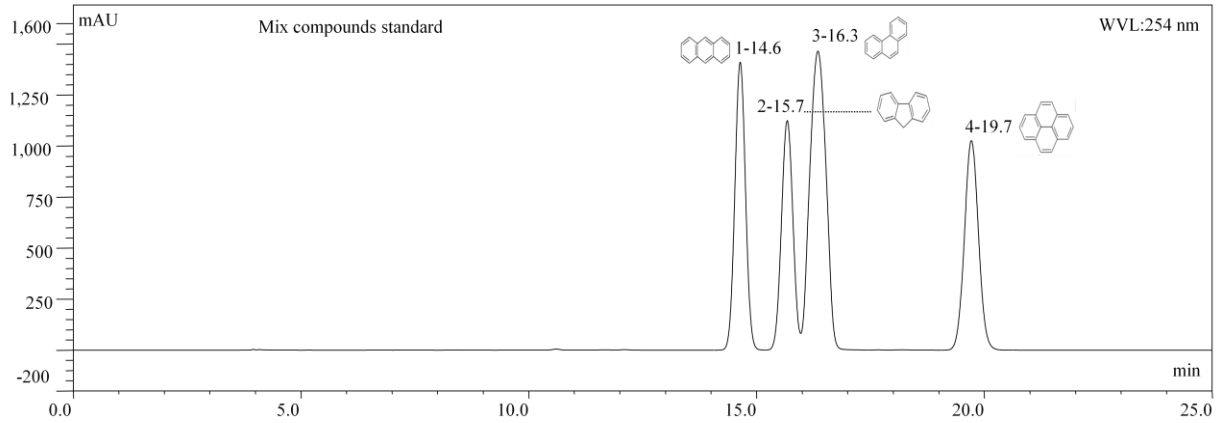


Fig.4.10: HPLC based separation profile of mixture of standard compounds; anthrecene (14.6), fluorene (15.7), phenanthrene (16.3) and pyrene (19.7) along with their retention times (*Rt*).

After standardization of mixture of compounds using HPLC, degradation analysis was performed. The study revealed that, although at lower concentrations, mixtures of compounds affect the degradation process. Here, in most of the strain, including UCPS2, degradation of pyrene (HMW) was decreased by two fold when compared with the degradation of an individual compound. As expected, due to presence of LMW PAHs (anthracene, fluorene), strains have not preferred the HMW PAHs (pyrene) and therefore its degradation was found less as compared to other compounds. Overall degradation percentage of each single compound by each strain is presented in figure 4.11 and table 4.1.

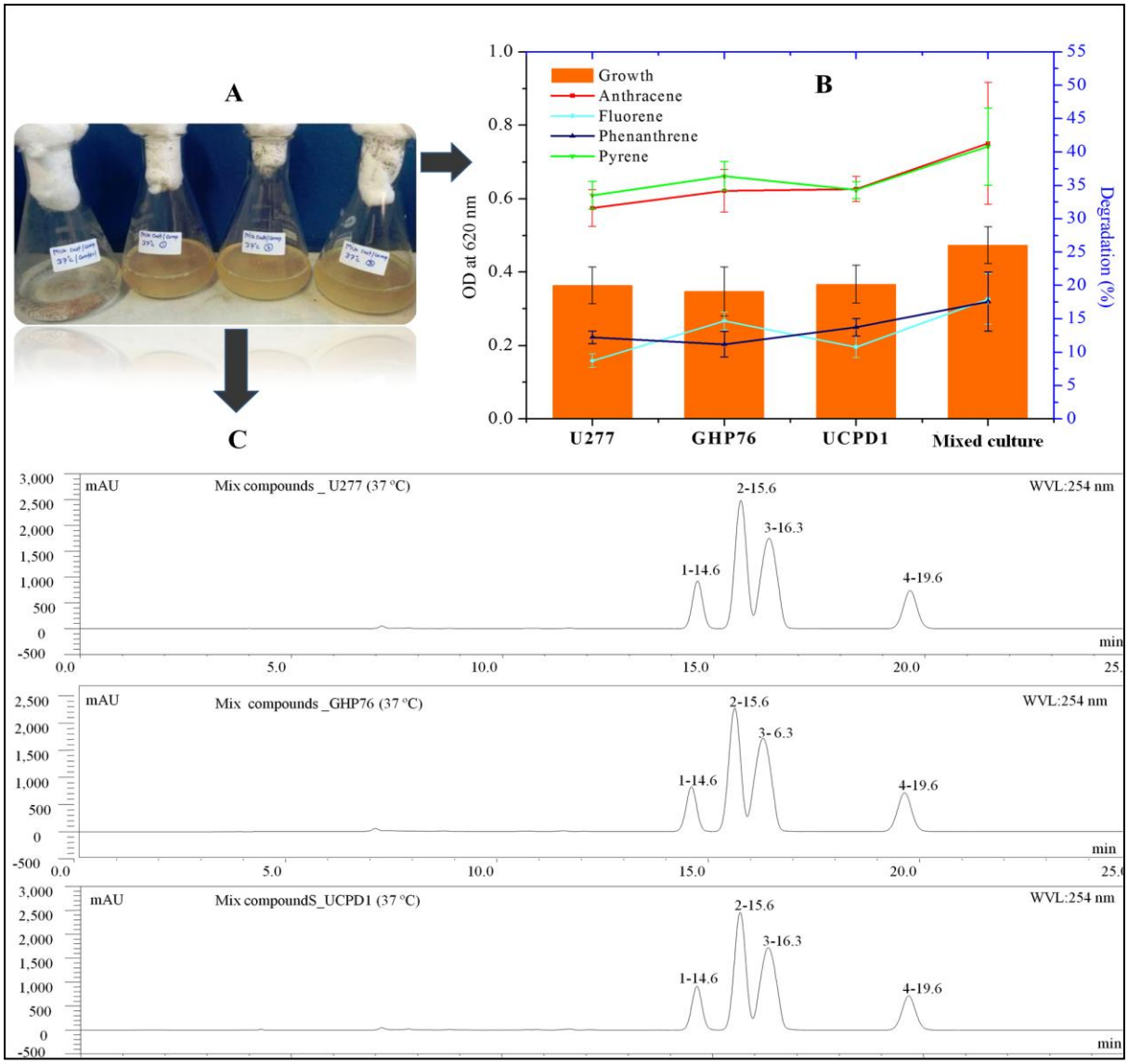


Fig.4.11: Degradation of mixed PAH compounds using individual strains and mixed culture at 37ºC. **A:** Flasks showing the growth of cultures, **B:** Graph showing the growth and degradation of PAHs, **C:** Representative HPLC chromatogram of degradation analysis.

Although the percentage of degradation of HMW PAHs was lowered in mixed compounds degradation strategy, however, all the compounds present in the medium were being used by bacterial strains. In case of mixed culture, a relatively higher degradation was observed as compared to the degradation occurred through the use of an individual strain. Interestingly, the degradation patterns for all four compound present in mixture was almost found similar in individual strains and mixed culture.

Table 4.1: Degradation analysis of mixed compounds using individual and mixed cultures

Sr.no	Strain	Growth	Anthracene	Fluorene	Phenanthrene	Pyrene
1	U277	0.36 ± 0.05	31.58 ± 2.73	08.73 ± 1.01	12.24 ± 0.95	33.51 ± 2.06
2	GHP76	0.34 ± 0.06	34.18 ± 3.18	14.67 ± 1.33	11.19 ± 1.91	36.37 ± 2.19
3	UCPD1	0.36 ± 0.05	34.45 ± 1.88	10.77 ± 1.59	13.75 ± 1.29	34.30 ± 1.24
4	Mixed culture	0.47 ± 0.05	41.28 ± 9.14	17.95 ± 3.78	17.59 ± 4.41	40.84 ± 5.78
5	UCPS2	0.52 ± 0.07	92.02 ± 0.15	83.77 ± 1.27	16.64 ± 0.57	51.66 ± 1.34
6	Mixed culture	0.56 ± 0.08	96.30 ± 0.64	86.75 ± 2.26	54. 26 ± 6.06	71.48 ± 1.33

4.3.4.3: Degradation of mixed PAHs using individual and mixed cultures at 50ºC

In case of degradation at 50ºC by UCPS2 and mixed culture, similar kinds of results were observed as observed at 37ºC. In this case also, a relatively higher degradation of LMW PAHs (anthracene and fluorene) was observed (Fig. 4.12). However, overall PAHs degradation found to be much higher than the degradation performed at 37ºC either as an individual strains or in a mixed cultures. This is may be due to temperature effect, as same type of observation was made during the degradation of single compounds at both the temperature sets.

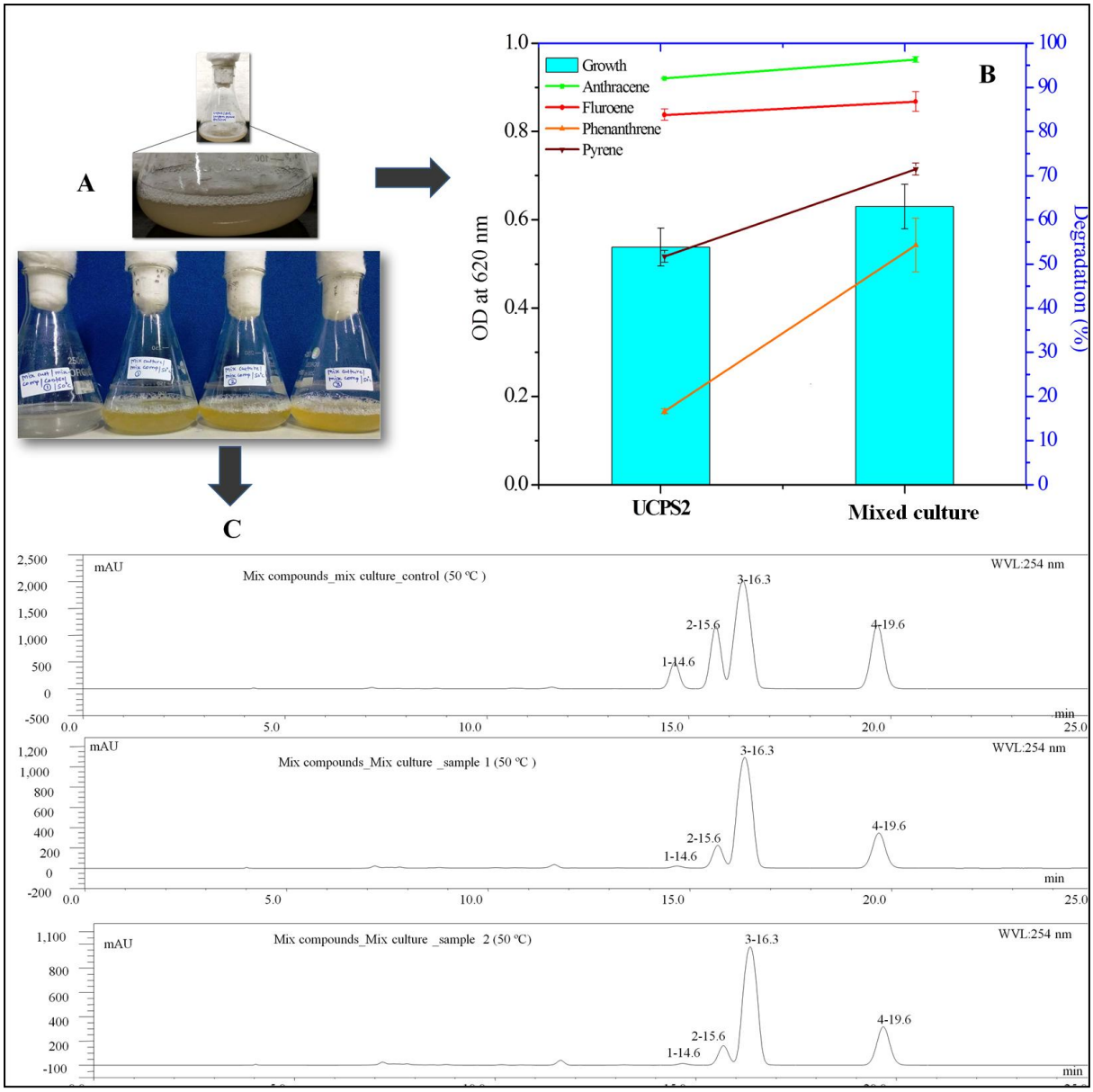


Fig.4.12: Degradation of mixed PAH compounds using individual strains UCPS2 and mixed culture at 50ºC. **A:** Flasks showing the growth of cultures, **B:** Graph showing the growth and degradation of PAHs, **C:** Representative HPLC chromatogram of degradation analysis.

A comparative account of UCPS2 strain during degradation of single compound (pyrene) and mixed compounds showed that, under mixed compound conditions, pyrene degradation was decreased. In all the cases, degradation of anthracene was found to be highest (92- 96%) either by individual strain and in a mixed culture under mixed compound condition.

Anthracene being a low molecular weight PAHs might be having relatively higher solubility at high temperatures and therefore readily gets degraded as compared to other HMW PAHs. A comparative account of mixed compound degradation studies of 37ºC and 50ºC sets revealed that at a higher temperature, high degradation could occur irrespective of individual culture, mixed culture and mixed compounds. On the other hand, it is also possible that the strain UCPS2 has inheritance PAHs degradation property.

4.3.4.4: Degradation of mixed PAHs using total enriched microbial community

In this case, degradation of mixed compounds was analysed using total enriched microbial community (TEMC). As the TEMC has different organisms, degradation was performed using different inoculums percentages (0.5% - 3%). The results revealed that the inoculums effect the degradation of LMW and HMW compounds differently (Fig. 4.13). In case of 0.5% inoculum, only the degradation of LMW PAH compounds observed. As the inoculums size increases, the degradation rates were also increased for both types of PAH compounds *i.e.* LMW and HMW compounds. Overall, around 69.11 ± 2.39 of anthracene, 63.38 ± 1.92, of fluorene 39.93 ± 1.01 of phenanthrene and 76.19 ± 1.99 of pyrene degradation was observed in 3% inoculums (Table 4.2). A comparative analysis with degradation by mixed and individual cultures showed that the mixed culture at 50ºC was found more effective for degradation of mixed PAHs than other sets including the total microbial community. Since degradation at 50ºC was found to higher than other cases, it indicates that the temperature has a profound effect on degradation of PAH compounds.

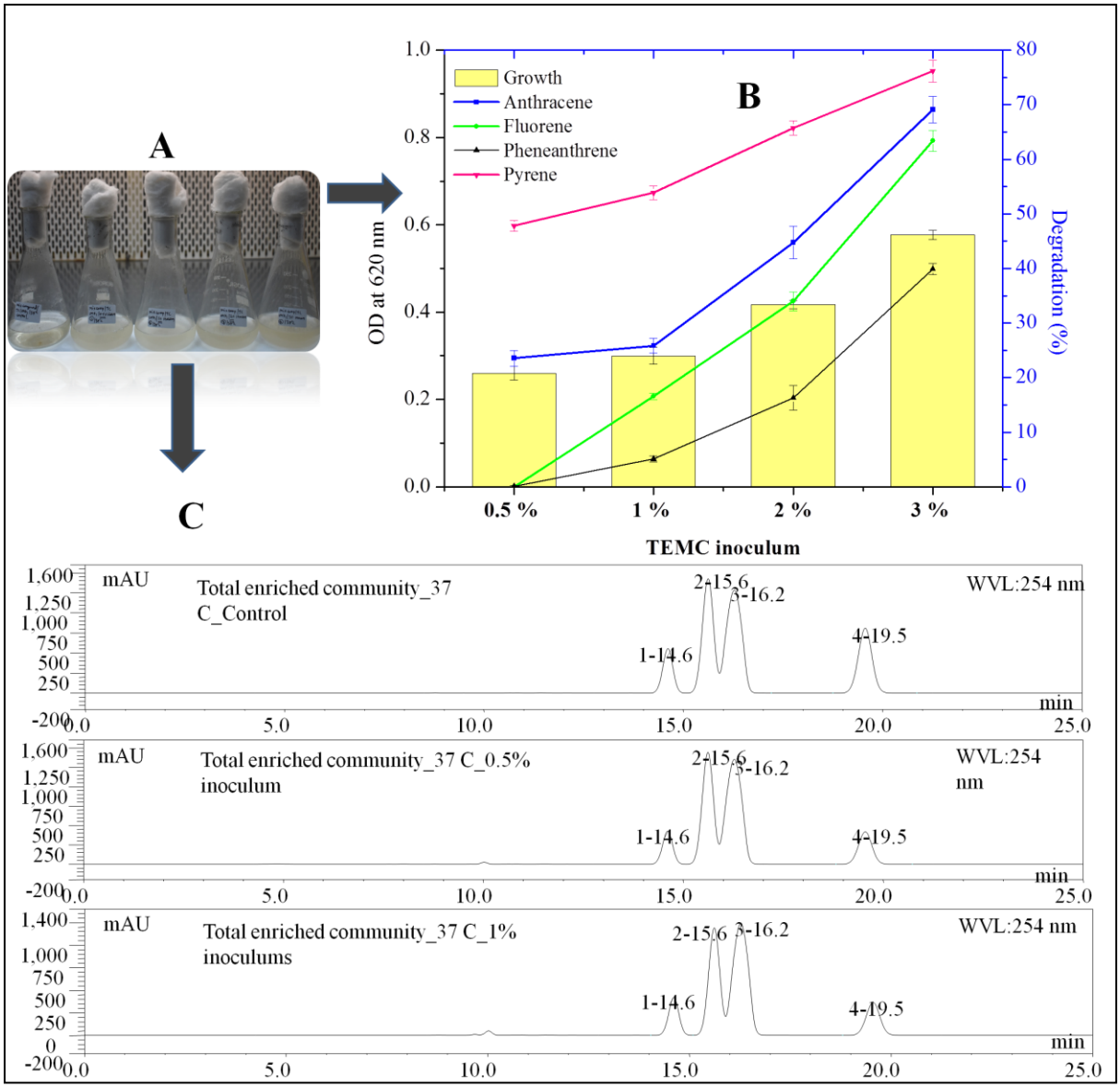


Fig.4.13: Degradation of mixed PAH compounds using different inoculums of TEMC at 37ºC **A:** Flasks showing the growth of cultures, **B:** Graph showing the growth and degradation of mixed PAHs, **C:** Representative HPLC chromatogram of degradation analysis

Table 4.2: Degradation analysis using total microbial community

Inoculums	Growth	Anthrecene	Fluorene	Phenanthrene	Pyrene
0.5%	0.26 ± 0.01	23.57 ± 1.42	ND	ND	47.81 ± 1.00
1%	0.29 ± 0.01	25.87 ± 1.37	16.5 ± 0.58	5.06 ± 0.55	53.87 ± 1.26
2%	0.41 ± 0.09	44.70 ± 3.06	33.98 ± 1.73	16.29 ± 2.24	65.75 ± 1.31
3%	0.57 ± 0.01	69.11 ± 2.39	63.38 ± 1.92	39.93 ± 1.01	76.19 ± 1.99

4.3.5: Strategies for enhancing degradation

Although it is important to select proper microbial strains for biodegradation of PAHs, however, the efficiency could be increased by implementing a proper strategy. Several strategies are being developed to make biodegradation a successful technology particularly in soils without degrading indigenous microorganisms. In these approaches, several techniques such as the use of microbial consortia, developments of genetically engineered strains as well as the use of various carriers are being used (Mrozik and Piotrowska-Seget, 2010). Here, we have used a carrier, which could also help to increase the bioavailability of PAHs to the microbial strain for more effective degradation with increased rates.

4.3.5.1: Enhancement by biochar amended mixed cultures

Degradation of mixed PAHs was found significantly higher at the 50ºC temperature set using a mixed culture approach. Therefore, to further enhance the degradation rates of this set, an addition of biochar was performed. Biochar is known to enhances the degradation of PAH compounds, it could act as a sorbed carrier for PAH compounds and therefore increases the availability of PAH compound molecules to the degrading organism (Kong et al. 2018). Biochar prepared from the solid agricultural waste could be more useful as it could also solve the agricultural waste problems, which ultimately helps in the reduction of environmental

pollutions. Here, biochar was prepared using one of the agricultural waste product *i.e.* corncob. Biochar was prepared using slow pyrolysis (Fig. 4.14) and used for degradation studies.

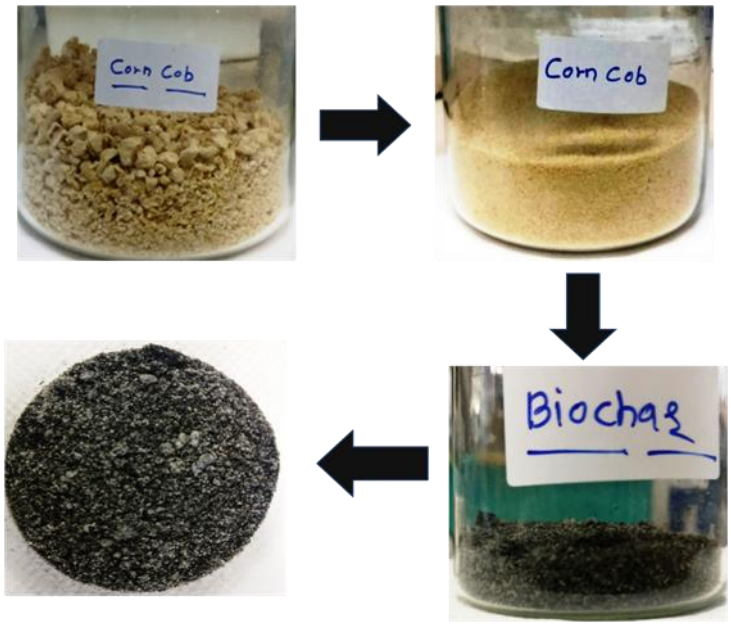


Fig. 4.14: Preparation of biochar from the agricultural waste product, corncob using slow pyrolysis method

Biochar could act as a strong sorbent which could increase the adsorption of PAHs compounds. At a high temperature, biochar shows high sorption capacity, therefore, could increase the bioavailability of PAH compounds to the molecules (Sigmund et al. 2018). In this experiment, with the 0.5% (w/v) biochar was used for enhancement of PAHs degradation. The result showed that, around 64.053 ± 2.38 of anthracene, 65.30 ± 5.10 of fluorene, 52. 28 ± 0.050 of phenanthrene and 76.56 ± 6.83 percentage of pyrene degradation has occurred within 15 days (Fig. 4.15). These results are surprising, as with the addition of biochar, the degradation of anthracene and fluorene was decreased and an increase in the degradation of phenanthrene and pyrene (HMW) was observed. This indicates that biochar could significantly increase the bioavailability of HMW PAHs in the aqueous medium.

However, it was also possible that biochar could adsorb differently the PAHs, reducing the bioavailability of LMW PAH in the solution.

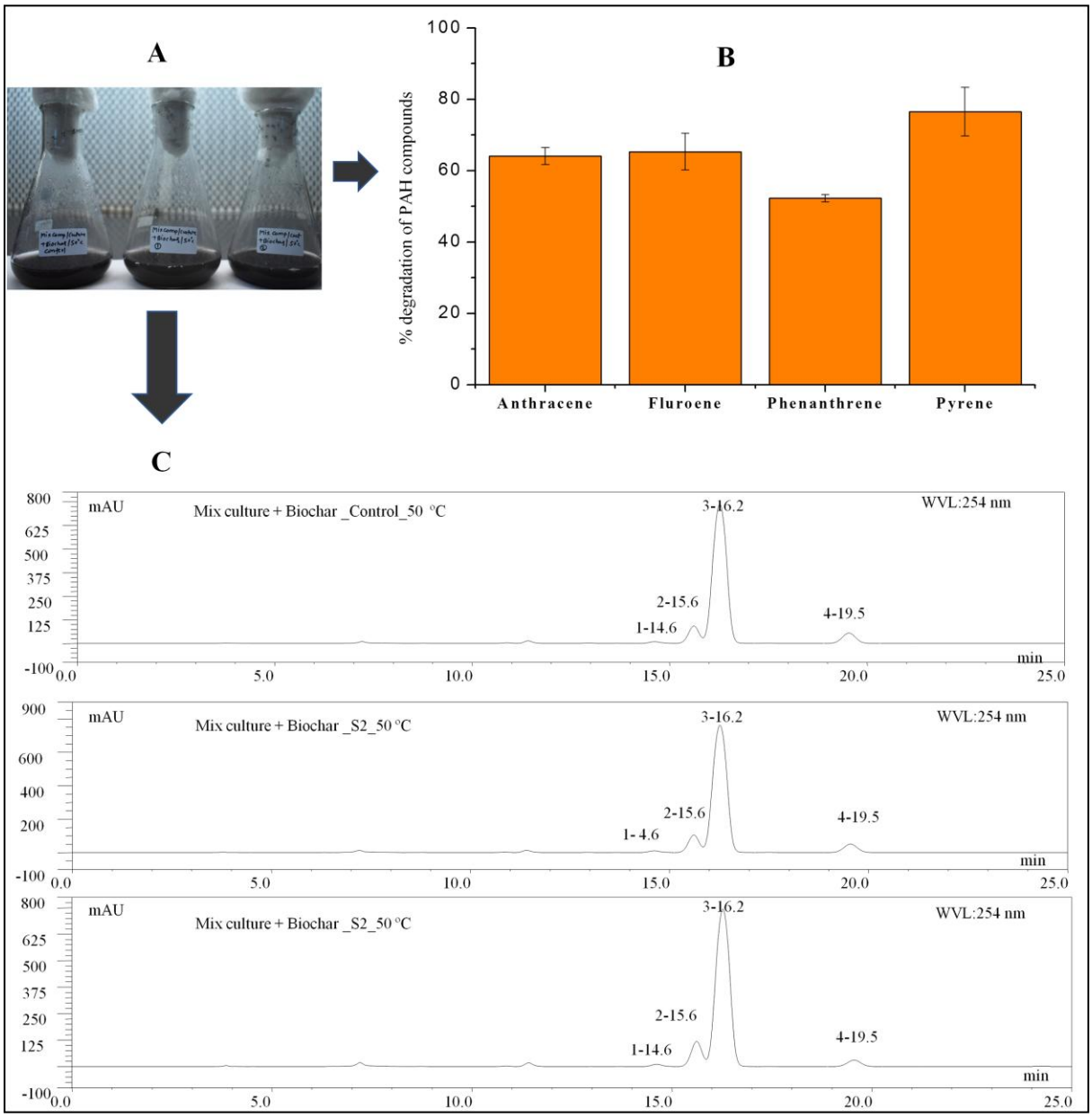


Fig.4.15: Degradation of mixed PAH compounds using biochar amended mixed culture at 37ºC **A:** Flasks showing the added biochar, **B:** Graph showing the degradation percentage of mixed PAHs, **C:** Representative HPLC chromatogram of degradation analysis.

4.3.6: Comparative account of biodegradation strategies

A comparative analysis of the degradation of mixed PAHs using different biodegradation strategies used in this study was performed. Analysis of degradation of each compound in the specified degradation strategy is shown table 4.3 and figure 4.16.

Table 4.3: Comparative account of PAHs degradation using biodegradation strategies

Strain	Anthracene	Fluorene	Phenanthrene	Pyrene
UCPS2	92.02 ± 0.15	83.77 ± 1.2	16.64 ± 0.57	51.66 ± 1.34
Mixed culture	96.30 ± 0.64	86.75 ± 2.26	54.26 ± 6.06	71.48 ± 1.33
TEMC	69.11 ± 2.39	63.38 ± 1.92	39.93 ± 1.01	76.19 ± 1.99
MCBC	64.05 ± 2.38	65.30 ± 5.10	52.28 ± 1.05	76.56 ± 6.83

The analysis revealed that along with the temperature, chemical nature of compounds, their bioavailability and stress of mixture of compounds as well as nature and number of microbial strains affect biodegradation processes. Degradation at 50ºC either by a single strain or in a mixed culture showed the highest degradation of LMW PAHs (Anthracene and fluorene). However, the addition of sorbing material *i.e.* biochar has affected their degradation rates with contracting observations. Here, HMW PAHs degradation was enhanced and degradation of LMW PAHs was reduced. Similarly, at 37ºC, degradation of PAHs using TEMC had shown an increase in the degradation of HMW PAHs. A comparison of individual strains, mixed culture, Biochar amended culture (MCBC) and TEMC irrespective of temperatures shows that in the TEMC and MCBC, a relative increase in the degradation of HMW PAHs, and a decrease in LMW PAHs was observed (Fig. 4.16). Such contracting results may be due to the differential solubility of LMW and HMW PAH compounds. More interestingly, among

the TEMC and MCBC, no significant difference was observed for overall mixed compound degradation. This is maybe because, TEMC contents various unknown microorganism, most of could not be cultured would be responsible for the production of extracellular substances which ultimately increases the solubility of PAH compounds. In general, HMW PAH compounds are less soluble and therefore remained persistence in the environments. Therefore, they are of great concern as compared to LMW PAHs. It, therefore, implies that the use of such sorbed carrier for removal of PAHs from contaminated environments would be recommended.

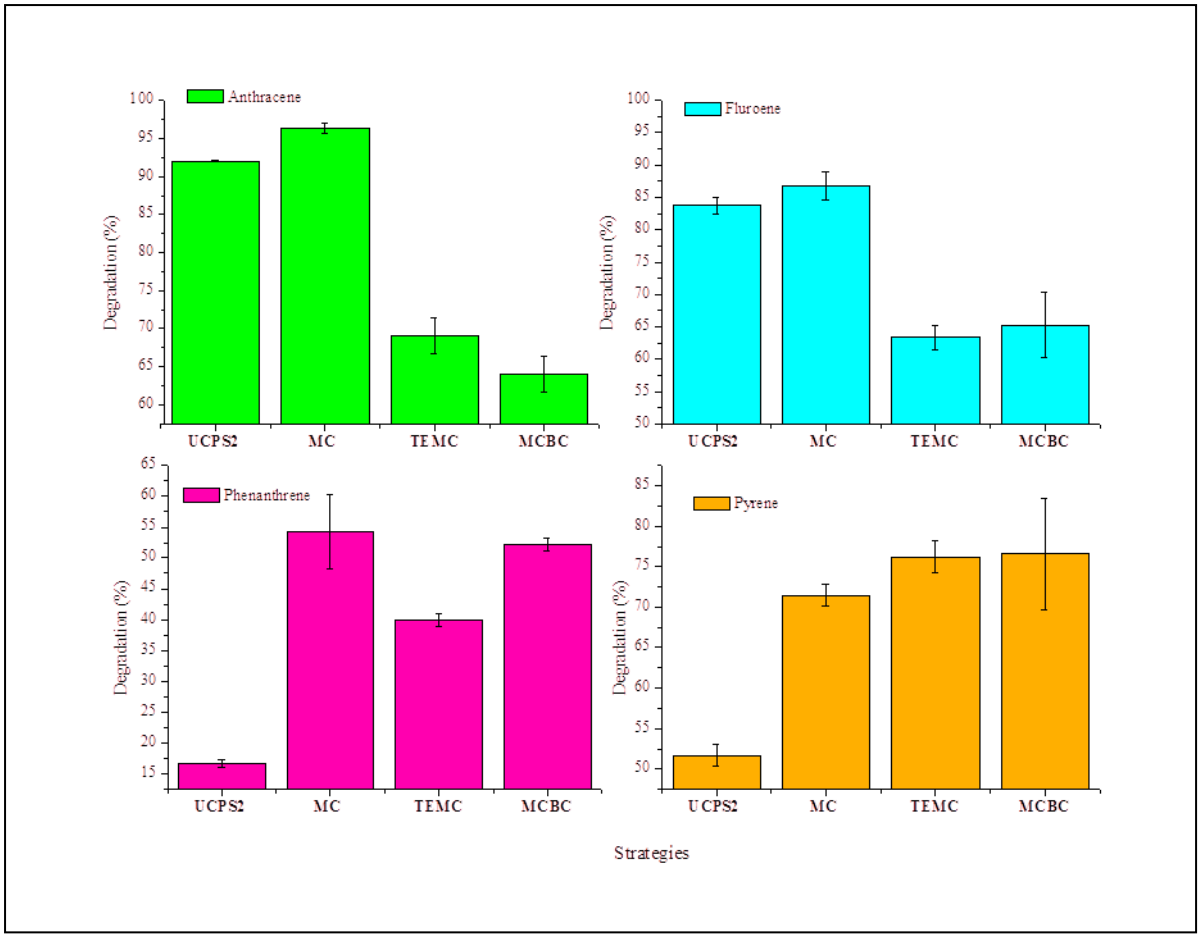
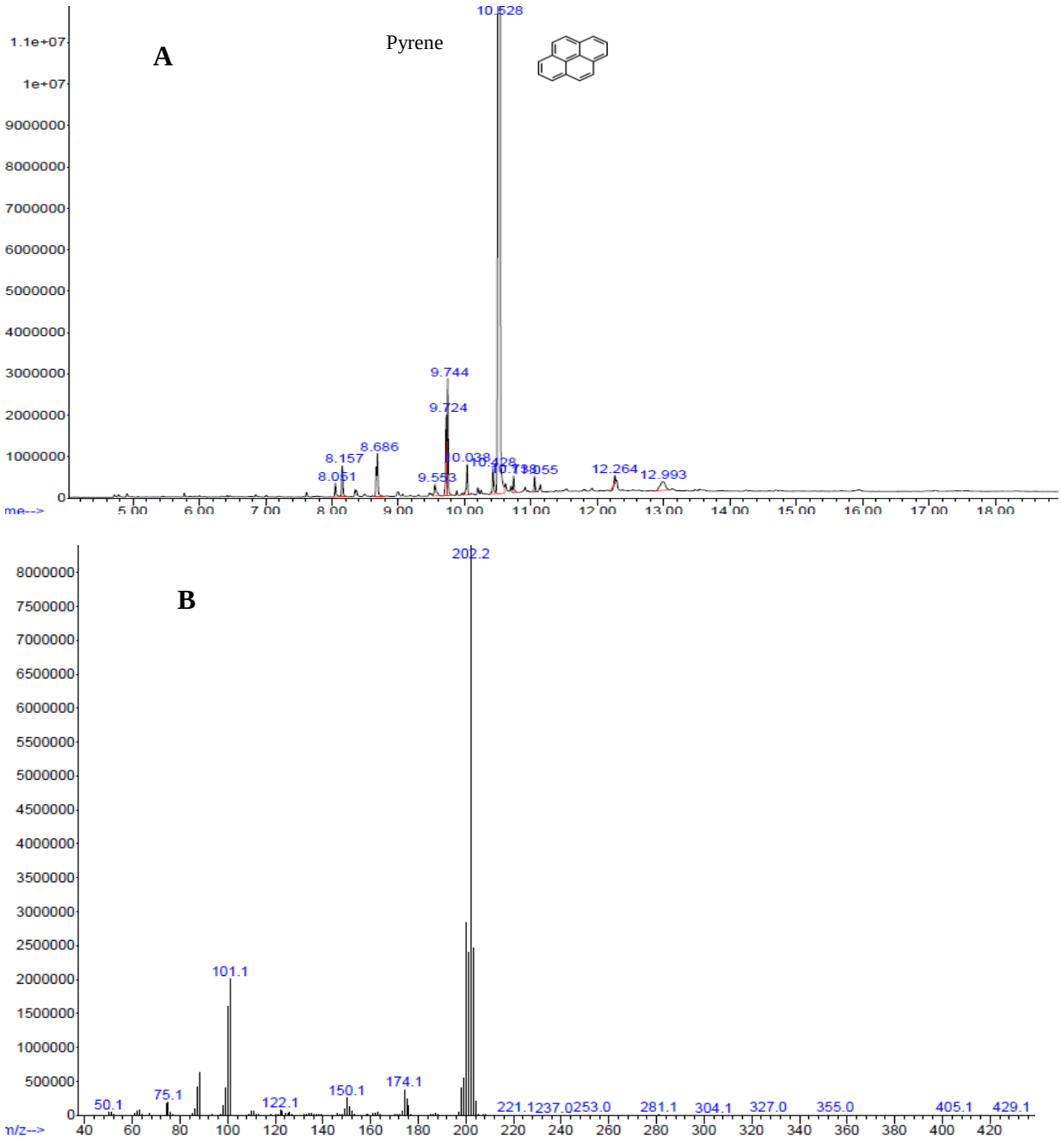


Fig.4.16: Degradation pattern of four PAHs, when used in a mixture of compounds by different degradation strategies.

4.3.7: Analysis of intermediate metabolites and pathways prediction

For the detection intermediates metabolites of pyrene degradation, GC-MS analysis was performed along with the standard compound (Fig.4.17A, B). In this analysis two different intermediate metabolites *i.e.* 9-methoxyphenanthrene (m/z ratio of 208) at retention time of 10.42 minutes and phenanthrene 4, 5-dicarboxylic acid (m/z ratio of 222) at retention time of 12.976 minutes were observed (Fig.4.17C, D).



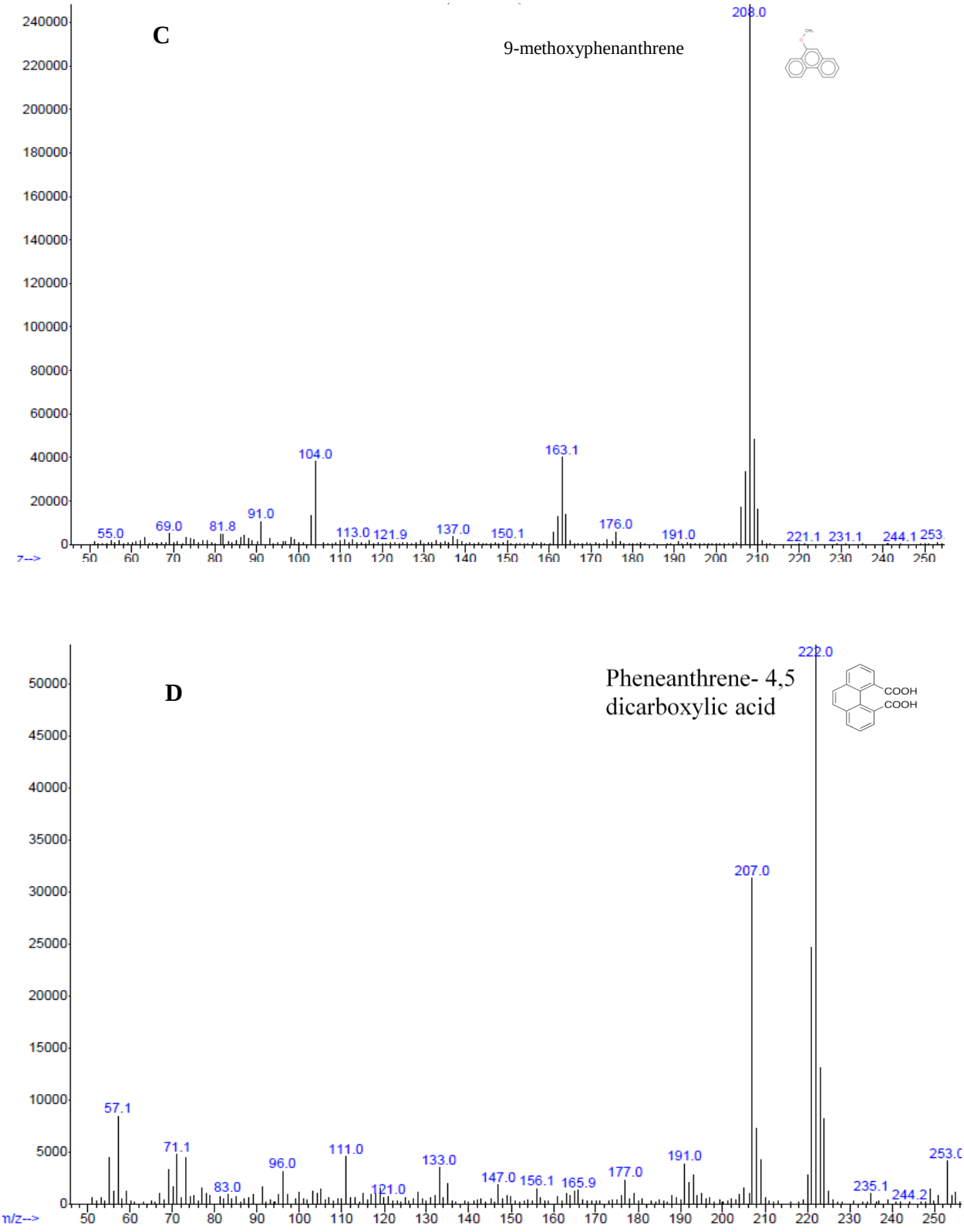


Fig. 4.17: Detection of pyrene degradation intermediate metabolites using GC-MS analysis (A and B: Pyrene with Rt., C and D, Intermediate metabolites)

In general, bacteria favour aerobic conditions for degradation of PAHs via oxygenase-mediated metabolism (involving either monooxygenase or dioxygenase enzymes). Usually, the first step in the aerobic bacterial degradation of PAHs is the hydroxylation of an aromatic ring via a dioxygenase, with the formation of a cis-dihydrodiol, which gets rearomatized to a diol intermediate by the action of a dehydrogenase, leading to intermediates such as catechols that are ultimately converted to TCA cycle intermediates (Ghoshal et al. 2016; Mallick et al. 2011). Bacteria can also degrade PAH via the cytochrome P450-mediated pathway, with the production of trans-dihydrodiols (Moody et al. 2004). Based on these intermediate metabolites, it was observed that the 9-Methoxyphenanthrene was thought to be an important intermediate product also reported previously studied *Bacillus* sp. (Khanna et al. 2011). Therefore, the strains might be using the cytochrome mediated route for degradation of PAH compounds (Fig. 4.18).

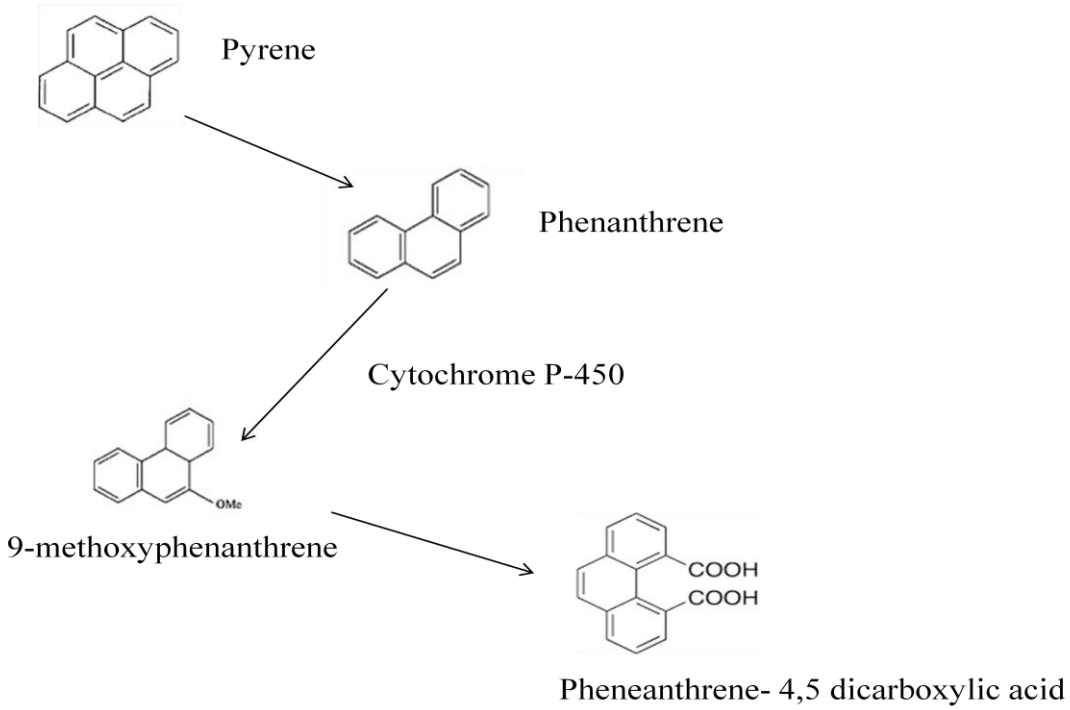


Fig. 4.18: Predictive pathway of pyrene degradation using *Bacillus* sp. (U277)

4.3.8: Degradation of PAHs in crude oil

The degradation of PAHs either as a single or a mixture of compounds could be achieved using efficient microbial strains. In the natural source, degradation of these compounds might be difficult as several other factors could affect their degradations. Crude oil is the major source of these compounds for the environmental contaminations. Therefore degradation of these compounds in crude oil could be more significant for the development of effective tools for bioremediations. Here, degradation of PAH compounds directly in the crude oil was analyzed. For knowing their *Rt* and separation profile of PAHs, method development was performed as described previously using HPLC. All the four PAHs were identified based on their separation profile and retention time (*Rt*) using a well-developed method (Fig. 4.19).

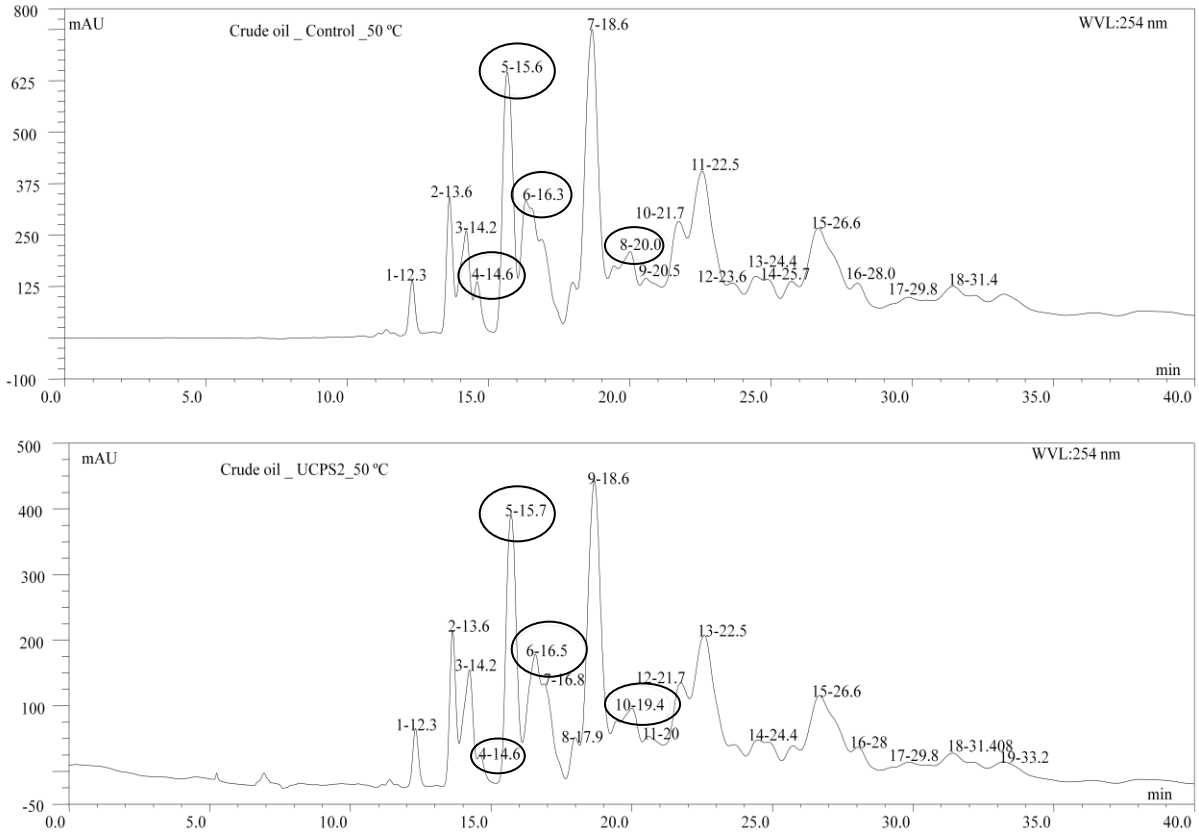


Fig. 4.19: HPLC chromatogram of degradation analysis of PAH compounds in crude oil showing degradation of anthracene, fluorene, phenanthrene and pyrene

A similar type of degradation pattern was observed as previously observed in case of mixed compound degradation analysis. However, in this case, comparative to that of mixed compound degradation strategies, less degradation of both LMW and HMW PAHs was observed (Fig. 4.20).

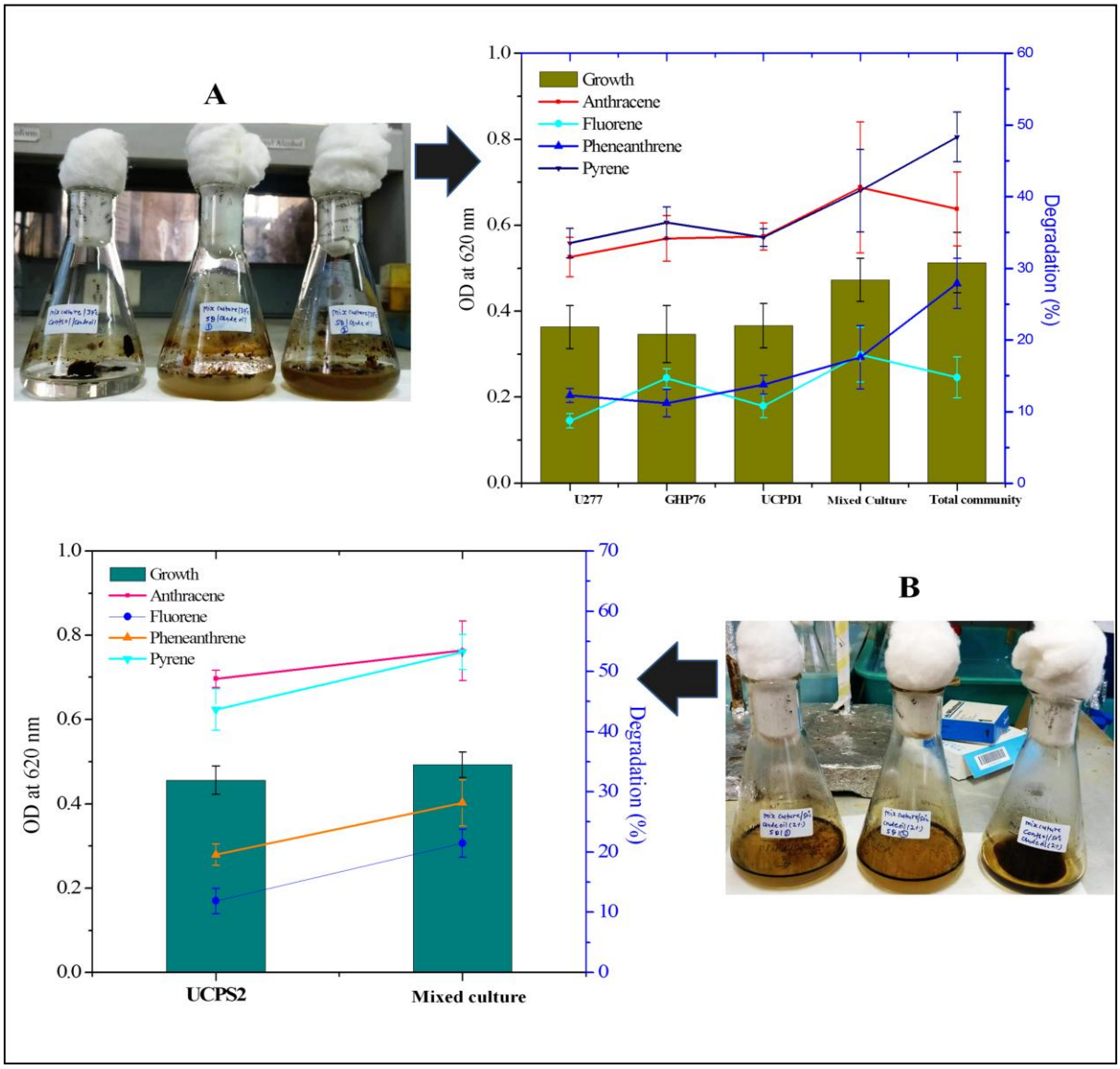


Fig. 4.20: Degradation of mixed PAH compounds in crude oil using individual and mixed cultures

Overall highest degradation has occurred in a mixed culture at 50ºC, however not any significant difference between the degradation of LMW and HMW PAHs was observed

(Table 4.4). The study infers that, although microbial mediated degradation of PAHs could occur with higher rates when used as a single or mixed compound. However, in a complex mixture of compounds present in crude oil affect the overall degradation process. As, crude oil also contains some aliphatic compounds, which are more soluble than PAHs and also easy to degrade, therefore microbial strains might be preferring such compounds, which has rendered the degradation of PHAs in crude oil.

Table 4.4: Degradation analysis of mixed PAH compounds in crude oil

Strain	Growth	Anthracene	Fluorene	Phenanthrene	Pyrene
U277	0.36 ± 0.05	31.58 ± 2.73	8.73 ± 1.01	12.24 ± 0.95	33.51 ± 2.06
GHP76	0.34 ± 0.06	34.18 ± 3.21	14.67 ± 1.33	11.19 ± 1.91	36.37± 2.19
UCPD1	0.36 ± 0.05	34.45 ± 1.88	10.77 ± 1.59	13.75 ± 1.29	34.3 ± 1.24
Mixed culture	0.47 ± 0.05	41.28 ± 9.14	17.95 ± 3.78	17.59 ± 4.41	40.84 ± 5.78
Total community	0.51 ± 0.07	38.28 ± 5.14	14.75 ± 2.88	27.89 ± 3.51	48.34 ± 3.48
UCPS2	0.45 ± 0.03	48.78 ± 1.43	11.89 ± 2.11	19.59 ± 1.75	43.67 ± 3.45
Mixed culture	0.49 ± 0.03	53.45 ± 4.94	21.47 ± 2.29	28.17 ± 3.81	53.21 ± 2.9

4.3.9: Screening for biosurfactants

The strains used in PAHs degradation studies were also evaluated for BS production. Biosurfactants are also known to increases the solubility of PAHs, therefore these strains might be producing such extracellular substances to solubilise and use PAHs efficiently.

Selected strains were grown in nutrient broth and the supernatant was used for detection of BS using different methods.

4.3.9.1: Drop collapse and oil spreading methods

The biosurfactant production ability was tested using drop collapse and oil spread methods. Based on both of these screenings, all the four strains (U277, GHP76, UCPD1 and UCPS2) were found positive for BS production as shown in figure 4.21. To further characterized, the supernatant obtained from these strains was also evaluated for surface tension reduction measurements.

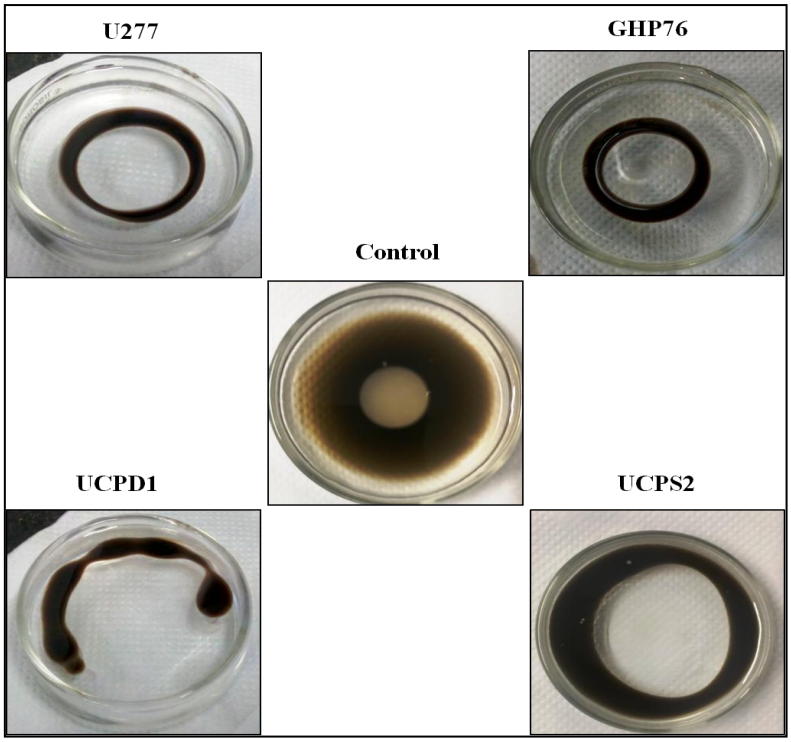


Fig.4.21: Oil displacement method showing the displacement of crude oil by BS produced by positive strains.

4.3.9.2: Surface tension measurement

Biosurfactant production efficiency was also evaluated by measuring the reduction in surface tension using a tensiometer. The surface tension reduction potential of the cell-free supernatant obtained from the positive strains was measured along with control as shown in

table 4.5. All these four bacterial strains showed a reduction of surface tension <45 mN/m, indicating their strong surface tension reduction potential.

Table 4.5: Surface tension reduction measurement of biosurfactant

Strain No	ST reduction
Control (Water)	72.8 dyne/cm
U277	30.65 dyne/cm
GHP76	26.75 dyne/cm
UCPD1	28.20 dyne/cm
UCPS2	27.31 dyne/cm

4.3.10: Production and purification of biosurfactants

4.3.10.1: Fermentation and process parameters

All the positive strains were used for production and characterization of BS. Production was carried out in a 3L fermentor under optimized conditions. For three strains of Bacillus sp (U277, GHP76, and UCPD1) fermentation was performed at 37ºC, while for UCPS2 strain, the temperature was set to a 60ºC (Fig. 4.22).

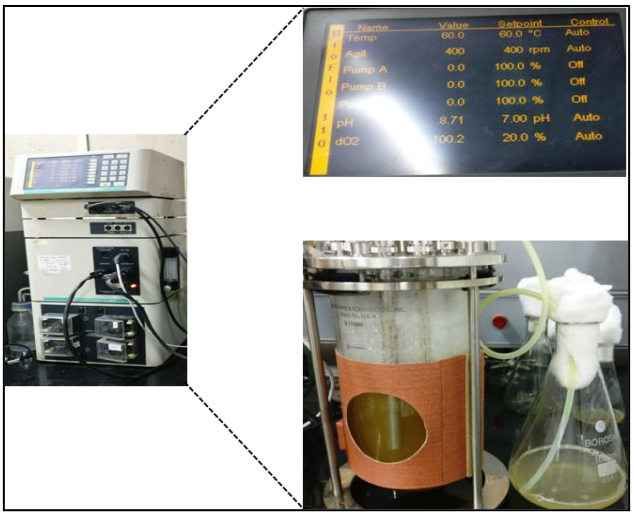


Fig. 4.22: Biosurfactant production using a 3L fermentor, showing process parameters

4.3.10.2: Extraction and purification

The extraction of BS from fermentation broth was performed by acidification followed by extraction with Chloroform: Methanol (2:1) solvent. The obtained crude extract was dehydrated by passing through the anhydrous sodium sulfate, freeze-dried and weight was measured. The yield of biosurfactant was calculated as the production of crude biosurfactant in one liter of fermentation media as shown in the table 4.6. The yield for GHP76, UCPS2 was relatively higher as compared to other strains.

Table 4.6: Yield of crude biosurfactant obtained after freeze-drying

Sr. No	Name of the strain	Yield (gm/lit)
1	U277	0.56
3	GHP76	0.72
4	UCPD1	0.68
5	UCPS2	0.91

The crude biosurfactant was dissolved in methanol and used for purification using reverse phase HPLC (RP-HPLC) in a preparative mode. Method development and optimization were carried out by adjusting the solvent gradient program and solvent flow rates. Initially, a fraction of major peaks observed at the retention time 13.2, 32.2 and 33.5 (Fig. 4.23A) were collected and the fractions showing positive activity were considered for further processing. The fraction eluted as 33.5 retention time has shown the positive activity in drop collapsed methods. Therefore, for elution of this fraction, further optimization in the method was done and this fraction was collected repeatedly using high injection volume. The purified fraction was further run using the same program, which has shown the appearance of the single major peak (Fig. 4.23B). The purified biosurfactant obtained by this method was used for characterization.

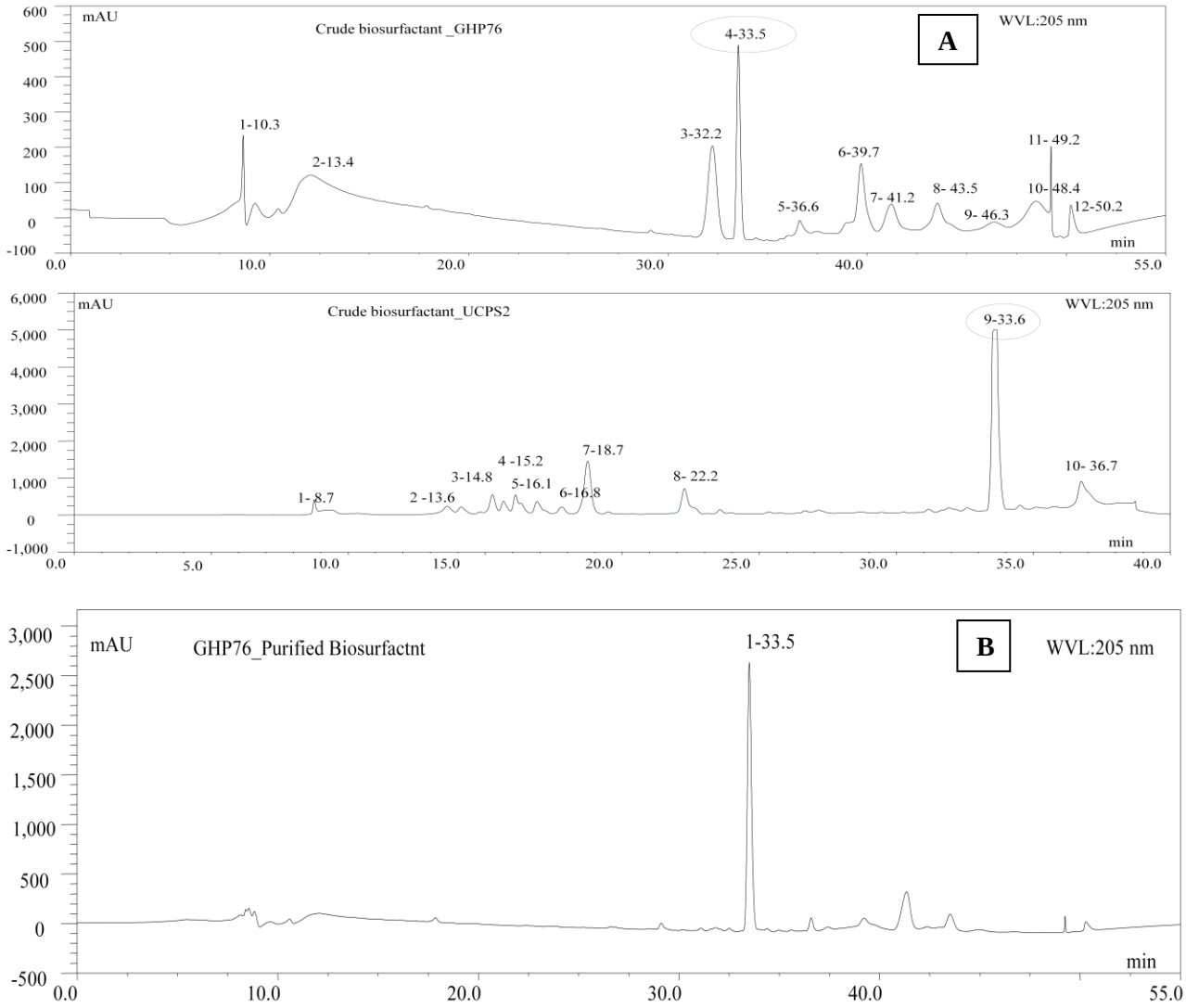


Fig. 4.23: HPLC chromatogram of crude (A) and purified (B) biosurfactant

4.3.11: Characterization of biosurfactant

The purified BS produced by bacterial strains was characterized using various analytical tools. In addition, molecular based characterization by sequencing and analysing the BS producing genes from the bacterial strains was also done.

4.3.11.1: Using molecular tools

PCR based identification of lipopeptide genes was performed. PCR amplification was done for four lipopeptides (Fengycin, Baccilomycin D, Iturin A and Surfactin). Based on PCR identification of lipopeptide producing genes, all the strains were found positive for surfactin,

while GHP76 was found positive for both surfactin as well as inturin producing genes (Fig. 4.24).

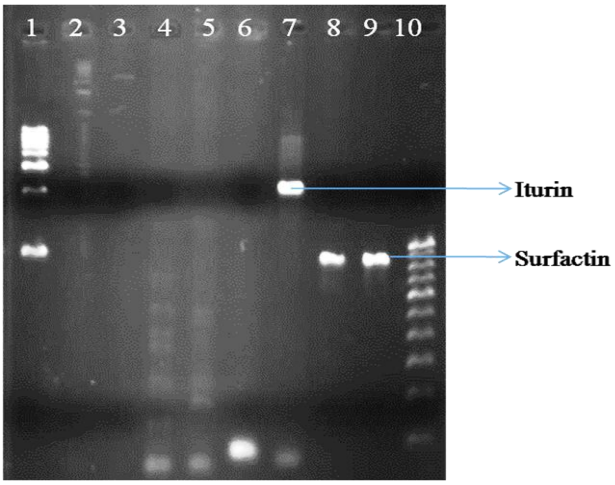


Fig. 4.24: Image of agarose gel electrophoresis showing the presence of surfatin and iturin genes in positive strains.

PCR products were purified and used for sequencing analysis, sequencing data was analysed by blastN and blastX with closest cultured sequences retrieved from the NCBI database (Altschul et al. 1990) as shown in table 4.7. The strain U277 shows highest sequence similarity (94%) with surfactin biosynthetic gene of *Bacillus subtilis* species, where as the strain GHP76 shows 99% with *Bacillus amyloliquificians* species. For the Iturin biosynthesis gene, the strain GHP76 had shown 94% similarity with *Bacillus subtilis* species. In the Phylogenetic analysis by neighbour-joining both the strains cluster with *Bacillus subtilis* species (Fig. 4.25).

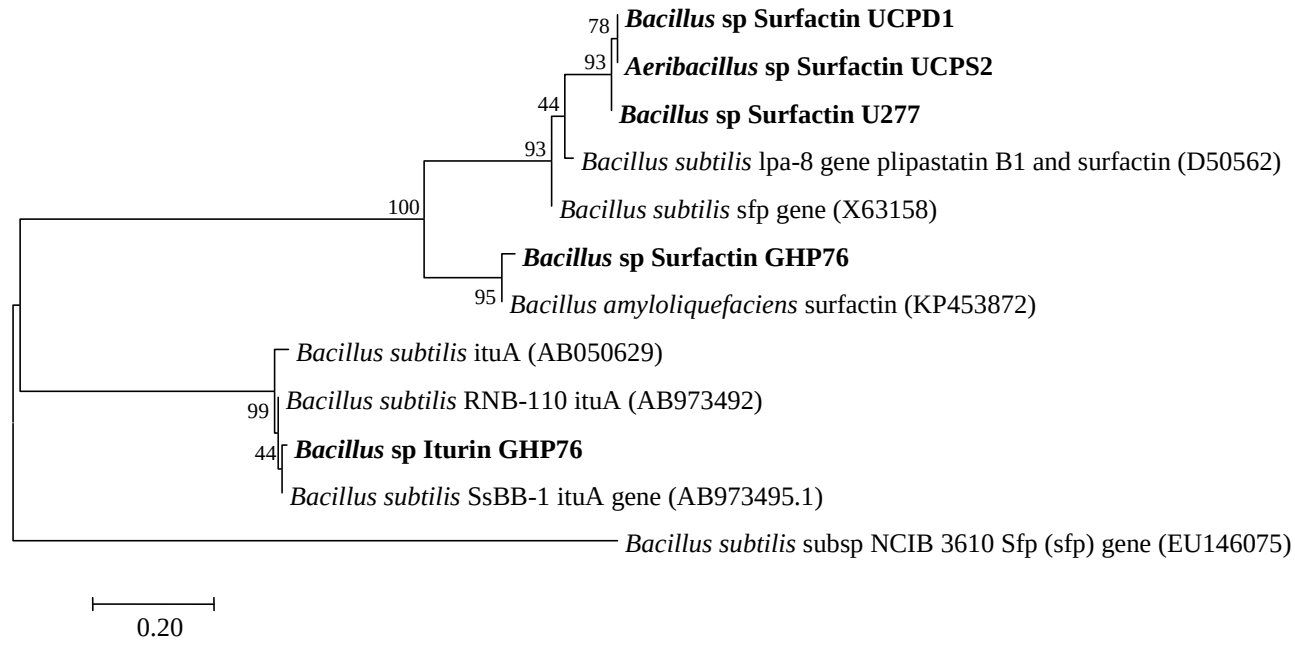


Fig. 4.25: The phylogenetic tree analysis of nucleotide sequences of Surfactin (*sfp)* gene and *Iturin (Itu A)* using neighbor-joining method with 500 bootstrap replicates. Scale bar represents the differences in conserved region of the gene between organisms and sequences obtained in the present study shown in bold with accession numbers.

Table 4.7: Sequences similarities of the biosurfactant synthesizing (BS) genes with NCBI BLASTN and BLASTX databases

Sr. No	Strain Name	Gene	BLASTn Identity	tBLASTx Identity	References
	Bacillus sp. (U277)	Surfactin	*Bacillus subtilis* subsp. *inaquosorum* strain CT43_2 sfp gene (KX099384) Identity: 582/616(94%)	*Bacillus subtilis* subsp. *inaquosorum* strain CT43_2 sfp gene (KX099384) Identity: 582/616(94%)	-

1	*Aeribacillus sp.* (UCPS2)	Surfactin	*Bacillus subtilis* lpa-8 gene essential for biosynthesis of the lipopeptide antibiotics *plipastatin* B1 and *surfactin* (D50562) Identity: 530/561(94%)	*Bacillus subtilis* lpa-8 gene essential for biosynthesis of the lipopeptide antibiotics *plipastatin* B1 and *surfactin* (D50562) Identity: 179/186 (96%)	Tsuge et al. 1996
2	*Bacillus sp.* (UCPD1)	Surfactin	*Bacillus subtilis sfp* gene (X63158) Identities: 516/547(94%)	*Bacillus subtilis sfp* gene (X63158) Identity: 172/181(95%)	Nakano et al. 1992
3	*Bacillus sp.* (GHP76)	Surfactin	*Bacillus amyloliquefaciens surfactin* synthetase gene (KP453872) Identity: 586/592 (99%)	*Bacillus amyloliquefaciens surfactin* synthetase gene (KP453872) Identities: 193/196 (98%)	Ben Abdallah et al. 2015
		Iturin	*Bacillus subtilis ituA* gene (AB050629) Identity: 678/735 (92%)	*Bacillus subtilis ituA* gene (AB050629) Identity: 214/244 (88%)	Tsuge et al. 2001

4.3.11.2: Using analytical tools

The molecular analysis revealed that the biosurfactant producing strains shows the presence of surfactin genes. Therefore, the biosurfactant produced by these strains must be a surfactin. However, it further confirmed it by analytical tools; a standard surfactin was procured from Sigma for a comparative analysis. All the analytical analysis was then performed using surfactin as a standard compound.

4.3.11.2.1: Thin layer chromatography

A thin layer chromatography (TLC) of purified BS was performed. The results showed a prominent single band similar to that of standard surfactin after staining with ninhydrin

(Fig.4.26). Thin layer chromatogram of purified BS of all the strains had an *Rf* value of 0.69 (band/solvent front ratio).

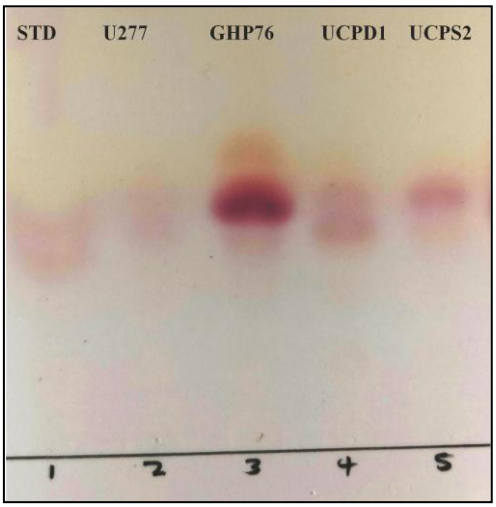


Fig. 4.26: TLC analysis of the purified BS along with the standard (Surfactin, Sigma), showing a prominent single band.

4.3.11. 2.2: FTIR analysis

Characterizations of the purified BS produced by these strains were performed using FTIR-ATR analysis. The spectra obtained for *Aeribacillus* sp. (UCPS2) as well as from the standard surfactin showed a great similarity between them (Fig. 4.27).

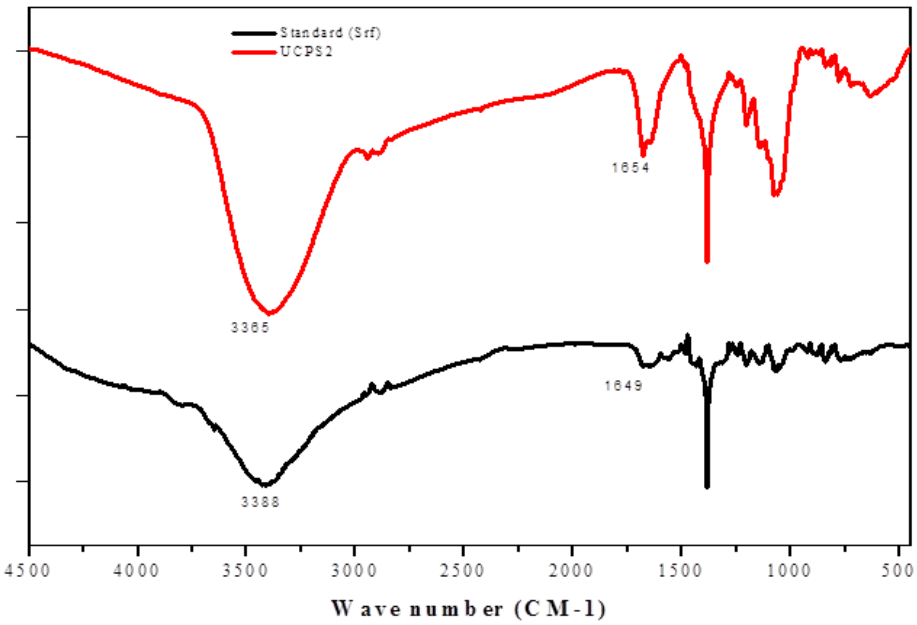
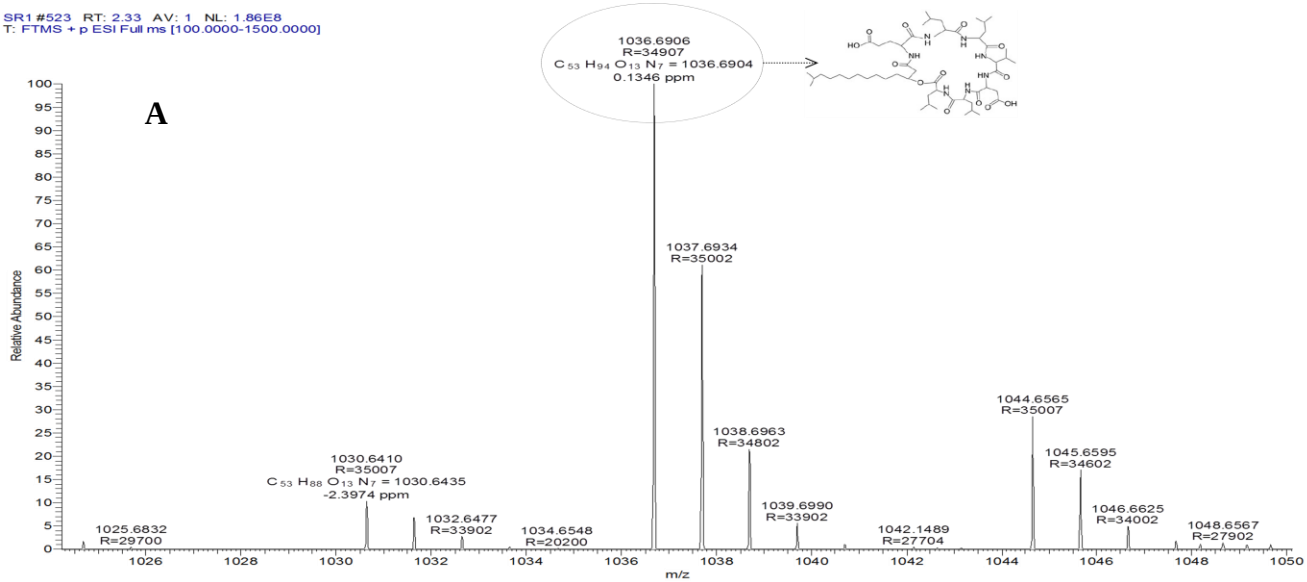


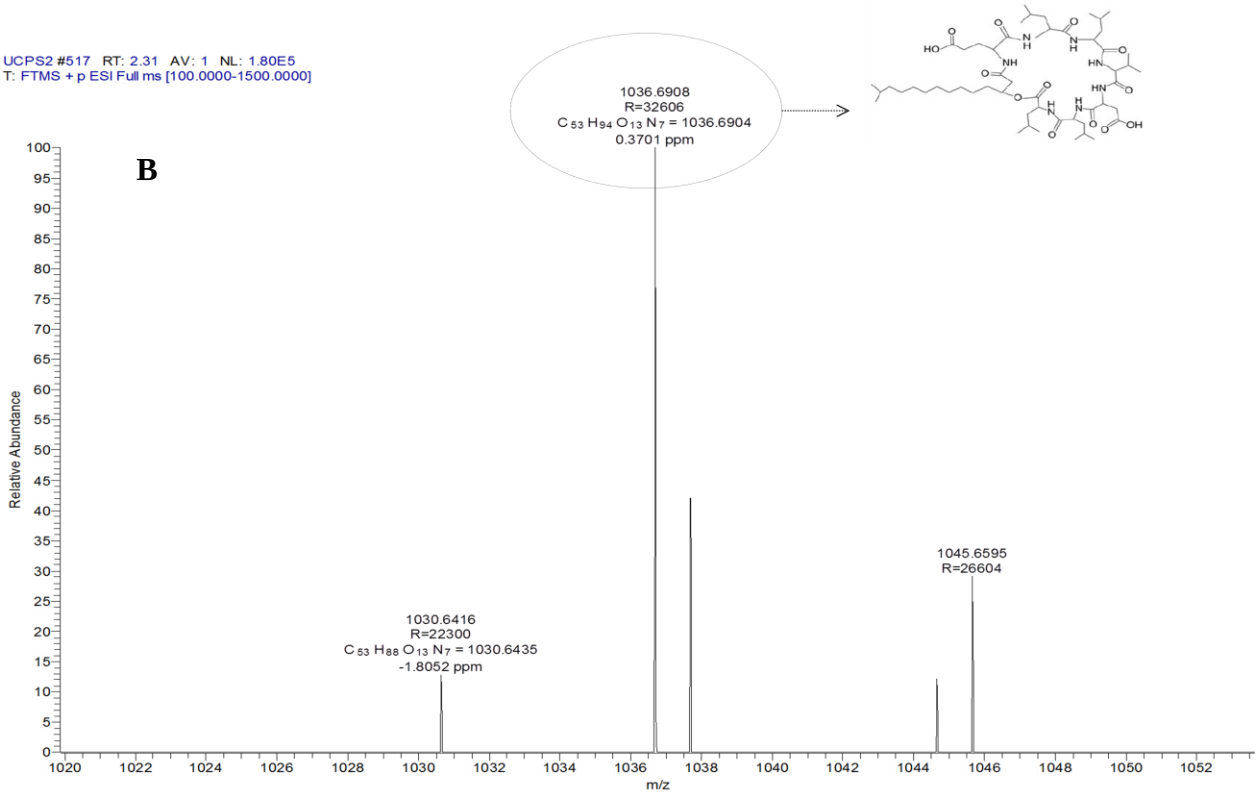
Fig. 4.27: FTIR analysis of purified BS from *Aeribacillus* sp. (UCPS2) strain.

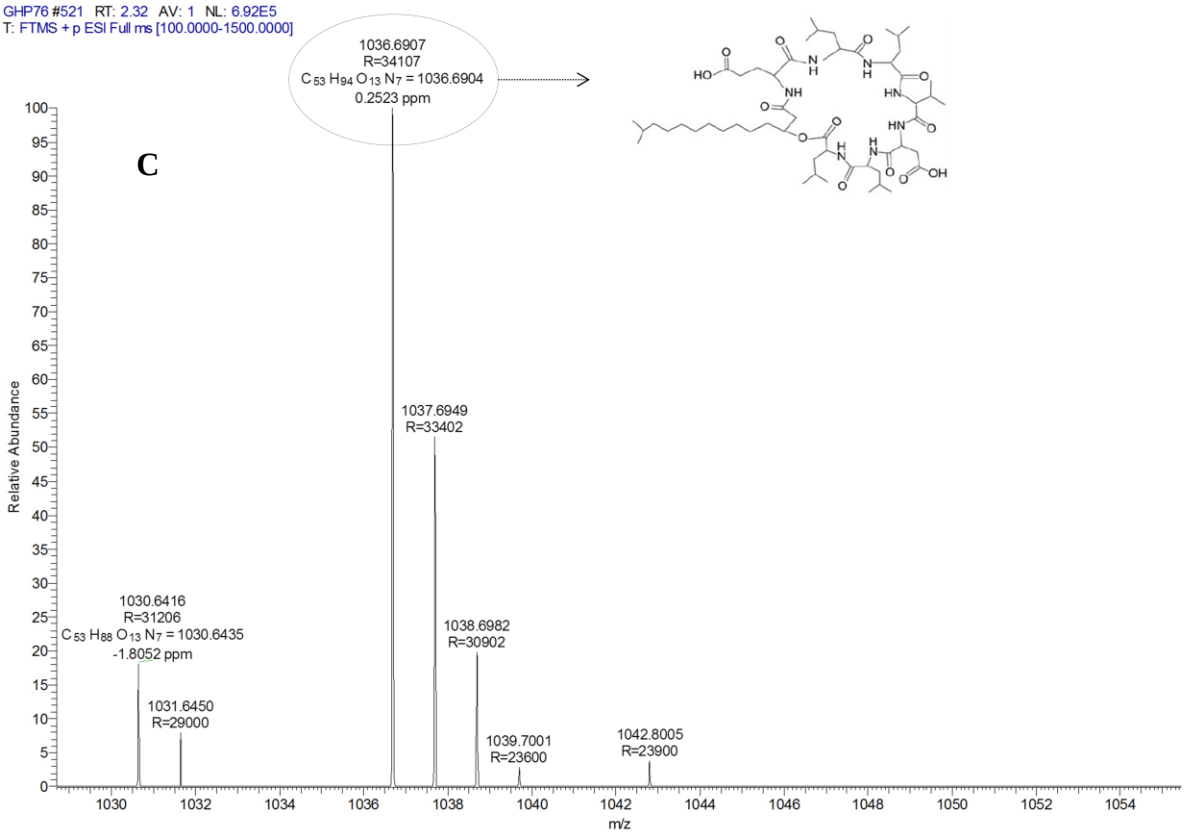
The FTIR spectrum of the UCPS2 strain has shown similarity with the surfactin compounds used as a standard. Both the spectra have displayed strong absorbing bands which are consistent with the presence of peptide and fatty acid components. The stretching modes at 3366-3388 and 1649-1654 cm^{-1} are related to N–H and CO–N bond, respectively. Based on these analyses, the chemical nature of HPLC purified BS revealed the presence of peptide indicated their lipopeptide nature.

4.3.11.2.3: HRMS analysis

The purified BS was analyzed using HR-MS, positive scans of the HRMS chromatogram of all the strains are shown in figure 4.28 (A-D). In the chromatogram of standard surfactin different variant of surfactin homologs were observed in all the strains. All the peaks were found to correspond to sodiated molecules $[M^{+}Na]^{+}$ m/z 1030, 1036, 1037 and 1044 in positive modality. At the same retention time, a similar pattern of homologs peaks having the same $[M^{+}Na]^{+}$ m/z was observed in all strains. The results confirmed that the BS produced by all the strains was surfactin in the form of four homologs that corresponded to surfactin generally produced by various *Bacillus* species.




UCPS2 #517 RT: 2.31 AV: 1 NL: 1.80E5
T: FTMS + p ESI Full ms [100.0000-1500.0000]
B
1036.6908
R=32606
C 53 H94 O13 N7 = 1036.6904
0.3701 ppm
1030.6416
R=22300
C 53 H88 O13 N7 = 1030.6435
-1.8052 ppm
1045.6595
R=26604
Relative Abundance
m/z


GHP76 #521 RT: 2.32 AV: 1 NL: 6.92E5
T: FTMS + p ESI Full ms [100.0000-1500.0000]
C
1036.6907
R=34107
C 53 H94 O13 N7 = 1036.6904
0.2523 ppm
1037.6949
R=33402
1030.6416
R=31206
C 53 H88 O13 N7 = 1030.6435
-1.8052 ppm
1038.6982
R=30902
1031.6450
R=29000
1039.7001
R=23600
1042.8005
R=23900
Relative Abundance
m/z

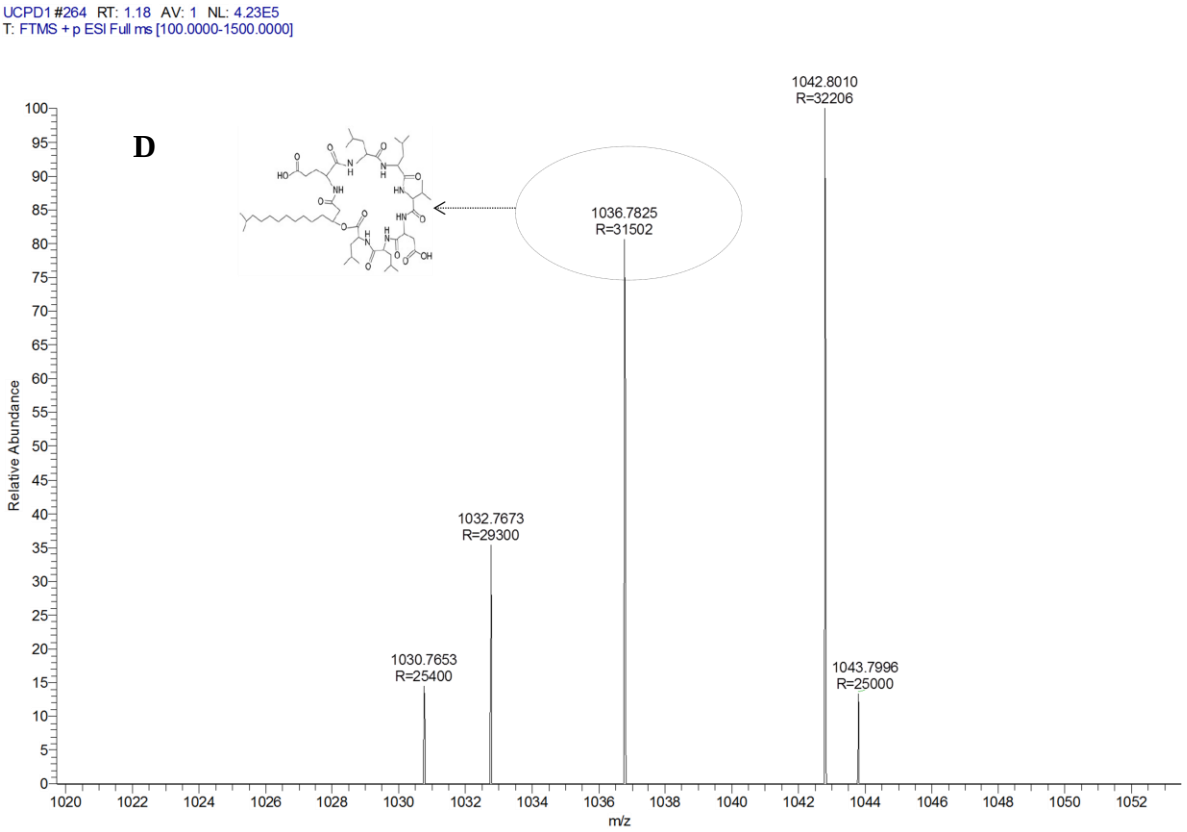


Fig. 4.28: HRMS profile of purified biosurfactant produced by positive strains

4.3.12: Activity assay: Emulsification index

The BS produced by these strains was evaluated for its emulsification activity index against various immiscible compounds including some hydrocarbons. The BS from all the strains has shown high emulsification activity especially against crude oil, and heavy oils such as vacuum oil, engine oils. With hydrocarbons such as benzene, xylene, hexane and hexa-deccane strong activity was recorded (Fig. 4.29 and Table 4.8). The purified BS could emulsify all the tested substrates namely n-hexadecane, hexane, olive oil, crude oil, engine oil which are highly immiscible in water. But the highest E24 was recorded with crude oil followed by olive oil. Most of the solvents such as n hexadecane, n-hexane, xylene, and benzene had shown approximately equal E24.

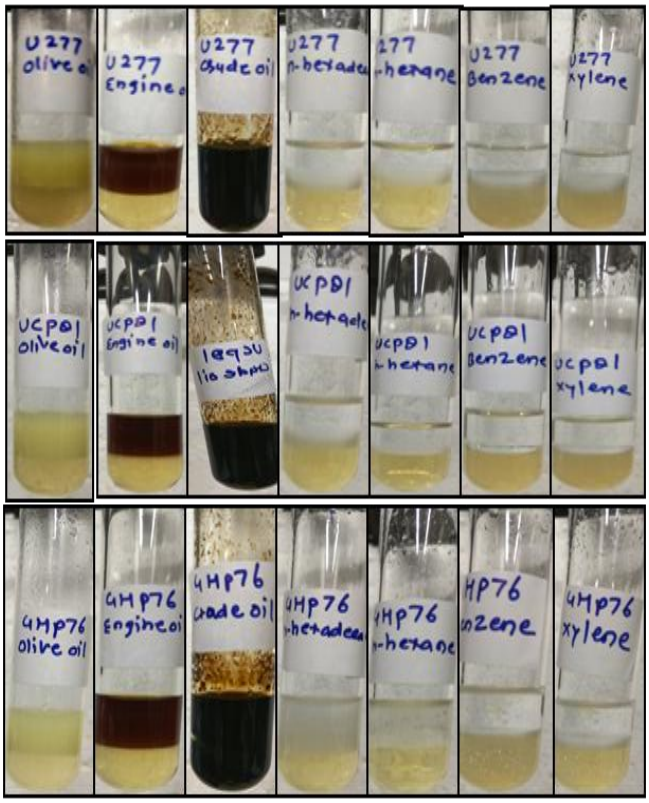


Fig. 4.29: Emulsification activity of purified BS against various immiscible substrates

Table 4.8: Emulsification index (E24) of biosurfactant against various substrates

Sr. no	Substrate	U277 (E24)	GHP76 (E24)	UCPD1 (E24)	UCPS2 (E24)
1	Olive oil	55. 55	50	65.0	65.0
2	Engine oil	42.85	47.61	52.68	52.68
3	Crude oil	95.65	95.51	99.56	100
4	n-Hexane	26.31	57.89	21.05	23.06
5	n-Hexadeccane	43.89	67.84	56.23	59.61
6	Benzene	49.58	67.49	58.34	89.40
7	Xylene	44.83	48.90	67.29	91.93

4.4: General discussions

Various environmental conditions such as temperature, nature of the compound affect the biodegradation of PAH compounds. Different studies have reported various aspects of PAHs degradation such as community strcture, enzymes and pathways involved in the processes, effect of various nutrienal and suppoting materials. However, appropriate use of potential of naturally occurring microorganisms needs to be evaluated by applying them for biodegradation of such compounds with optimizing certain factors (Ghoshal et al. 2016). Therefore in the present study, thermophilic and thero-tolerant microorganisms for possible degradations of some aromatic hydrocarbon along with their biosrufactant production abilities were tested. Some of the previous studies indicated that the temperature and some other factors directly influence the activities and diversity of indigenous microbial populations of petroleum contaminated desert soils (Abed et al. 2015). Addition of exogenous thermophilic microbial strains in such sites could enhance the degradation rates and may overcome the constraint of high temperature in these environments. Some studies also showed that the thermophilic hydrocarbon degraders of *Bacillus*, *Thermus*, *Thermococcus* and *Thermotoga* species from the natural high temperature or sulphur-rich environments are of special significance as they could be efficiently used for bioremediation of oil-polluted desert soil and other arid regions (Meintanis et al. 2006). In addition, microorganisms prevailing in hot springs environments were reported to possess the hydrocarbon degradation potentials which could be exploited for PAHs compound degradations (Saxena et al. 2017). Although hot springs are not considered as a PAH contaminated environments, but the thermophilic properties of hot springs could be an advantage for biodegradation studies. Since the last few years, various microbes isolated from contaminated environments were potentially thought to be efficient degrader, but at the field levels most of them fails. Microbial isolation for bioremediation from the pristine environment and their utilities for

better clean-up of oil spills, petroleum hydrocarbons and other hazardous waste are gaining attentions (Schwarz et al. 2018).

In the present study, a wide variety of bacterial strains were isolated using enrichments and screening for various PAHs degradation. Enrichments under PAH stress conditions is often considered as a useful technique for isolating efficient microbial strains for degradation of PAHs compounds rather than only demonstrating the presence of potential microorganisms in environmental samples (Boonchan et al. 2000; Hilyard et al. 2008). Selection of enrichments and isolation strategies are considered as important tools for isolation of efficient microorganisms. In general, isolation of potent microbes from enriched cultures is more critical and requires special techniques. The identification and characterization of the microorganisms involved in hydrocarbon degradation is an important step for the development of effective bioremediation strategies. However, little is known about the changes in the microbial community to be isolated and the selectivity of the culture medium, which influences the diversity of the existing community when cultivation is directed towards bioremediation (Guerra et al. 2018). Here, we used two different types of isolation techniques, first one was the direct use of standard spread plate technique, while other involved the use of hydrophobic membrane. Although both were found useful but great difference observed among the growth and types of bacteria recovered. In standard spread plate techniques, comparatively few bacterial colonies were obtained. In case of membrane method, few selected bacteria were grown in lawn indicating that, membrane hold the spiked compounds and make them available for organisms. Previously use of such membrane based methods were used for screening to isolates more efficient strains and reported that these techniques were more useful for isolations (Bastiaens et al. 2000).

Taxonomic analysis of the isolated strains revealed that, the isolated bacteria belong to different bacterial phyla; *Firmicutes*, *Actinobacteria* and *Proteobacteria*. However, *Bacillus*

species (*Firmicutes*) were observed in more dominance followed by different genera of *Actinobacteria* such as *Janibacter*, *Cellulomonas*, *Arthobacter*, *Rothia*, *Dietzia*, and *Micrococcus*. The species of the bacterial genera obtained in this study were found to be closely related to the species which were reported for degradation abilities and were isolated from natural or petroleum hydrocarbon contaminated sites. For example, the strains belonging to *B. amyloliquefaciens* were reported for pyrene degradation (Nagger et al. 2010), *B. pumilus* (Patowary et al. 2016) and *B. subtilis* (Hunter et al. 2005) were reported for degradation studies and biosurfactant production. The members of actinobacterial were also found to be reported for degradation studies such as, *Micrococcus* (Rahman et al. 2002), *Rhodococcus* (Song et al. 2011). Therefore, the close association between the strains obtained in this study and those previously reported for degradation may reveal that these genera possess such inheriting degradation potential, albeit they have been not studied from naturally occurring extreme environments. Importantly, among strains obtained from the 50ºC enrichment, thermophilic strains of *Aeribacillus* sp. was previously reported from different natural as well as contaminated thermal environments.

Based on screening and growth analysis, the strains (mainly from Gram-positive and *Bacillus* group) were selected for biodegradation studies. In general, Gram-positive bacteria are known to have broad ranges of adaptability to various environmental stresses. The predominance of *Firimicutes* in isolated cultures reveals that the enriched microbial community majorly dominated by gram-positive bacteria, may be play a significant role in the breakdown of recalcitrant organic matter. Endospore-forming bacteria like *Bacillus* or *Aeribacillus* groups can survive in the highly variable environments (Wang et al. 2006). Therefore, Gram-positive bacteria may be playing a key role in PAH degradation.

Some important parameters such as culture media, temperature conditions for the selected strains should be considered before analysing for degradation at the lab scale.

Generally, bioremediation processes are found to be affected by several such factors such as nutrient availability and optimization of such nutritional parameters were also necessary (Breedvelt and Sparrevik 2000). Optimization of nutrient parameters for different microbial strains possibly will also found useful for enhancement of metabolic activities of degrading indigenous microorganisms by adding the stimulating nutrients in contaminated sites for enhancing growth and bioremediation (Wong et al. 2002; Adams et al. 2015). Apparently, it was also observed that low nutrient containing culture media such as BH medium which is a specially designed medium for the isolation of hydrocarbon degrading bacteria was found to more effective for isolation and degradation analysis of PAH compounds. In previous studies, BH medium was utilized for isolation of various microbial strains from petroleum contaminated sites (Eriksson et al. 2003; Hilyard et al. 2008).

Biodegradation analysis was performed using various strategies such as individual strains, mixed cultures, total enriched microbial community, individual compounds or mixed compounds at different temperatures. Furthermore, attempts were also made to enhance the degradation using biochar addition for biodegradation showed higher degradation. Among these strategies, a mixed culture at 50ºC found more effective for degradation. This mixed culture consists of four bacterial strains, three Bacilli from 37ºC enrichment set and one strain of *Aeribacillus* sp. selected from 50ºC set. The higher degradation by this mixed culture majorly portrait to *Aeribacillus* sp, as in the individual cultures set this strain has performed well as compared to other bacterial strains. Most of the previous studies have isolated the strains of *Aeribacillus* sp from various natural environments such as hot springs, petroleum reservoirs, compost, marine hydrothermal vents and from the oil fields with the abilities to degrade different petroleum hydrocarbon and other compounds at higher temperatures (Mnif et al. 2014; Wang et al. 2006). In general, it has been observed that biodegradation of PAH compounds under aerobic conditions is more favourable as compared to anaerobic

degradations (Olajire and Essien 2014). In this respect, *Aeribacillus* sp. were found to aerobic, which could also add further advantages for more efficient degradations. Similarly, it was also reported that the oxygen mass transfer rates into aqueous media have been found to be higher at elevated temperatures and are even enhanced in the presence of hydrocarbons which could compensate for lower oxygen solubility at higher temperatures (Viamajala et al. 2007).

Use of mixed culture for degradation of PAH compounds was also previously reported, where, a mixed culture capable of degrading a range of other PAHs, including benzo[*a*]pyrene, anthracene, phenanthrene, acenaphthene, and fluorene was reported (Trzesicka-Mlynarz and ward, 1995). In case of mixed culture with addition of biochar, degradation of HMW PAHs was found higher than the original mixed culture. In previous studies also biochar was used for enhancing the degradation of PAH compounds. Biochar has a high sorption potential for organic contaminants and is thus well suited for the immobilization of PAHs. The effect of adding biochar in combination with compost to soil to assess the contaminant mobility and bioavailability has been compared. Modern researches have demonstrated that biochar is one such fraction that might create an enhancement in the biodegradation of organic pollutants and there has a growing interest in utilizing biochar to remediate contaminated soils. Several researchers have reported that prepared at a high temperature has high sorption capacity similar to activated carbon that once commonly used in soil remediation (Sigmund et al. 2018). In our comparative analysis of biodegradation using individual cultures, mixed cultures, mixed culture amended with biochar, total enriched microbial community has shown interesting results. Better degradation of a defined PAH mixture was observed with the mixed culture than with individual isolates. A mixed culture containing four individual strains after amended with biochar (MCBC) manifested a similar PAH biodegradation performance as observed for total enriched microbial community

(TEMC) mixed culture. When compared with the mixed culture, individual strains especially at 50ºC, exhibited a relatively good capacity to remove LMW PAHs (anthracene, fluorene, phenanthrene). In contrast, removal of HMW PAHs (pyrene) was low with the individual strains compared with the mixed culture. In Biochar amended mixed culture (MCBC) and total enriched microbial community (TEMC), degradation of anthracene and fluorene was delayed or found to be reduced, while HMW PAHs, pyrene degradation was enhanced. These results indicate that addition of biochar increases the availability of PAH compounds, as HMW are highly hydrophobic, they may have higher attraction for biochar and they became readily available for microorganism in the medium. While in case, enriched community, diverse members were present, could be able use HMW PAHs as a sole source of carbon, or may be obtaining their energy by cometabolic degradation of these compounds. Overall, it could be inferred from the sets of mixed cultures and degradation of compounds that composition of mixed compounds could affect the degradation process and depends on the types of microbial strain, environmental factors such as temperatures. Therefore, to further investigate such effects of mixed compounds on degradation, degradation of PAH compounds present in the crude oil was examined. In case of crude oil, almost similar types of results were obtained, where higher degradation was observed in mixed cultures than the individual cultures. The study indicates that the use of mixed cultures or microbial consortia is more effective for degradation of mixed PAH compounds than the individual strains.

In general, hydrocarbon utilizing bacterial strains were known to produce biosurfactants to uptake the hydrocarbon by changing cell-surface hydrophobicity (Franzetti et al. 2008). Therefore, biosurfactants play important role in the remediation of hydrocarbon compounds thereby increasing bioavailability of these compounds through mobilization and emulsification.The biosurfactants are surface-active amphiphilic compounds which could enhance the rates of biodegradation of PAHs compounds by increasing the solubility of these

compounds and numerous studies have been conducted to evaluate the effects and mechanisms of surfactants on the bioavailability and biodegradation of pollutants (Cameotra et al. 2003; Bordoloi et al. 2009). In this study the four strains used in mixed cultures were tested for their biosurfactant production ability. All the four strains were found to produce biosrfactants, which infers that these strains could be solubilising the PAH compounds for their degradation and utilization as a carbon and energy source. Molecular characterization revealed that all the strains showed the presence of surfactin synthetase gene revealing that the strain produces lipopeptide type of biosurfactants. An independent comparison of homology using BLASTN and BLASTX programme with the known sequences retrieved from the database showed 94% to 96% similarities at the nucleotide and amino acid level with the previously deposited *Bacillus* strain in the database. The production of surfactin type of biosurfactant by *Bacillus spp.* was reported from various environments (Cochrane and Vederas 2016). Many strains of *Bacillus* genus have the ability to produce biological surface active compounds belonged to lipopeptide (LPs) group. Lipopeptides have higher ability to reduce surface and could act as an excellent emulsifiers (Desai and Banat, 1997). These lipopeptides type of biosurfactants are synthesized by a biosynthetic process involving NRPs, the genes responsible for these enzymes have been observed somewhat conserved in various *Bacillus* species (Yang et al. 2015). Overall, the study showed that the identification and characterization of the microorganisms involved in hydrocarbon degradation and biosurfactant production are important to develop bioremediation strategies. Although information about the microorganisms involved in these processes is available, the changes in the microbial community to be isolated and the selectivity of the culture medium, temperatures conditions were also necessary. Thermo-tolerant strains have such broad tolerance properties and therefore could aid in effective bioremediation of PAH compounds.

4.5: Conclusion and future prospects

Thermophiles which have PAH degradation abilities could be employed in the bioremediation strategies being developed for PAHs removal from the contaminated sites, considering their high adaptability towards extreme conditions. In addition, these microorganisms were also known to produces biosurfactants, which may further aid in the enhancement of degradation rates. The present study shows that microorganisms from extreme environments have the potentials for bioremediations of PAHs compounds probably may be due to their higher metabolic capacities and survivals at extreme conditions. The study also promotes the explorations of microbial resources of various extreme habitats for possible environmental applications.

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CHAPTER 5

Summary, conclusion and Future Prospects

5.1: Summary

The main aim of the present work was to investigate the diversity, ecology and functional potential of the microbial communities of Unkeshwar hot springs in a systematic and precise manner and to establish a link between taxonomic composition and impacts of various environmental, anthropogenic factors on it. For this purpose, we designed our study in three major parts, in the first part; we used culture-independent approaches for microbial diversity analyses. While part second and third focused on cultivation and bioprospecting activities.

In the first part of the study, a comprehensive analysis of microbial community composition and functions was performed using high-throughput sequencing techniques. Using such techniques, we found that Unkeshwar microbial communities are dominated by Firmicutes, Proteobacteria, Bacteroidetes, Deinococcus-Thermus, Cyanobacteria, and Saccharibacteria and some candidate phyla. The bacterial community in the hot spring showed more significant variation than that in the nearby borehole and anthropogenically impacted sites possibly indicating that microbial community in the Unkeshwar hot spring is more prone to environmental disturbances. However, functional analysis revealed that the hot springs have high metabolic capacities in terms of metabolisms of xenobiotic compounds, suphur, and methane, indicating that both their natural role in biogeochemical cycling and also in the degradations of toxic pollutants. Overall the study has shown an improved census of microbial community structure in this hot spring, which could provide a foundation for exploring microbial biogeochemical cycling and microbial function in Indian hot spring ecosystems. However, the impact of human interferences, rain influx, and other abiotic factors on microbial communities had shown that there is a need to conserves such valuable resources for future bioprospecting for useful applications.

In the second part of the study, studied microbial communities using a cultivation approach, which is more useful for harnessing the metabolic potentials of the microbes. We

reported the isolation and characterization of diverse and previously underexplored members of bacteria using various cultivation strategies. This study highlighted an interesting aspect in basic microbiology that the multiple cultivation strategies till holds a useful tool for characterizing microbial diversities form extreme environments. Using cultivation approaches a total of 57 bacterial genera were isolated including some rare bacteria. Metabolic potential of observed diversity revealed that many bacterial genera have various properties such as the production of industrially important enzymes, tolerance of heavy metal stresses and also found resistance to most of the antibiotics. The occurrences of the antibiotics resistance microbes in hot springs show the impact of anthropogenic load on microbial communities, which further suggest for the conservation of such pristine natural environments. In addition, the study also promotes the use of larger scale microbial cultivation studies for isolating the unexplored species for deeper understanding and to get an increased access to the bacterial communities in hot spring environments. In the last part of the study, we have a prospect the microbes of Unkeshwar for bioremediation applications. The study showed that hot springs microorganisms have the potentials for bioremediations of PAHs compounds probably may be due to their higher metabolic capacities and survivals at extreme conditions. The study also promotes the explorations of microbial resources of various extreme habitats for possible environmental applications. Overall few of the key findings were observed in the present study which is provided below.

5.2: Major Highlights of the study:

- Present study generated valuable information and scientific knowledge of microbial community structure and dynamics in un-noticed and least studied Unkeshwar hot spring located in Godavari geothermal region over a period of time with respect to geochemistry.
- Temporal variations observed in the microbial communities of this basaltic spring might be due to the influences of anthropogenic activities, which suggest the need of conservation of the physical state of the Indian hot springs.
- The present study revealed the advantages of classical microbiological cultivation methods using a comprehensive multiple isolation approach for exploring untapped and unique bacterial diversity from this Indian hot spring, and also prospects for various industrial microbiology applications.
- Key bacterial strains isolated in this study are efficient for the degradation of high molecular weight PAH compounds (along with biosurfactants synthesis) at high temperatures that could aid in the development of effective bioremediation strategy.

5.3: Concluding remark and future prospects

A combination of classical microbiology and advanced high throughput sequencing technologies enabled study of Unkeshwar hot spring (India) revealed that the hot spring host high diversity of interesting groups of bacteria, which is somewhat similar to other hot springs of India (with similar temperatures) indicating harmony among microbial communities. Natural microbial population of Unkeshwar holds a high metabolic potential, which could be harnessed for biotechnological potential, and biodegradation of PAHs compounds. Also, like other hot springs, Unkeshwar spring also experience impact of environmental factors and human activities as documented throughout study. The natural composition of microbial assemblages is getting fluctuated over the time and season and introduction of the non-indigenous microflora in hot spring could cause a threat to the microbial compositions of such hot springs. In summary, we could say that there is ample scope for improvement and magnification of existing practices of hot spring microbiological studies in India to make it more attractive for commercial exploitation of moderately thermophilic bacteria. Nonetheless, major attention should be directed towards the preservation of the natural ecosystem of the springs, which represent ancient life forms on earth. Furthermore, traditional microbiological approaches have already shown how useful microbes can be; the new approach of metagenomics should be applied and extend for functional analysis to discover and benefit from microbial capabilities of hot springs. Therefore, with a more research and better public awareness regarding the use of hot spring water possibly will help to conserve these natural resources for valuable future biotechnological applications.

Appendix I

List of Publications

1. **Mehetre GT**, Shah MB, Dastager SG, Dharne MS (2018) Untapped bacterial diversity and metabolic potential within Unkeshwar hot springs, India. Arch Microbiol 200: 753:770.

2. **Mehetre GT**, Paranjpe AS, Dastager SG, Dharne MS (2016). Complete metagenome sequencing based bacterial diversity and functional insights from basaltic hot spring of Unkeshwar, Maharashtra, India. Genom Data 7: 140-143.

3. **Mehetre GT**, Paranjpe AS, Dastager SG, Dharne MS (2016). Investigation of microbial diversity in geothermal hot springs in Unkeshwar, India, based on 16S rRNA amplicon metagenome sequencing. Genome Announc 4: e01766-15.

4. **Mehetre GT**, Shah MB, Dastager SG, Dharne MS. Profiling and assessment of microbial communities within naturally occurring and borehole hot spring systems. Geomicrobiology (**Under Revision**).

5. **Mehetre GT**, Shah MB, Dastager SG, Dharne MS. Exploration of microbial diversity in waters, sediment and mats of Unkeshwar hot spring using high throughput sequencing approach (unpublished manuscript)

6. **Mehetre GT**, Shah MB, Dastager SG, Dharne MS. Anthropogenic disturbances influences the distribution of microbial communities in the water and sediment of Unkeshwar hot springs (unpublished manuscript).

7. **Mehetre GT**, Shah MB, Dastager SG, Dharne MS. Comparative analysis of whole metagenome based Illumina and MinIOn sequence for microbial community structure and function in the basaltic hot Springs of India (unpublished manuscript).

8. **Mehetre GT**, Dastager SG, Dharne MS. Heavy metal and antimicrobial resistance patterns of bacterial strains isolated form hot water aquatic ecosystems (hot springs), India (unpublished manuscript).
9. **Mehetre GT**, Dastager SG, Dharne MS. Evaluation of the biodegradation and biosurfactant potentials of thermophilic bacteria for polycyclic aromatic hydrocarbon degradation (unpublished manuscript).
10. **Mehetre GT**, Dastager SG, Dharne MS. Evaluation of the PAH degradation by the biodegradation strategies using mixed culture, enriched microbial community and biochar on degradation rates (unpublished manuscript).

Other than PhD thesis work

1. Dhotre SV, **Mehetre GT**, Dharne MS, Suryawanshi NM, Nagoba BS (2014) Isolation of *Streptococcus tigurinus* – a novel member of *Streptococcus mitis* group from a case of periodontitis FEMS Microbiol Lett 357:131–135.

2. Ganesh M, Sheikh NK, Shah P, **Mehetre GT**, Dharne M, Nagoba BS (2015) Detection of *Clostridium tetani* in human clinical samples using *tetX* specific primers targeting the neurotoxin, J Infect Public Health., (2015) 9 (1): 105-109.

3. **Mehetre GT**, Jaya Chakraborty, Monika Dhote, Vibhavari Sapkale, Dharne MS. Actinobacteria: Promising source for bioremediation of polycyclic aromatic hydrocarbon (PAH) compounds. Environmental science and pollution research (**Under Revision**)

4. **Mehetre GT**, Vinodh J S, Bhushan Burkul Santhakumari B, Mahesh S. Dharne, Syed G. Dastager. Taxonomic diversity and biosynthetic potentials of Actinobacteria associated with Indian geothermal springs (unpublished Manuscript)

APPENDIX II

List of conference attended/presentations

Presentations:

1. **Mehetre GT**, Dastager SG, Dharne MS. Biodegradation of Polycyclic aromatic compounds (PAHs) using Thermophiles. **14th Biotech Research Society, India (BRSI) Convention and International Conference CSIR-NEERI, Nagpur, India,** Oct. 8-10, 2017 (poster).
2. **Mehetre GT**, Dastager SG, Dharne MS. Bacterial Diversity and Biochemical Potential in Geothermal Hot Springs of India: **National Science Day, CSIR-NCL, Pune, India,** Feb. 25-28, 2016 (poster).
3. **Mehetre GT**, Dastager SG, Dharne MS. Microbiology and metagenome snapshot of basaltic thermal spring of Unkeshwar, Maharashtra India. **56th Annual Conference of the Association of Microbiologists of India (AMI), New Delhi, India**, Dec. 7-10, 2015 (poster).
4. **Mehetre GT**, Kumbhar Y, Ramama V, Shouche Y, Dharne MS. Need of conservation of microbial biodiversity of Lonar lake: Microbial view. **National Science Day, CSIR-NCL, Pune, India,** Feb. 25-28, 2014 (poster).

Appendix III

Summary of CSIR 800 (Societal program)

Title: Microbiological study of Drinking Water quality at local railway stations in PMC and PCMC area: Challenges and possible solutions

Summary:

The availability of clean water to all is becoming a challenging task for authorities in India. The poor quality of drinking water has resulted in a health problem which affects, directly and indirectly, the livelihood of people in both urban and rural India. Drinking water sources at the public places are highly neglected in terms of quantitative risk assessment. Railway stations are most crowded and accessed by most of the people. In view of this, the aim of the present study is to analyze the drinking water quality with respect to microbial contamination of water provided at public places like railway stations.

Local railway stations on the route of Pune to Lonavala were investigated for inspection of availability of drinking water facilities and its maintenance status. It was found that, at Pune junction, water purification units were installed (VM, RO unit). While in the case of other stations, only tap and water coolers are present but without the regular maintenance, most taps were not in working condition. Micorbiolgical examination was performed using the standards methods for all the collected samples. The result revealed that the total heterotrophic count and total coliform count was higher than the permissible limits according to BIS Indian standards in water of most of the stations. Only railway stations those come under urban zone were found to provide potable quality of drinking water than the rural region stations. It, therefore, insists on finding the solution to this highly neglected problem. There is need to create awareness among commuters and also the installation of maintenance free, robust water purifiers at very convenient charges/free developed by CSIR-NCL. This will save money, and also will be more useful for poor people.

Appendix VI

Name of antibiotics

LE	:	Levofloxacin
AMC	:	Augmentin
NIT	:	Nitrofurantoin
CX	:	Cefoxitin
CPD	:	Cefpodoxime
CAZ	:	Ceftazidime
NA	:	Nalidixic acid
CTR	:	Ceftriaxone
CL	:	Colistin
CIP	:	Ciproflaxicin
IMP	:	Imipenem
COT	:	Co-trimoxazole
MO	:	Moxifloxacin
GEN	:	Gentamicin
GAT	:	Gatifloxacin
TOB	:	Tobramycin
OF	:	Ofloxacin
NZ	:	Norfloxacin
AK	:	Amikacin
AT	:	Aztreonam

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Data in Brief

Complete metagenome sequencing based bacterial diversity and functional insights from basaltic hot spring of Unkeshwar, Maharashtra, India

Gajanan T. Mehetre [a,b], Aditi S. Paranjpe [a], Syed G. Dastager [a,b], Mahesh S. Dharne [a,b,*]

[a] National Collection of Industrial Microorganisms (NCIM), CSIR-National Chemical Laboratory (NCL), Pune, India
[b] Academy of Scientific and Innovative Research (AcSIR), New Delhi, India

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ABSTRACT

Unkeshwar hot springs are located at geographical South East Deccan Continental basalt of India. Here, we report the microbial community analysis of this hot spring using whole metagenome shotgun sequencing approach. The analysis revealed a total of 848,096 reads with 212.87 Mbps with 50.87% G + C content. Metagenomic sequences were deposited in SRA database with accession number (SUB1242219). Community analysis revealed 99.98% sequences belonging to bacteria and 0.01% to archaea and 0.01% to Viruses. The data obtained revealed 41 phyla including bacteria and Archaea and including 719 different species. In taxonomic analysis, the dominant phyla were found as, Actinobacteria (56%), Verrucomicrobia (24%), Bacteriodes (13%), Deinococcus-Thermus (3%) and firmicutes (2%) and Viruses (2%). Furthermore, functional annotation using pathway information revealed dynamic potential of hot spring community in terms of metabolism, environmental information processing, cellular processes and other important aspects. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of each contig sequence by assigning KEGG Orthology (KO) numbers revealed contig sequences that were assigned to metabolism, organismal system, Environmental Information Processing, cellular processes and human diseases with some unclassified sequences. The Unkeshwar hot springs offer rich phylogenetic diversity and metabolic potential for biotechnological applications.

Specifications	
Organism/cell line/tissue	Unkeshwar hot spring metagenome
Sex	not applicable
Sequencer or array type	Illumina Hi seq 2500
Data format	raw data: fastq
Experimental factors	environmental sample
Experimental features	shotgun metagenome sequencing followed by microbial community and taxonomic analysis using KEGG pathways
Consent	not applicable
Sample source location	water sample, Unkeshwar hot spring, Maharashtra State, India

1. Direct link to deposited data

http://www.ncbi.nlm.nih.gov/sra/SRX1499016

2. Experimental design, materials and methods

Thermopiles have been discovered in geothermal features all over the world, leading to the discovery of many novel environmental microorganisms with important applications in biotechnology, medicine, and bioremediation [1]. Microbes inhabiting hot springs are dictated by environmental physicochemical characteristics such as pH, redox potential, temperature and concentration of trace elements [2,3]. Among these factors, water temperature is the major factor in controlling microbial distribution within hot springs [4]. Hot springs microorganisms thrive under multiple environmental stresses and to survive under such stresses, microbial communities use mutualistic or communalistic symbiotic relationships [5].

Metagenomic analyses using high throughput sequencing have been extremely a valuable tool for describing microbial community structure and function in extreme ecosystems [6,7]. Using such high throughput techniques most of the terrestrial hot springs all over the world, hosting diverse thermophilic microorganisms has been investigated. Majorly studied regions such as Yellowstone National Park, USA [8], Great Basin, USA [4], Philippines [9], Canada and New Zealand [10] and China [11] revealed many novel microbial lineages with promising applications in biotechnology. Metagenome sequencing techniques

* Corresponding author at: Academy of Scientific and Innovative Research (AcSIR), New Delhi, India.
E-mail address: ms.dharne@ncl.res.in (M.S. Dharne).

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are also useful for identifying novel genes with novel bioactive molecule production capabilities [12,13].

India has some high as well as low temperature geothermal springs in different geographic regions. In India thermal springs are found scattered throughout the country and occur either as solitarily or in groups up to 3000 M above sea level [14]. From hot springs of basaltic region of Deccan plateau of India, scanty and superficial reports have been documented [15,16]. Therefore we investigated microbial diversity of Unkeshwar hot spring located in such region in Maharashtra state of India. Unkeshwar is located at geographical South East Deccan Continental basalt of India (19°34′–19°40'N and 78°22′–78°34'E) from mean sea level. The hot springs in this area emerge through basalt. The main Unkeshwar hot spring is within Unkeshwar temple in the form of kund. The water has sulphurous smell with feeble gaseous activity and discharge through the jointed Deccan basalts. Water samples were collected in sterile containers from Mukhya kund location of Unkeshwar hot spring during November 2012. The discharged water (10,000 l/h) is used by visitors to take bath for a therapeutic belief. Samples were processed for metagenomic and physioco-chemical analysis.

Temperature and pH were measured for the sample at the site. The physiochemical parameters of the samples were analysed at a certified chemical testing laboratory (Accurate Analytical Laboratory Pvt. Ltd., Pune, India) using standard methods of American Public Health Association [17]. The temperature of water sampled was between 50 °C–60 °C and the pH 7.3. The physiological parameters of the water sample tested shown that the total dissolved solids (0.0507%), total volatile solids (0.0180%), Phosphorus (0.008724%), and Sulphates (SO_4^{2-}) (0.004375%) were present in higher amount as compared to other elements. Other elements like Calcium, Cobalt, Iron, and Copper were not detected in the tests. Unlike other hot springs, Unkeshwar hot springs content high Phosphorus and Sulphates content. The chemical stress like availability of high phosphorus, sulphur concentrations and slightly higher organic content enriches the microbial diversity of this hot spring.

One litre of water sample was filtered using a 0.45 μm (to remove debris) followed by 0.22 μm filter membrane (MOBIO, USA). The 0.22 μm filter membrane was then sliced and subjected to simultaneous metagenomic DNA and RNA extraction using RNA PowerSoil® Total RNA Isolation Kit (MO BIO), according to the manufacturer's protocol. The quantitative analysis of the DNA was done using a Nanodrop and the integrity of DNA samples was checked on 1% agarose gel using a gel documentation system (Protein Simple). The low abundant metagenomic DNA was enriched by Multiple Annealing and Looping Based Amplification Cycles (MALBAC) amplification protocol as described earlier [18]. Enriched metagenomic DNA was extracted and analysed by whole shotgun metagenome analysis for microbial community structure and functional annotations.

Whole metagenome shotgun sequencing of 12UM sample was performed using the Illumina HiSeq 2500 sequencer (Illumina, USA). Metagenome sample library quantification was done using Bioanalyzer 2000 (Agilent, USA). For sequencing, a dual-indexed Paired-End sequencing (2 × 251base pairs) strategy with a total of 250 cycles, six bp index sequence was used. The entire sequencing run was completed in 39 h. Based on quality report of fastq files sequence reads were trimmed wherever necessary to only retain high quality sequence for further analysis. Assembled contigs size >150 bp was considered for

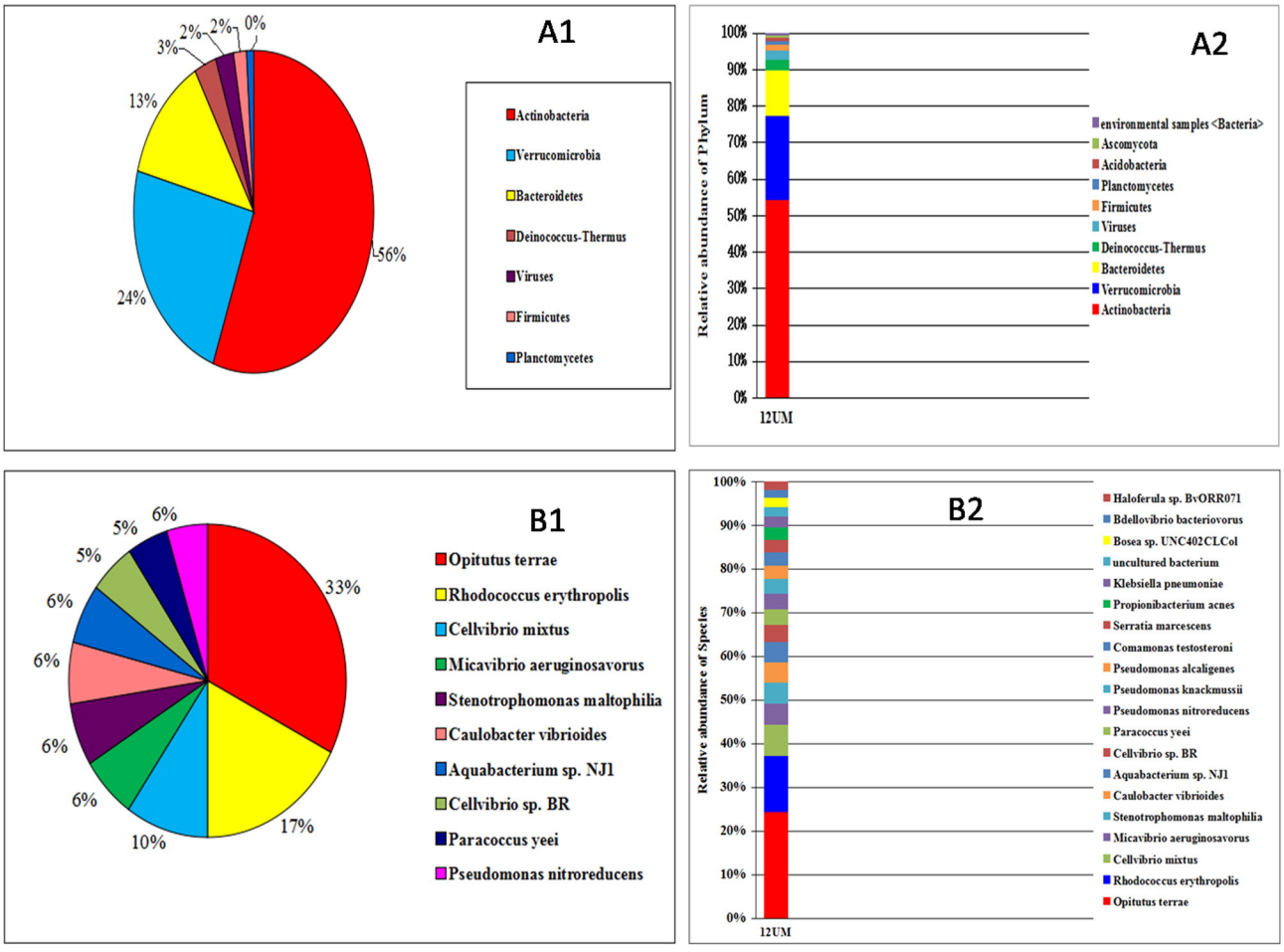

Fig. 1. Taxonomic profiling and microbial community structure in Unkeshwar hot spring using NCBI taxonomy data sets A1: Phylum abundance; A2: Relative abundance phylum of samples; B1: Species abundance; and B2: Relative abundance of species of hot springs (Only top 10 species hits were highlighted).

further analysis. Assembly was performed with default k-mer length (21-size) using de-bruijn graph method. In-house PERL and Python code were used to parse the fastq files for further analysis. Taxonomic profiling was performed using NCBI taxonomy data sets. The taxonomy tree was generated based on neighbour-joining method using MEGAN software [19].

Whole metagenome shotgun sequencing revealed a total of 848,096 reads with 50.87% G + C content. After trimming and assembly, a total 34,123 contigs were obtained. Taxonomic profiling was performed using NCBI taxonomy data sets for 21,424 reads and revealed 41 phyla including bacteria and archaea. The contig sequences presented, Actinobacteria (56%), Verrucomicrobia (24%), Bacteriodes (13%), Deinococcus-Thermus (3%) and firmicutes (2%). At the species level, *Opitutus terrae* (33%), *Rhodococcus erythropolis* (17%), *Cellovibrio mixtus* (10%) were found dominant species (Fig. 1).

The prediction of Open reading frames (ORFs) in the assembled contigs was done using a Glimmer-MG tool [20] and the complete functional annotation along with contig ID, gene function and sequences were carried out. For functional annotation the contigs of 12UM were queried to BLASTX programme with optimum e value of $1e^{-10}$. The gene or protein functions of all the contigs from BLASTX output were parsed using in-house PERL (Practical Extraction and Report Language) script. Further, functional annotation was carried out by doing KEGG (Kyoto encyclopedia of genes and genomes) analysis based on taxons. Functional annotation of all the contigs is carried out by SEED Classification [21]. MEGAN software was used to assign the function of each contig. The protein function of each contig having highest alignment score from BLASTX results was considered for functional assignment and revealed functions like protein metabolism (1), cell wall and capsule (1), unassigned (14,281) and not hits (5794).

Pathway annotation was done using KEGG pathway analysis performed for each contig sequence by assigning KEGG Orthology (KO) numbers obtained from known reference hits. Around 65% contig sequences were assigned to metabolism, 20% unclassified sequences, 9% organismal system, 3% Environmental Information Processing, 1% cellular processes and 1% human diseases (Fig. 2). Sample read counts of KEGG annotation were metabolism (1990), Organismal systems (283), Environmental information processing (81), Human diseases (37), Genetic information processing (36), cellular processes (11) and unclassified (616).

Taxonomic and functional diversity of a community quantified using whole metagenome shotgun sequencing revealed the dominance of bacterial population and their metabolisms. At the phylum level dominant bacterial phyla were Actinobacteria, Bacteriodes, Deinococcus-

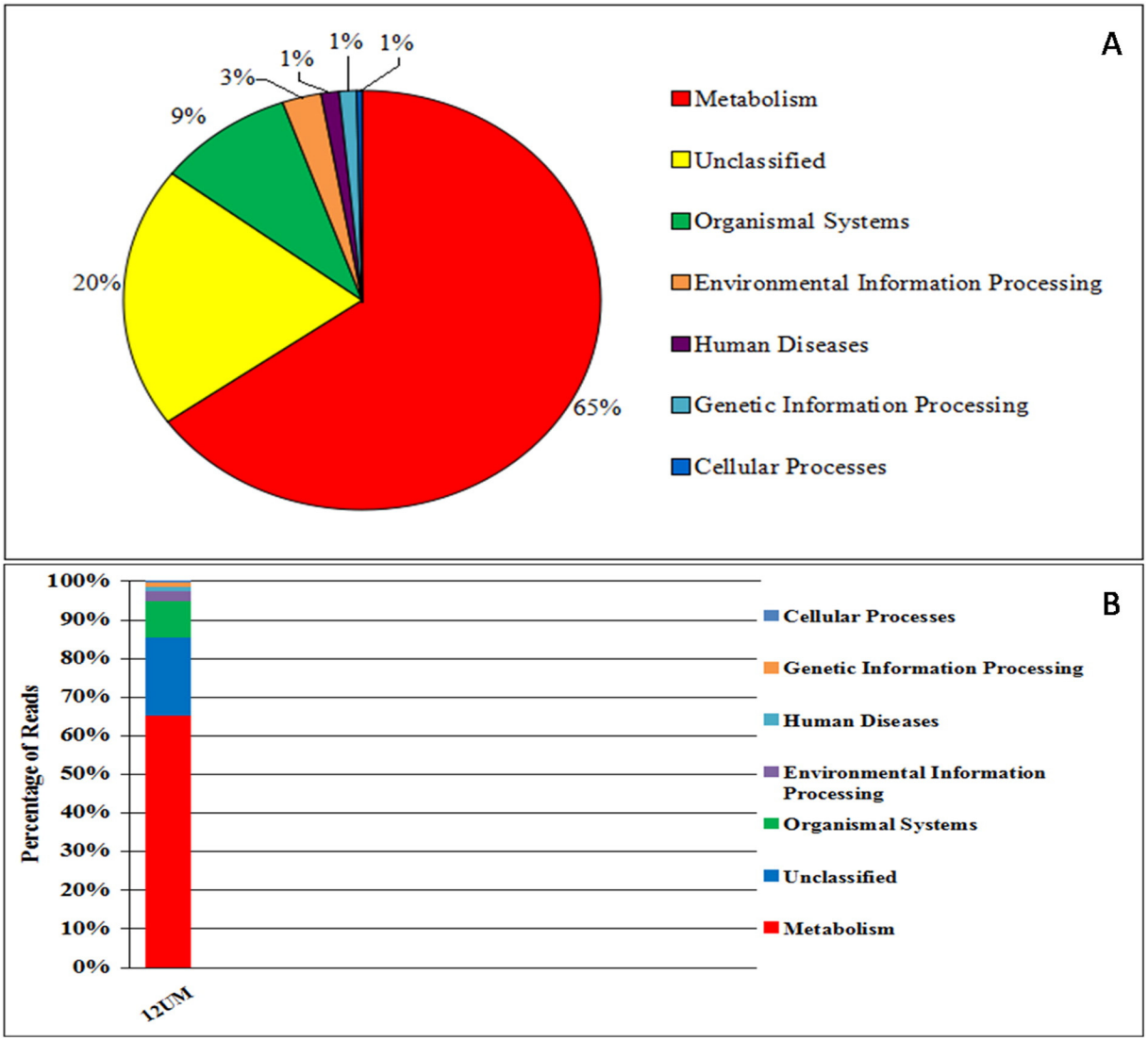

Fig. 2. Functional annotation based on KEGG-pathway analysis of Unkeshwar hot spring. A: number of sequence assigned to the pathway; and B: KEGG Relative abundance of Sample.

Thermus, Firmicutes, and Planctomycetes. Bacterial genera like *Rhodococcus*, *Microbacterium*, *propionibacterium*, *Flavobacterium*, *Deinococcus*, *Caulobacter*, *Brevundimonas*, *Methylobacterium*, *Paracoccus*, *Roseomonas*, *Novosphingobium*, *Sphingomonas*, *Achromobacter*, *Acidovorax*, and *Aquabacterium* were also dominant. KEGG pathway analysis shows that higher number of sequences was contributed to the metabolism as shown in the larger edge size. A number of DNA sequences remained unassigned with respect to taxonomic and functional coherence. To the best of our knowledge, this is the first study that deals with the description of complete profiling of microbial diversity from Unkeshwar hot spring using next generation massively parallel sequencing approach. Metagenome sequencing analysis may significantly provide important breakthroughs in depicting taxonomic structure and functional and/or metabolic pathways of basaltic hot spring of unkeshwar with the promise of novel genes and novel microbes for biotechnological applications.

3. Nucleotide sequence accession number

Metagenome sequence data is available in NCBI SRA under accession number http://www.ncbi.nlm.nih.gov/sra/SRX1499016.

Competing interest

Authors declare that there are no competing interests.

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AMERICAN SOCIETY FOR MICROBIOLOGY | genomeAnnouncements

Investigation of Microbial Diversity in Geothermal Hot Springs in Unkeshwar, India, Based on 16S rRNA Amplicon Metagenome Sequencing

Gajanan T. Mehetre,[a,b] Aditi Paranjpe,[a] Syed G. Dastager,[a,b] Mahesh S. Dharne[a,b]

National Collection of Industrial Microorganisms (NCIM), CSIR-National Chemical Laboratory (NCL), Pune, India[a]; Academy of Scientific and Innovative Research (AcSIR), New Delhi, India[b]

Microbial diversity in geothermal waters of the Unkeshwar hot springs in Maharashtra, India, was studied using 16S rRNA amplicon metagenomic sequencing. Taxonomic analysis revealed the presence of *Bacteroidetes*, *Proteobacteria*, *Cyanobacteria*, *Actinobacteria*, *Archeae*, and OD1 phyla. Metabolic function prediction analysis indicated a battery of biological information systems indicating rich and novel microbial diversity, with potential biotechnological applications in this niche.

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Address correspondence to Mahesh S. Dharne, ms.dharne@ncl.res.in.

Deccan basaltic geothermal hot springs are rich in sulfur and yet are unexploited for microbial ecology (1). The geographical location of Unkeshwar is latitude 19°34′ to 19°40′N and 78°22′ to 78°34′E longitude, with water temperatures ranging from 42°C to 60°C, located in Maharashtra, India.

Replicate water samples were collected during December 2012 in sterile containers, filtered through 0.22-μm-pore-size filters (Merck Millipore, India), and DNA extraction was performed using the RNA PowerSoil total RNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA), according to the manufacturer's protocol. DNA was enriched by the Multiple Annealing and Loop-Based Amplification Cycles (MALBAC) protocol (2) and then amplified by using primers spanning the V3 to V4 region of the 16S rRNA gene (3).

Paired-end sequencing of the library was performed on an Illumina MiSeq platform using 2 × 251-bp chemistry. The quality parameters for the obtained sequences were checked using the FASTQ quality filter (Phred quality [Q] <20). The resulting good-quality sequences were then overlapped into single longer reads using SeqPrep QIIME (4). Chimeras were removed using the program UCHIME, and all nonchimeric sequences were taken for picking operational taxonomic units (OTUs) using the program Uclust, with a threshold of 97% similarity (5, 6). A representative sequence was identified for each OTU and aligned against a Greengenes core set of sequences using the PyNAST program (7, 8) Taxonomic classification was performed using the RDP Classifier (9) and Greengenes (7) OTU databases. Further, alpha diversity was determined by calculating Shannon, Chao1, and observed species metrics (4). The rarefaction curve was generated for each of the metrics, and metric calculations were performed using the QIIME software (8).

A total of 1,360,637 raw reads were obtained, and 873,631 reads were considered for analysis (after filtering), from which a total of 6,684 OTUs were detected. They were checked for singleton OTUs (i.e., OTU has single reads), and 4,935 singletons were identified and removed. A total of 1,749 OTUs were used for taxonomy classification showing the dominant phyla of *Bacteroidetes*, *Proteobacteria*, *Cyanobacteria*, and *Actinobacteria*. Around 80% of the reads were assigned to *Bacteroidetes* and the other 20% to all other phyla. Two of the reads were also assigned to *Archaea* belonging to the *Cenarchaeum* genus (phylum *Thaumarchaeota*). Interestingly, some of the reads mapped to the OD1 phylum, which is known for small genome size (0.7 to 1.2Mbp) and large inventories of novel proteins (10). The rest of the OTUs were mapped to other and unknown phyla. The microbial diversity within the samples was also calculated by Shannon, Chao1, and observed species metrics used to measure the estimated observed OTU abundances, accounting for both richness and evenness.

To determine the accuracy of functional predictions using a Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), inferences were compared (11) to understand survival strategies and adaptation in extreme niches. The results revealed a wider range of genetic diversity involved in various essential processes, like genetic (translation, transcription, and repair) and environmental information signaling and processing, cellular processes (cell growth and death, cell communication, cell motility, transport, and catabolism), signal transduction and metabolism (of carbohydrates, amino acids, lipids, terpenoids, polyketides, cofactors, vitamins, xenobiotics, energy, and proteins and biosynthesis of secondary metabolites) and organismal systems.

Nucleotide sequence accession number. The sequence reads obtained in this study were deposited in the Sequence Read Archive (SRA) accession no. SRX1499015.

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ORIGINAL PAPER

Untapped bacterial diversity and metabolic potential within Unkeshwar hot springs, India

Gajanan Mehetre[1,2] · Manan Shah[1] · Syed G. Dastager[1,2] · Mahesh S. Dharne[1,2]

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Abstract

Hot springs support diverse and interesting groups of microorganisms adapted to extreme conditions and gaining attention in biotechnological applications. However, due to limitations of cultivation methods, a majority of such extremophiles remain uncultivated and unexplored. The advent of multiple cultivation conditions and specialized culture media could possibly aid to access the unexplored microbial portion of hot springs. In the present study, different media and isolation strategies were applied to isolate hitherto unexplored bacterial taxa in the water samples collected from Unkeshwar hot springs, India. Molecular, phylogenetic and predictive functional characterization of the isolated bacterial population was done using 16S rRNA sequencing coupled with Tax4Fun tools. Furthermore, representative isolates were screened for important enzymes (cellulase, xylanase, amylase, and protease) and heavy metal tolerance (chromium, arsenic) properties. A total of 454 bacterial isolates obtained were mapped into 57 unique bacterial genera and 4 different bacterial phyla. Interestingly, 37 genera not previously isolated from Indian hot springs, were isolated for the first time in the present study. However, most of these genera (23 out of 37) were reported only in metagenomics studies from Indian and global hot springs. Furthermore, around 14 genera not previously cultivated and not detected in metagenomics studies of hot springs are documented here. The metabolic potential was ascertained by determining the abundance of specific genes using in silico based Tax4Fun tool, which identified around 315 metabolic pathways for metabolism of carbohydrates, synthesis of secondary metabolites and degradation of xenobiotic compounds. Bioprospection study revealed that 33 and 25 bacterial genera were positive for enzyme production and resistance to the heavy metals, respectively. The present study revealed the advantages of cultivation methods using a comprehensive multiple isolation approach for exploring untapped and unique bacterial diversity, and also utilities for various biotechnological and environmental applications.

Keywords Untapped · Bacterial diversity · Cultivation · Bioprospecting · Heavy metals

Introduction

Revolution in sequencing techniques has dramatically changed the trend of microbial studies of extreme environments, where sequencing instead of culturing became a major research focus for microbiological investigations (Gutleben et al. 2017). For the past few decades, natural hot spring habitats mostly have been studied for microbial analysis through metagenomic approaches, which lead to the identification of an astonishing microbial diversities of thermophiles (Hall et al. 2008; Miller et al. 2009; Costa et al. 2009; Kubo et al. 2011; Tobler and Benning 2011; Badhai et al. 2015). Furthermore, such studies have greatly expanded our understanding of the microbial ecology of previously uncultured microbes that can be present in extreme environments (DeLong and Pace 2001; Lewin et al. 2013;

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✉ Mahesh S. Dharne
ms.dharne@ncl.res.in

[1] National Collection of Industrial Microorganisms (NCIM), CSIR-National Chemical Laboratory, Pune, Maharashtra 411008, India

[2] Academy of Scientific and Innovative Research (AcSIR), New Delhi, India

Song et al. 2013). Nevertheless, pure microbial cultures are needed to decipher microbial physiology and to test the omics-based ecological hypotheses, which have enlightened the importance of culture-based methods in the 'omics' era (Gutleben et al. 2017). Besides, culture-based methods are useful for isolation and identification of taxonomically interesting groups of bacteria with high biotechnological potential (Prakash et al. 2013; Grivalský et al. 2016). The metabolic potential of an isolated organism can be more readily exploited through genome manipulation for use in different biotechnological applications (Pontes et al. 2007; Pham and Kim 2012). For instance, the remarkable discovery of thermophiles and subsequent use of thermo-stable enzymes for molecular techniques have dramatically changed the field of biotechnology (Brock and Freeze 1969; Podar and Reysenbach 2006). However, research efforts still seek isolation and exploitation of novel and uncultured microbial species from extreme environments such as geothermal springs. Though culturing of the microbial members of an uncultured fraction of microbial communities seems to be difficult, the use of diverse culture conditions possibly may provide an access to such previously hidden metabolic diversity (Grivalský et al. 2016). In addition, advanced molecular techniques used in combination with such diversified and high throughput culture-based methods may lead to the isolation of previously unexplored microbial community members (Vartoukian et al. 2010; Stewart 2012; Lagier et al. 2015).

India hosts around 400 hot springs and some of the hot springs studied were analysed for microbial diversity (Singh et al. 2016). Recently, some of the metagenomics based studies have identified and characterized microbial diversities of various hot springs present in different geothermal regions of India (Badhai et al. 2015; Mehetre et al. 2016a, b; Saxena et al. 2017; Gupta et al. 2017; Poddar and Das 2017). A few culture-based studies were also performed, mostly from Himalayan geothermal belt; such as Soldhar and Ringigad (Kumar et al. 2004; Pandey et al. 2015), Himachal Pradesh (Kumar et al. 2014a, b; Sahay et al. 2017), and from one other region, Tulsi Shyam from Gujarat (Kikani et al. 2015). However, these previous studies were not performed in a coordinated manner and more comprehensive studies are necessary to gain access to the microbial community composition and for understanding the microbial ecology of Indian hot springs (Poddar and Das 2017).

In the present study, an attempt was done to survey the bacterial diversity present in Unkeshwar hot springs located in the central part of India using varied culture conditions/media which may lead to identification and isolation of previously unexplored microbes from hot springs and for the future preservation of potential diversity for bioprospecting of such unique environments. Integration of sequence-based tools for predictive functional analysis and further screening for enzyme production, heavy metal tolerance properties were also evaluated. Furthermore, comparative analyses were performed with the previous metagenomics studies to analyse the results of both such culture-dependent and independent methods, which may be helpful for designing future studies in a coordinated manner.

Materials and methods

Site description and sampling

Unkeshwar hot spring is located at a geographical South East Deccan Continental basalt of India (19°34′–19°40′N and 78°22′–78°34′E) as shown (Fig. 1). This region falls within the Deccan volcanic province of India, and the hot spring lies at the eastern periphery within the Godavari rift valley, where hot water emerges through the basalt rock (Varun et al. 2012). Four water samples from the different portions of the hot spring (1 l each) were collected in November 2012 in sterile containers and transported to the laboratory within 12 h and stored at 4 °C for further analysis. Around 100 ml of water from each sample was mixed in a sterile bottle under sterile conditions and used for microbiological analysis. The same water sample was used for each microbiological analysis starting from the first day to around the 60th day of isolation experiments. For physicochemical analysis, 300 ml water from each sample was mixed and used for analysis.

Physicochemical analysis

During the collection of water samples, temperature and pH of the water were measured at the site using a digital thermometer and pH meter. The analysis of other physicochemical parameters such as total dissolved solids, organic matter content and ionic concentration of different elements was performed at a certified chemical testing laboratory (Accurate Analytical Laboratory Pvt. Ltd., Pune, India) following standard methods according to American Public Health Association (APHA 2012).

Direct isolation using multiple culture media

To isolate a diverse range of bacteria, varieties of culture media (from high nutrient content to low nutrient) were used. Commercially available low nutrient-containing media were also used. The following are the media used for bacterial isolation; Nutrient agar, International Streptomyces Project (ISP) medium (ISP1-ISP7), Reasoner's 2A agar (R2A), Starch Casein agar, Streptomyces agar, Bushnell Haas agar, Mueller Hinton agar, Thermus agar (peptone 0.5%, yeast extract 0.2%, beef extract 0.4%, NaCl 0.5%, agar 1.8%), Thermus agar dilutions (1:10; 1:100; 1:1000) and nitrogen

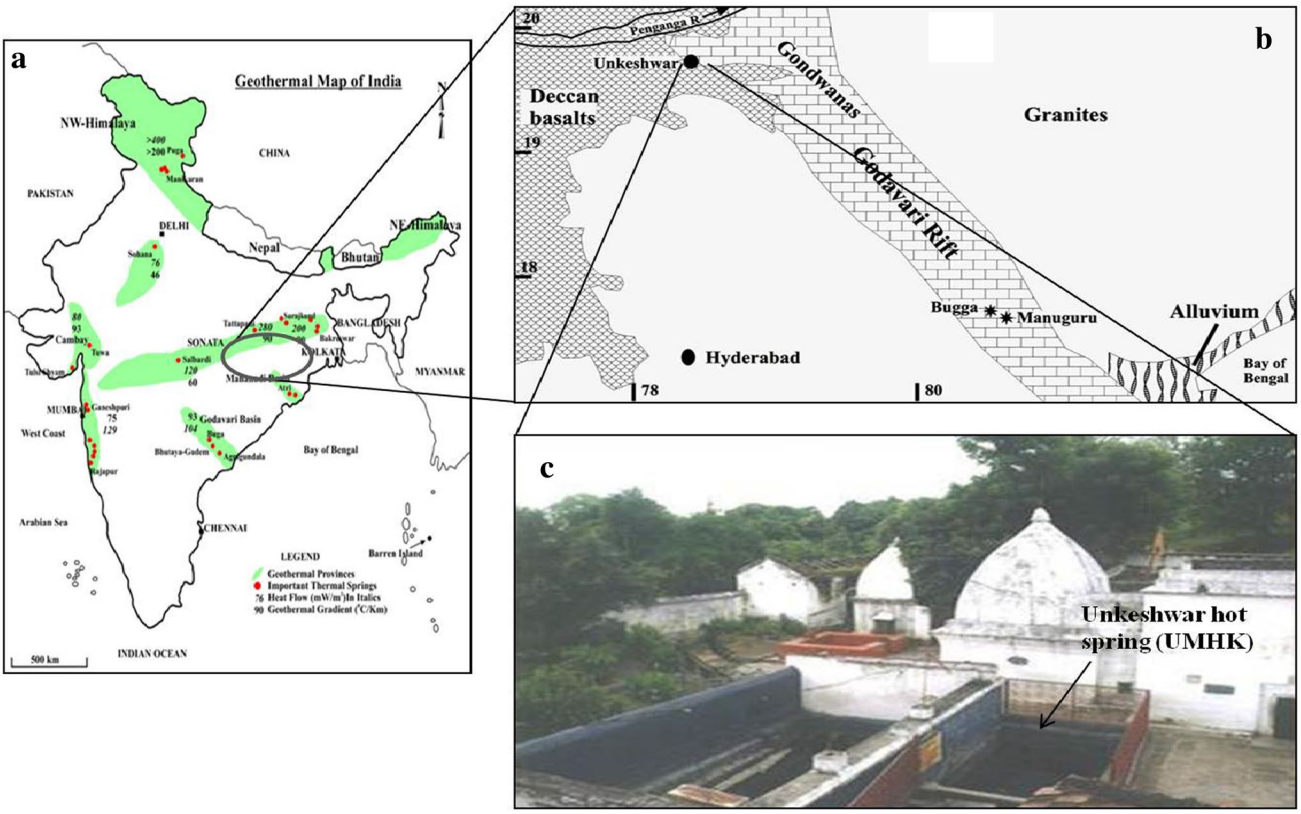

Fig. 1 Location of Unkeshwar hot spring. **a** Geothermal provinces of India. **b**: Deccan Basaltic region. **c** Sampling site of Unkeshwar hot spring (UMHK) (Modified from google.com)

fixing growth (NFG) medium (glucose 1%, calcium chloride 0.01%, magnesium sulphate 0.01%, dipotassium phosphate 0.09%, monopotassium phosphate 0.01%, calcium carbonate 0.5%, ferric chloride 0.001%, sodium molybdate 0.0005%, agar 1.8%). All the media used in the study were purchased from HiMedia (India) except Thermus agar and NFG medium. Chemical compounds: pyrene, anthracene, phenanthrene, fluorene were purchased from Sigma Aldrich (India) and all other chemicals used in this study were analytical grade.

Isolation using enrichment methods

Three enrichment methods were used; for physiological stress (temperature), artificial nutrient depletion (media autoclaving in each growth cycle) and chemical stress (phenol and polycyclic aromatic compounds like pyrene, anthracene, phenanthrene, and fluorene were employed). Briefly, for temperature enrichment, Luria Bertani (LB) broth was used. The water sample was pre-heated in a water bath at 50 °C for one hour before a 100 µl inoculum was added to flasks containing 100 ml LB broth. Flasks were incubated at 45 °C for up to 7 days. Subcultures from the same flask (100 µl broth) were added to fresh LB broth and incubated at 50 °C; the same procedure was followed up to 75 °C with 5 °C increments at each step and after every step 100 µl broth was used for plating. For nutrient depletion enrichment, 100 µl water samples were added in 100 ml LB broth and incubated at 37 °C up to 24 h and growth was observed visually as turbidity in the flasks. Subsequently, the same broth was autoclaved and utilized repetitively through the same procedure up to 3–4 times and the final broth was used for plating. Chemical stress enrichment was carried out using peptone water phenol medium (WP) containing (0.05–0.5% phenol) (Kanekar et al. 1998). The polycyclic aromatic hydrocarbon compounds (PAHs) enrichment was done in 100 ml mineral salt medium (MSM) (Singh et al. 2013) containing each pyrene (100 ppm), anthracene (10 ppm), phenanthrene (10 ppm), fluorene (10 ppm) and flasks were incubated at 37 °C for 15 days, and then 100 µl broth was used for plating.

While performing every experiment (direct and enrichment), incubated plates were observed for distinct colony morphologies after every 24 h of incubation up to the 7 days of incubations. Based on differences in colony morphology, new visible colonies that appeared on plates were selected for isolation. Distinct morphotypes were selected from

different plates and subsequently subcultured on respective media and stored as a glycerol stock.

Molecular identification and phylogenetic analysis

From the glycerol stock, isolates were subcultured, purity was checked using Gram staining and processed for DNA isolation using a standard phenol/chloroform/isoamyl alcohol (25:24:1) method (Sambrook et al. 1989). The isolated DNA was used for amplification of 16S rRNA gene using universal bacterial primers 16F27 (5′CCAGAGTTTGATCMTGGCTCAG3′) and 16R1525 (5′TTCTGCAGTCTAGAAGGGGTGWTCCAGCC3′). The PCR was carried out in a 50 µl reaction mixture containing 10 nM (each) primer (Eurofins), 200 µM (each) deoxynucleoside triphosphate (dNTP) (Genei), 1 U of Taq polymerase (Genei) in the appropriate reaction buffer, and 100 ng of DNA as a template. PCR conditions were 34 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min 30 s at 72 °C. PCR amplicons were purified using Exo-rSAP (USB) and sequenced for complete 16S rRNA gene in ABI 3500xl genetic analyzer (Invitrogen/Life Technologies) using internal primers to get appropriate overlaps of contigs (Weisburg et al. 1991). The 16S rRNA sequencing data of all the strains was edited using CHROMASLITE (version 1.5) and analyzed by Basic Local Alignment Search Tool (BLAST) with the closest cultured sequences retrieved from the National Center for Biotechnology Information (NCBI) database (Altschul et al. 1990) and EzTaxon server (Chun et al. 2007). The phylogenetic tree was constructed by a neighbor-joining method using Molecular Evolutionary Genetic Analysis (MEGA) 7 software (Tamura et al. 2013) of representative strains to determine the relationship among these strains. Representative sequences obtained in the present study were deposited in GenBank.

Predictive functional analysis using Tax4Fun

Predictive functional analysis based on 16S rRNA sequences of all the 454 isolates was performed using the Tax4Fun software package (Aßhauer et al. 2015). It uses its own precomputed association matrix, KEGG (Kyoto Encyclopedia of Genes and Genomes) organism functional profiles and 16S rRNA copy numbers obtained from NCBI database. An empirical pathways profile was created using Tax4Fun output by retrieving the complete list of pathways associated with each protein from the KEGG dataset and dividing the abundance from the Tax4Fun output. Then a class and subclass distribution of the predicted pathways was calculated using the pathways profile in a similar way by retrieving the class of each pathway from KEGG. Pathways profile, class and sub-class profiles were created using in-house scripts relying on KEGG REST API written in Perl and made available to the public on GitHub at https://github.com/ncim-ncl/metgenomics.

Screening for industrially important hydrolytic enzymes

Representative strains of 57 bacterial genera were screened qualitatively for production of important enzymes such as cellulase, xylanase, amylase, and protease. The following media were used for screening activity of each enzyme; Nutrient agar containing 1% carboxymethylcellulose was used for cellulase screening; for xylanase, Xylan agar (0.2% KNO_3, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.3% $CaCO_3$, 0.001% $FeSO_4$, 0.08% xylan. 1.5% agar) was used; for amylase nutrient agar with 1% starch (1% yeast extract, 1% peptone, 1% beef extract, 0.5% NaCl, 1% starch, 1.8% agar) and Casein agar (1% skimmed milk, 2% agar) was used for protease screening. The cultures were streaked on respective media plates and incubated at 37 °C for 48 h. The clear zone around the colony was considered as positive strains. However, for cellulase and xylanase clear zone was visualized after flooding the plates with an aqueous solution of 0.1% Congo red for 15 min and then washed with 1 M NaCl solution and for amylase, plates were flooded with Gram's iodine solution.

Screening for heavy metal tolerance

Heavy metal tolerance tests of all the representative strains were performed against chromium (Cr^{6+}) and arsenic (As^{3+}) based on the determination of minimum inhibitory concentration (MIC). The stock solution of both the heavy metals (potassium dichromate, sodium arsenate) was prepared and filter sterilized. Different concentrations (chromium: 50–1000 µg/ml and arsenic: 1–350 mg/ml) were added separately to individual tubes of 10 ml LB broth. The tubes were inoculated with 50 µl microbial cultures [1 optical density (OD) at 620 nm] and incubated at 37 °C for 24 h. Further, MIC was determined by checking the growth (OD) at 620 nm using a spectrophotometer (Systronics, India).

Results

Physicochemical analysis

The temperature of the water of the hot spring was 52 °C and the pH was 6.9. Analysis of other factors (Table 1) revealed that this hot spring has a higher concentration of phosphorus (87.24 mg/l) and sodium (234.72 mg/l) compared to the other hot springs as reported by Badhai et al. (2015), where the concentration of phosphorous was reported to be in the range of 2.3–3.9 mg/l and sodium was found in the range of 36–195 mg/l. However, other elemental concentrations

Table 1 Physicochemical properties of water samples collected from Unkeshwar hot spring

Sr. no.	Physicochemical parameters	Unkeshwar hot spring (UMHK)
1	Temperature (°C)	46
2	pH	7.1
3	Volatile dissolved solids at 550 °C (mg/l)	173
4	Total volatile solids at 550 °C (mg/l)	180
5	Total dissolved solids at 105 °C (mg/l)	507
6	Total solids at 105 °C (mg/l)	513
7	Volatile acids as acetic acid (mg/l)	157
8	Total organic carbon (TOC) (mg/l)	2.6
9	Total Kjeldhal nitrogen (mg/l)	2.59
10	Total phosphorus (PO_4^{3-}) (mg/l)	87.24
11	Nitrates as (NO_3^-) (mg/l)	1.91
12	Sodium as NaCl (mg/l)	234.72
13	Carbonates (CO_3^-) (mg/l)	57.03
14	Chlorides (Cl^-) (mg/l)	28.06
15	Ammonia (NH_3)(mg/l)	0.91
16	Sulphates (SO_4^-) (mg/l)	43.75
17	Sulphates as (S) (mg/l)	14.58
18	Calcium (Ca) (mg/l)	20.24
19	Cobalt (Co) (mg/l)	00
20	Nickel (Ni) (mg/l)	00
21	Boron (B) (mg/l)	0.0156
22	Magnesium (Mg) (mg/l)	1.47
23	Sodium (Na) (mg/l)	92.286
24	Potassium (K) (mg/l)	3.5
25	Iron (Fe) (mg/l)	00
26	Copper (Cu) (mg/l)	00

were found in similar ranges. Along with these two geochemical factors, other parameters such as total dissolved solids (507 mg/l), total volatile solids (180 mg/l), carbonates (57.03 mg/l), sulphur (43.75 mg/l) and magnesium (1.47 mg/l) were also found in higher concentrations as compared to the concentration of nitrogen (2.59 mg/l) and ammonia (0.91 mg/l).

Isolation and characterization of bacterial isolates

Among a total of 454 isolates, 290 were isolated with the use of multiple media with the remaining from enrichment techniques (temperature stress: 46, nutrient stress: 55; chemical stress: 63; phenol, 44 + PAH compounds, 19). The 16S rRNA gene sequencing and BLAST analysis at NCBI as well as EzTaxon database mapped all the isolates into 57 unique bacterial genera. All the representative sequences were deposited in NCBI database. The detailed analysis of representative strains with accession number is shown in (Table S1). In general, low nutrient-containing media (dilute nutrient media) were found to be useful for capturing the maximum number of genera (data not shown), whereas high nutrient media favoured the growth of few fast-growing bacteria, ultimately suppressing the growth of other slow-growing bacteria.

The taxonomic position of all the 57 bacterial genera showed that 56 genera (one genus was from an unassigned order) belong to 17 different taxonomic orders under 6 different classes in the four bacterial phyla, *Proteobacteria, Firmicutes, Actinobacteria*, and *Deinococcus–Thermus*. The relative abundance was highest for *Bacillales, Rhizobiales, Burkholderiales, Rhodospirillales, Caulobacterales, Micrococcales*, and *Streptomycetales* (Table 2). The phylum level distribution also showed that *Firmicutes* are more dominant (197 isolates, 7 genera); constituting a majority of the *Bacillus* spp. The second most dominant group was *Proteobacteria* with great taxonomic varieties (200 isolates, 30 genera); *Rhizobium, Azospirillum, Caulobacter, Pseudomonas, Cupriavidus, Hydrogenophaga, Phenylobacterium* comprised the maximum number of isolates along with some rare genera such as *Ramlibacter, Phretobacter, Methyloversatilis, Rhodobacter, Caldimonas, Methaloterigenea*. While in the case of *Actinobacteria*, the proportion of isolates was found to be more evenly distributed among genera (54 isolates, 24 genera) among which *Streptomyces, Arthrobacter, Micromonospora, Micrococcus, Microbacterium* and *Nocardioides* were found with a relatively higher number of isolates. Few genera with the least number of isolates were also obtained such as *Actinomodura, Cellulomonas, Cellulisimicrobium, Corynebacterium, Barrentisimonas* and *Dietzia*. Lastly, isolates belonging to phylum *Deinococcus–Thermus* were found in a low number (3 isolates) and within only one genus (Fig. 2).

The observed diversity obtained from this hot spring was compared with the past reports of microbial studies of other hot springs environments. The result indicated that, among these 57 genera, only 20 bacterial genera were well documented as cultivated from other Indian hot springs. However, the other 37 genera were not found to be reported as cultivated from hot springs and still were not explored for application studies from India. But, most of them were found in the reports of metagenomic analysis, which assures they are inhabitants of hot springs environments. More specific analysis revealed that, among these 37 genera, 13 were reported previously from Indian hot springs metagenomes, 10 were reported from other global hot springs metagenomes and 14 constitute some rare genera not previously found in microbiological analyses of hot springs (Table S2 and S3).

Phylogenetic analysis

Phylogenetic analysis revealed that all strains showed around 97–100% identity with sequences present in curated

Table 2 Relative abundance of the isolates with respect to the phylum, class and order at taxonomic levels

Phylum	Class	Order	No. of strains	Relative abundance (%)
Proteobacteria	α-Proteobacteria	*Rhizobiales*	82	17.62
		Rhodospirillales	26	5.7
		Caulobacterales	25	5.5
		Rhodobacterales	3	0.66
		Sphingomonadales	6	1.32
		Unassigned	1	0.22
	β-Proteobacteria	*Burkholderiales*	37	8.14
		Rhodocyclales	1	0.22
	γ-Proteobacteria	*Pseudomonadales*	16	3.52
		Enterobacterales	7	1.54
Actinobacteria	Actinobacteria	*Actinomycetales*	9	1.98
		Micrococcales	20	4.40
		Corynebacteriales	6	1.32
		Micromonosporales	5	1.10
		Propionibacteriales	4	0.88
		Streptomycetales	10	2.20
Firmicutes	Bacilli	*Bacillales*	197	42.95
Deinococcus–Thermus	Deinococci	*Deinococcales*	3	0.66

databases as shown in (Table S1). The phylogenetic relationship of the representative isolates of both Gram-positive and Gram-negative groups are shown (Fig. 3a, b). Here, we found an almost equal number of genera from both Gram-positive (27 genera) and Gram-negative (30 genera) bacteria. Among Gram-negative bacteria, 16S rRNA sequences of 3 strains showed 100% similarity to the type strains present in the database, 14 strains shared > 99% similarity, 8 strains shared > 98% similarity, whereas 4 strains shared > 97% similarity and one strain shared > 96% with cultured type strains present in the database (EzTaxon). In case of Gram-positive bacteria, strains belonging to *Firmicutes* shared 99–100% similarity, whereas 13 actinobacterial strains shared > 99%, 5 strains shared > 98% and 1 strain shared > 97% similarity with the cultured type strains present in the database.

Predicted functional analysis

Sequence analysis predicted the presence of 6235 molecules, of which 3364 molecules were assigned the KEGG ID. Our own in-house scripts identified 315 pathways from the KEGG database. Subsystem level classification of the pathways showed that metabolism being the most abundant followed by environmental information processing and genetic information processing. Further level categorization of metabolism, showed that carbohydrate metabolism was more dominant, very closely followed by amino acid metabolism. Interestingly, some of the KEGG subsystems were also mapped to the pathways for degradation of xenobiotic compounds and biosynthesis of secondary metabolites (Fig. 4), which indicates the strong potential of these microorganisms in biotechnological applications. Also, some KEGG subsystems were found associated with the metabolism of sulphur and methane, indicating a possible role of these organisms in biogeochemical cycling processes.

Screening for industrially important hydrolytic enzymes

Screening for hydrolytic enzyme production showed that around 33 genera were found positive for production of at least one enzyme (Fig. 5). Interestingly, among these 33 positive genera, 18 genera were from a set of 37 genera (Previously unexplored). Among these 18 genera, 11 were positive for cellulase, 5 for xylanase, 7 for amylase and 8 for protease. Overall results showed that *Bacillus* (U277) and *Streptomyces* (GH39) were found to produce all four enzymes, while other strains such as *Bhargavaea* (SA34), *Paenibacillus* (EM34), *Microbacterium* (UAC17), *Aeromicrobium* (GH118) and *Micromonospora* (GH99) were found to produce more than one enzyme. The enzymes cellulase and xylanase were mostly produced by actinobacterial strains, whereas *Bacillus* and related genera were found to produce amylases and proteases.

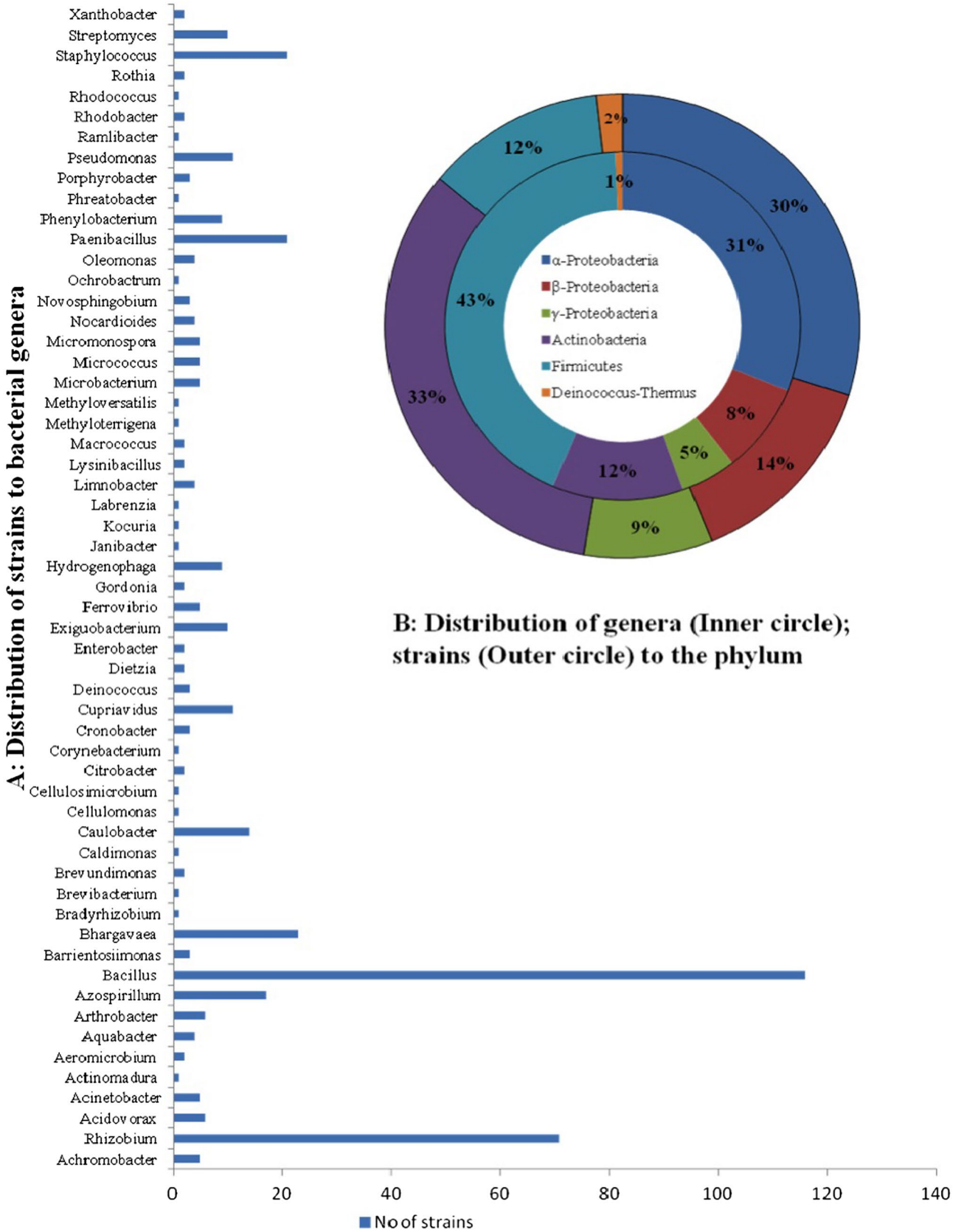

Fig. 2 Distribution of number of strains to each genus and phylum, **a** showing number of strains obtained for each genus; **b** percentage distribution of genus and strains to each phylum

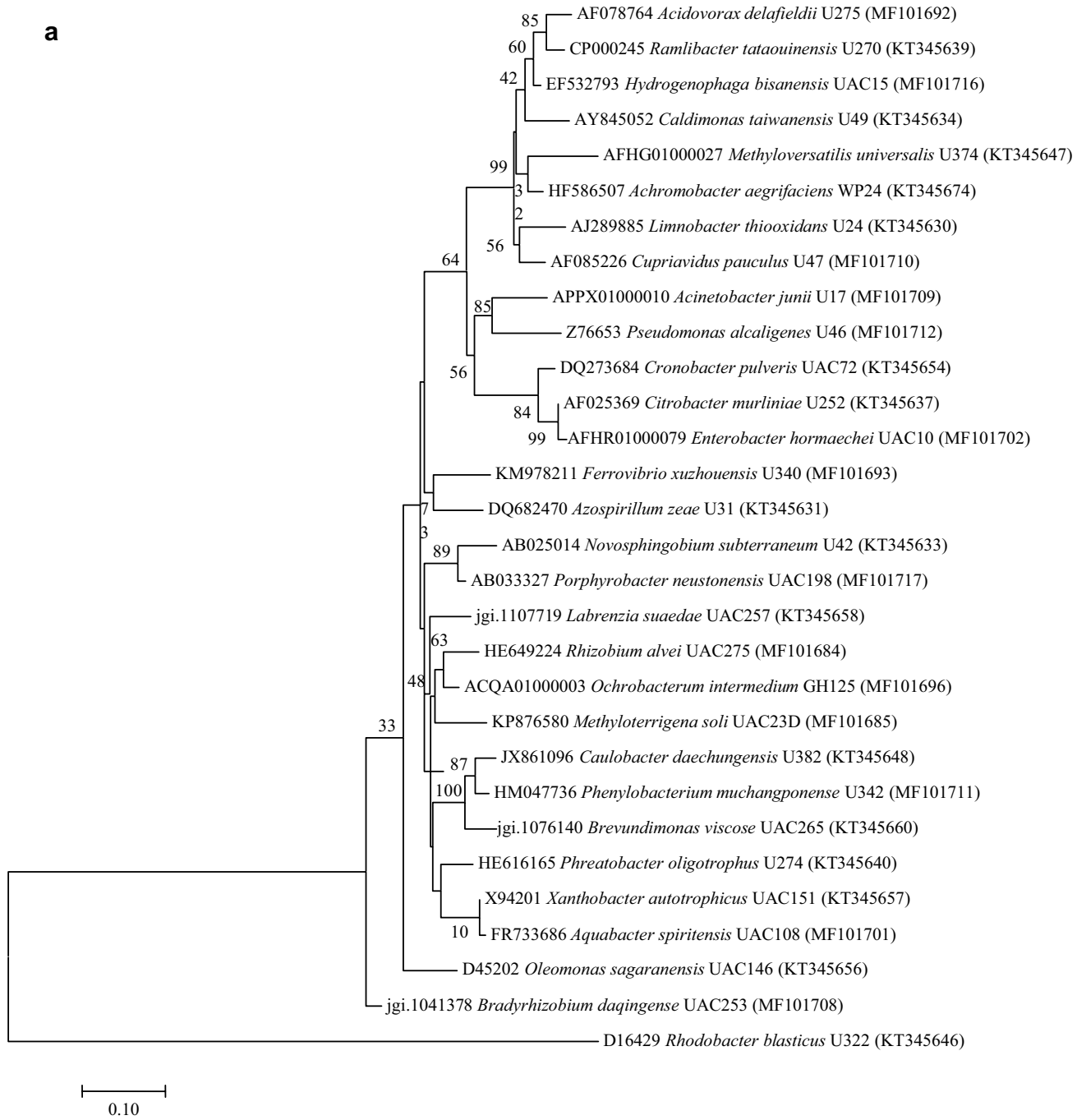

Fig. 3 **a** The Phylogenetic tree of the representative Gram-negative bacterial genera, constructed using the Neighbour-Joining method with bootstrap test (500 replicates) using MEGA7 software. **b** Phylogenetic tree of the representative Gram-positive bacterial genera, constructed using the Neighbour-Joining method with bootstrap test (500 replicates) using MEGA7 software

Screening for heavy metal tolerance

In the case of heavy metal tolerance, 21 genera were found to tolerate > 500 µg/ml chromium and 25 were able to tolerate > 10 mg/ml arsenic (Fig. 6). However, among these 12 and 17 were from a set of 37 genera which were underexplored taxa reported for tolerance of chromium and arsenic, respectively, for the first time. Overall, *Staphylococcus* (U23) (910 µg/ml) *Pseudomonas* (UAC46) (900 µg/ml), *Caldimonas* (U49) (830 µg/ml), *Hydrogenophaga* (UAC15) (700 µg/ml), *Streptomyces* (GH39) (700 µg/ml), *Exiguobacterium* (SA64) (700 µg/ml) and *Caulobacter* (U382) (650 µg/ml) were found to be tolerant to chromium. For arsenic, strains such as *Microbacterium* (UAC17) (190 mg/ml),

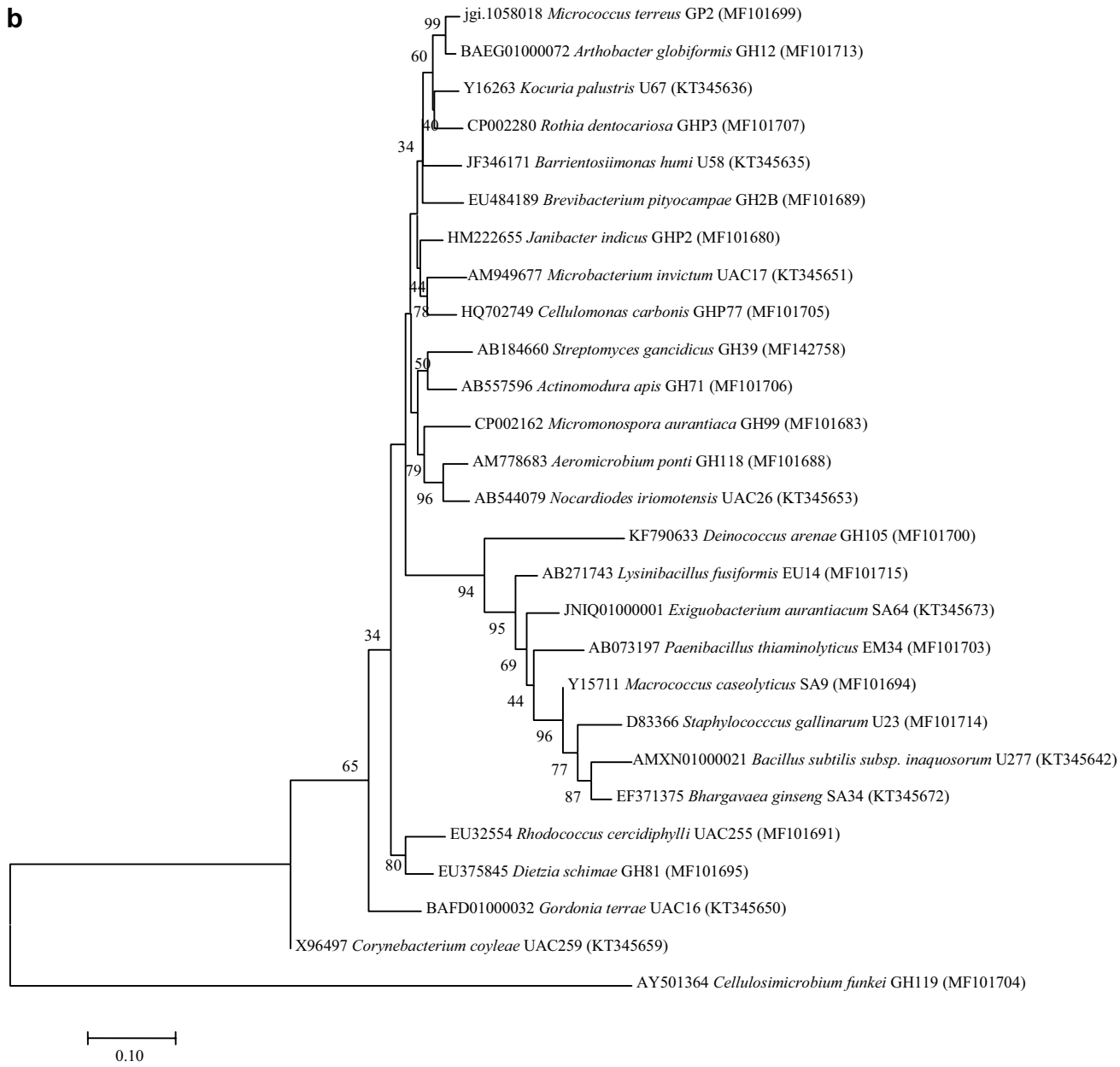

Fig. 3 (continued)

Acinetobacter (U17) (145 mg/ml), *Pseudomonas* (UAC46) (125 mg/ml), *Azospririllum* (U31) (105 mg/ml), *Rhodococcus* (UAC255) (105 mg/ml), *Streptomyces* (GH39) (100 mg/ml) and *Cupriavidus* (U47) (100 mg/ml) were found to be the most highly tolerant strains.

Discussion

Exploring the culturable diversity from hot springs is an important aspect of the understanding microbial ecology of such environments and also holds a great interest for bioprospecting. In the present study, an attempt was done to investigate the microbial diversity using multiple cultivation techniques from Unkeshwar hot springs and in an effort to compare it with recent metagenomics based studies reported from other Indian hot springs. Unkeshwar hot spring was found to be rich in minerals with a moderate level of temperature (50–60 °C). The temperature and other physicochemical factors were known to have a greater impact on microbial compositions in hot springs environments. Generally, with an increase in temperature, microbial diversity decreases (Wang et al. 2013; Sharp et al. 2014; Badhai et al. 2015) and moderate temperature range hot springs could support

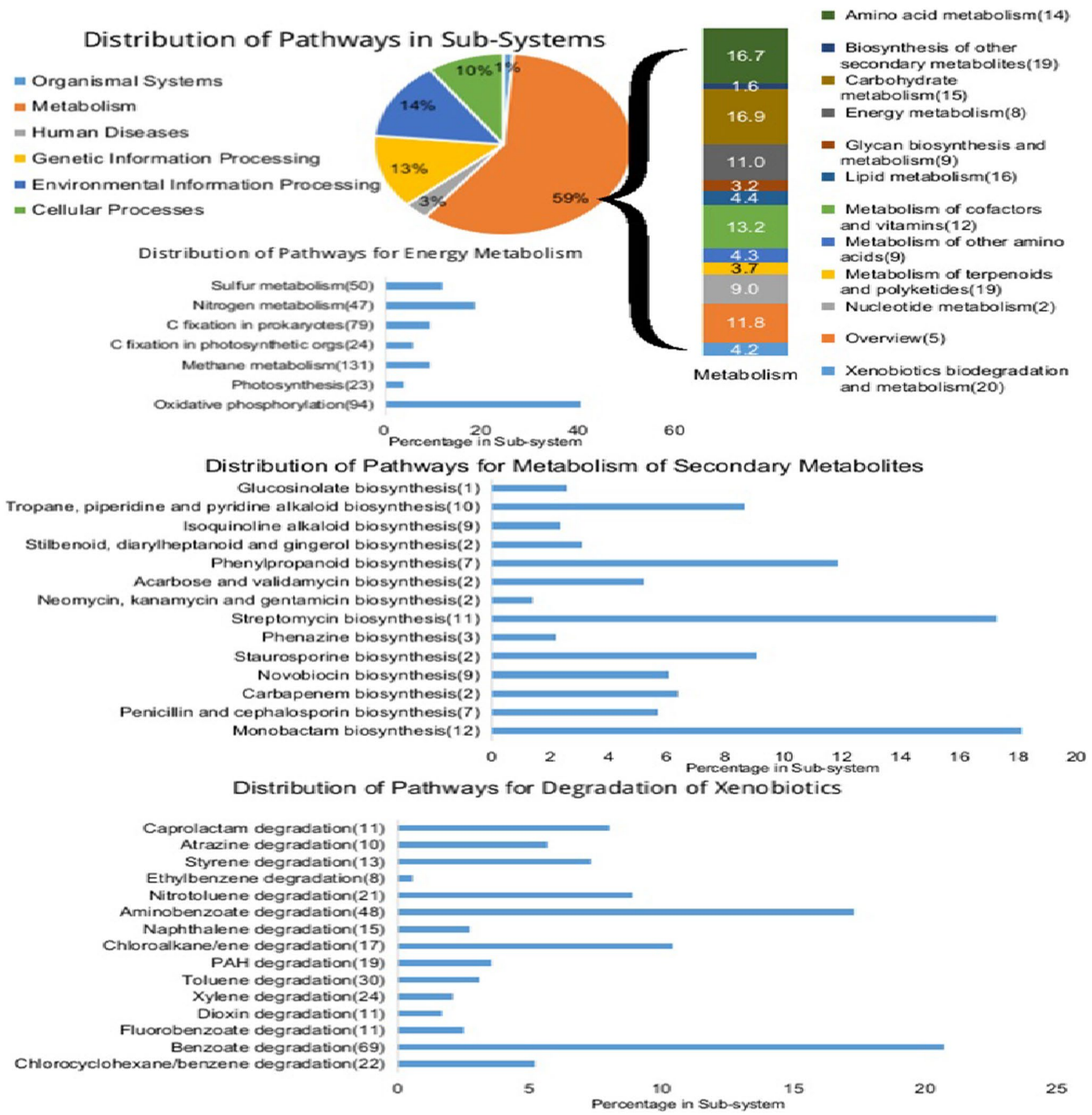

Fig. 4 Predictive functional analysis using Tax4Fun tool, showing relative abundance of assigned KEGG identity to the pathways of metabolisms at subsystem levels

high microbial diversities. In this study also great varieties of bacterial genera were isolated which could indicate that the Unkeshwar hot spring holds a taxonomically diverse bacterial diversity that may be due to its moderate temperature range. For example, most of the bacterial genera cultured in the present study were found to be moderately thermophilic to mesophilic in nature, and such bacterial populations were frequently observed in the terrestrial hot springs having a temperature of less than 75 °C (Lau et al. 2009; Wang et al. 2013). Furthermore, other physicochemical factors such as total organic carbon, sulphur and magnesium which were found with relatively higher concentrations may also have contributed to the presence of diverse microbial flora. For example, total organic carbon and magnesium may have supported the growth of hydrolytic enzyme-producing bacteria. The organic content can support the growth of heterotrophic

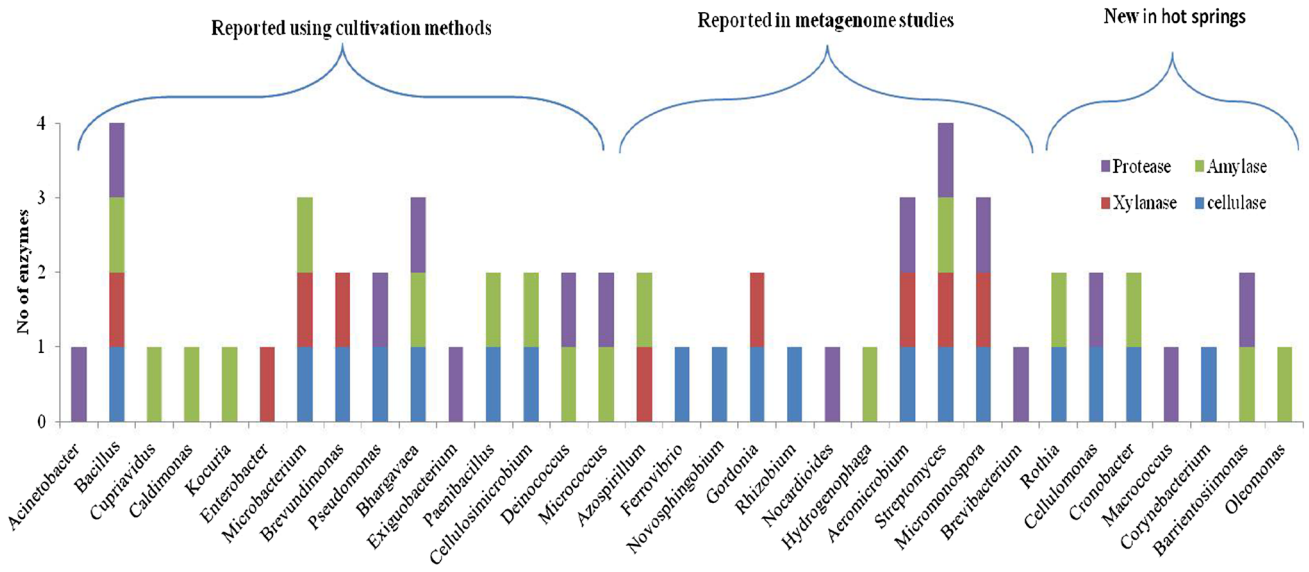

Fig. 5 Positive bacterial genera for production of important hydrolytic enzymes; cellulase, xylanase, amylase and protease production

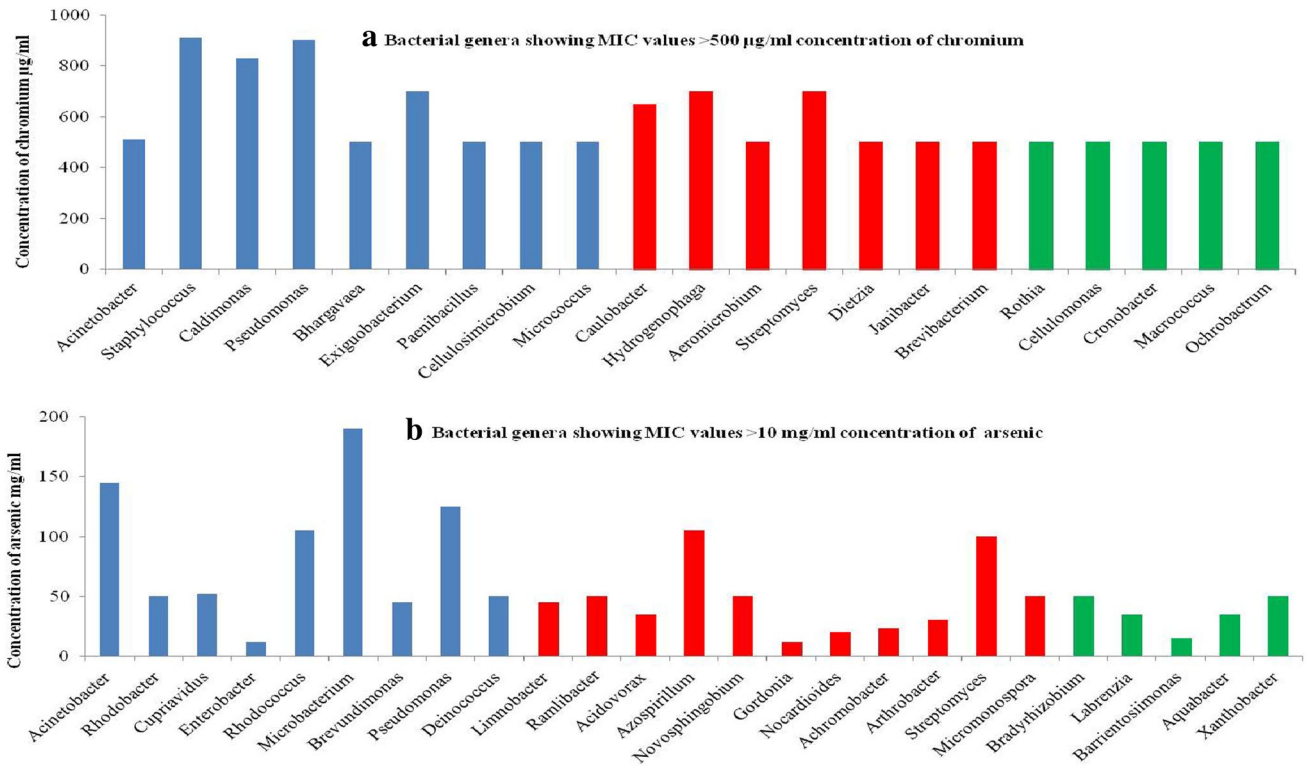

Fig. 6 Representative bacterial genera with MIC values for chromium and arsenic tolerance; blue colour bars represent isolates which were also previously cultivated from other hot springs, red colour represents those only detected in culture independent previous studies from other hot springs, green were first time isolates reported from hot springs. (Colour figure online)

bacteria in the hot springs as revealed in some of the previous studies (Chan et al. 2015; Saxena et al. 2017). The enzymatic activities of some of the microorganism may require magnesium ions, which, therefore, may affect the compositions of such enzyme-producing microbes in hot springs (Grivalský et al. 2016). However, the sulphur generally present in the form of sulphate or sulphite in hot spring environments have reported greater impact on microbial

composition in such environments (Purcell et al. 2007). The genus; *Limnobacter* a thiosulfate-oxidizing heterotrophic bacterium was also found to be present in this hot spring. Such correlations among physicochemical factors and microbial diversity could help in the understanding of the microbial ecology of these environments. Though the culture-based studies could not possibly explain such correlations in a better ways, such information could aid for better amendment of media compositions for isolations of different bacterial groups from such hot environments. With culturing, media composition and laboratory conditions are the most important factors for isolations of microbial groups from environmental samples. In addition, most of the bacterial genera were recovered using low nutrient media compositions such as diluted and R2A agars. Such low nutrient media were often found to be useful for isolations of rare bacteria genera from environmental samples (Vartoukian et al. 2010).

The general occurrence of the genera isolated from this hot spring was evaluated by comparison with the previous studies performed using both culture-dependent and independent methods. Such comparisons revealed some interesting attributes of microbial diversities of hot spring environments. Most of the genera were found to be common inhabitants of hot spring environments as many of them were detected by previous metagenomic analysis of such environments but still were not cultivated. Among all the bacterial genera obtained, only 20 different genera were found to have been previously isolated from hot springs. Some were only detected by culture-independent analysis, while others were reported for the first time in this study from hot spring environments. However, in the present study, those genera that matched with previously cultivated hot spring isolates were mostly found to belong to the *Firmicutes*, and other members of *Actinobacteria* and some *Proteobacteria*. Among them, *Firmicutes* mostly were found to be previously cultivated from high-temperature hot springs. For example, genera such as *Bacillus, Paenibacillus* (Singh et al. 2010; Pandey et al. 2015; Sahay et al. 2017); *Lysinibacillus, Exiguobacterium, Bhargavaea* and *Staphylococcus* (Kumar et al. 2014a, b) have been isolated from Himalayan geothermal hot springs with the temperature range of 90–95 °C. The predominant occurrence of *Firmicutes* and some other actinobacterial members such as *Microbacterium, Kocuria, Micrococcus* and *Cellulosimicrobium* in such high-temperature hot springs reveals that these groups of bacteria possess high-stress tolerance properties (Cerritos et al. 2011; Kumar et al. 2014a; Sharma et al. 2014). Among the members of *Proteobacteria, Acinetobacter* and *Pseudomonas* are the ubiquitous genera and they were also found to be present in hot springs environments. However, the genus; *Caldimonas* is a thermophilic bacterium and still now isolated only from hot springs and represented only by two valid species (Takeda et al. 2002; Chen et al. 2005). However, some other thermophilic species were also reported from hot springs having the moderate temperature range (Rakshak et al. 2013). The genus *Caldimonas* particularly has attracted more attention as members of *Caldimonas* are thermophilic and have various metabolic properties; for example, one of the species reported by Takeda et al. (2002) has the poly (3-hydroxybutyrate) (PHB) degradation as well as manganese oxidation abilities, while other species are reported to produce amylase enzyme (Chen et al. 2005). Another genus, *Rhodobacter*, is rarely found to be associated with hot springs environments. However, they can obtain energy through photosynthesis, and therefore, some species of this genus are used as model organisms to study bacterial photosynthesis (Imam et al. 2013).

Interestingly, with the help of diverse culture media and conditions around 37 bacterial genera were cultivated for the first time from hot springs of India. Most of them found to belongs to *Proteobacteria* (22 genera) and *Actinobacteria* (14 genera) and only one was found from *Firmicutes*. However, some of these genera were found to be present in other hot springs but were reported by metagenomics studies. For instance, 13 genera; *Ramlibacter, Acidovorax, Caulobacter, Novoshingobium, Gordonia, Nocardioides, Hydrogenophaga, Porphyrobacter, Achromobacter, Arthobacter, Dietzia, Streptomyces*, and *Brevibacterium* were previously identified from different Indian hot springs using omics-based techniques. Our previous such metagenomics studies (Mehetre et al. 2016b) of the same hot spring showed a match of only four genera; *Caulobacter, Achromobacter, Acidovorax*, and *Novosphingobium* of the present study. Interestingly, members of the group *Actinobacteria* such as *Gordonia, Nocardioides, Arthobacter, Dietzia, Streptomyces* and *Brevibacterium* were reported in the metagenomics analysis of high temperature (90–95 °C) hot springs (Sharma et al. 2017). The temperature tolerance range of these genera seems to be wide, as they were isolated in this moderate level temperature hot spring which possibly could reveal that the moderate temperature and dilute nutrient media might be the necessary conditions for their isolation from hot spring environments. Among the group members of *Proteobacteria*, genera such as *Hydrogenophaga, Porphyrobacter, Achromobacter* and *Ramlibacter* were found to be present in hot springs of wide ranges of temperature (Sangwan et al. 2015; Badhai et al. 2015; Panda et al. 2016). Among them, *Porphyrobacter* has been reported from various habitats but mostly from freshwater and hot spring environments. The members of this genus are known to contain bacteriochlorophyll and some species were found to have toxic environmental pollutant degradation potentials (Hiraishi et al. 2002). Another genus, *Ramlibacter*, was also detected in higher abundance in hot springs present in central India (Saxena et al. 2017). Presently the genus is represented by four valid species and

most of them were isolated from sub-desert soil or forest soil. The species isolated from desert environments were found to form a cyst and reported as a chemoautotrophic bacterium (Heulin et al. 2003). However, their dominant occurrence in hot spring habitats revealed in recent studies by Saxena et al. (2017) and their isolation from the present hot spring indicate that they may have some ecological role in such hot environments.

Although out of 37 bacterial genera, only 13 were found to be present in other Indian hot springs which was revealed through metagenomics studies. Surprisingly, another ten genera were matched with the microbial composition at the genus level of recently studied hot springs of Pakistan ranging in temperature from 60 to 95 °C (Amin et al. 2017). Interestingly, these hot springs of Pakistan were also mapped in the Himalayan geothermal belt, in which other Indian hot springs were located; however, we could not find these ten genera in these Indian hot springs. The possible reason for such a difference in abundance of microbial communities of hot springs may be due to the physicochemical factors, as some of the sites of these Pakistan hot springs were reported with high bicarbonates, which was also, evidenced the Unkeshwar hot springs through the previous lithological studies (Varun et al. 2012). However, such results also suggest that the sampling size could be one of the reasons for such differences, for instance, during the analysis of Pakistan hot springs the authors used around nine different samples from three different sites for metagenomics analysis.

Among those genera that were matched with Pakistan hot springs were members of *Proteobacteria* and *Actinobacteria*. Some of the genera such as *Limnobacter, Phenylobacterium, Methyloversatilis*, and *Rhizobium* of *Proteobacteria* were found to be interesting, as they possibly play important role in biogeochemical cycling in hot springs environments. For example, still now only two species of *Limnobacter* were reported and both are heterotrophic bacteria with the ability to oxidize thiosulfate to sulphate, one was isolated from the littoral zone of a freshwater lake and another from volcanic deposits (Lu et al. 2011). Another genus; *Methyloversatilis* is a facultative methylotrophic bacterium and can use methanol or methylamine as the source of carbon and energy (Kalyuzhnaya et al. 2006). Most of the strains of *Phenylobacterium* were isolated from soil and they can grow optimally only on artificial chemical compounds like chloridazon and antipyrin. However, one thermophilic species was isolated from the subsurface thermal aquifer and grows best with yeast extract as the sole carbon and energy source (Kanso and Patel 2004; Eberspächer and Lingens 2006). Among the actinobacterial genera; *Aeromicrobium, Micromonospora*, and *Janibacter*, the species of *Janibacter* were isolated from various extreme habitats including hydrothermal sediments of the ocean (Zhang et al. 2009) to the upper atmosphere (Shivaji et al. 2009). Overall, out of 57 genera, only 20 genera were found to be previously isolated from other hot springs and the remaining 37 genera were isolated for the first time from Indian hot spring environments among which 13 were detected in the previously performed metagenomics studies of other Indian hot springs. However, around ten different genera were detected in recently studied hot springs of Pakistan, several factors such as temperature, sampling size; geographical locations may explain their absence in the previous culture-based and metagenome analysis of Indian hot springs.

The overall analysis, to the best of our knowledge, revealed that around 14 bacterial genera were reported for the first time from hot springs environments. Although some of them may not be endemic to the hot springs environments, as they might have migrated from other sources to the hot spring. However, their fraction was found to be very low compared to the overall number of bacterial genera isolated. Interestingly, other genera were found to be rare genera, and therefore, could be natural bacterial inhabitants of such environments, but were missed in previous studies of both culture-based and metagenomics approaches. Several factors might account for their absence in previous studies. One possible reason is that they may represent a minor fraction of microbial communities in such environments and still were reported with a very low number of strains. For example, *Methyloterrigena*, which is a newly isolated genus, has only one species. The genus *Barrientosiimonas* is also found to be represented by only one species and was isolated from Barrientos Island in the Antarctic (Lee et al. 2013). An increase in the number of species and strains of such rare genera could help to assign precise taxonomy to such species which might be possible through the use of culturomics techniques. It was also observed that some other genera were taxonomic reshuffled genera with no proper presence of type species which could also limit their identification and isolations of closely associated species of such taxa. For instance, *Aquabacter, Labrenzia* and *Phreatobacter* and *Oleomonas* are taxonomically reshuffled genera and most of them were found to be represented by few numbers of species. However, most of them were reported from various types of environments and with different metabolic abilities. The genera *Methyloterrigena* and *Aquabacter* are members of the *Hyphomicrobiaceae* family and *Methyloterrigena* can metabolize C1 (methanol) compounds (Kim et al. 2016). Another genus, *Phreatobacter*, has not yet been assigned to any family, and species of this genus were reported to be oligotrophic in nature, which makes isolations from environmental samples difficult. So far, only one species has been isolated from ultrapure water samples (Tóth et al. 2014). *Oleomonas* species, isolated from the oil fields environment, were reported for their abilities to grow on aromatic compounds such as toluene, benzene and phenol, and aliphatic hydrocarbons (Kanamori et al. 2002). Another genus, *Cellulomonas* has

the ability to degrade cellulose and was isolated from alkaline environments (Jones et al. 2005).

During comparative analysis, it was also observed that both the culture-dependent and independent methods capture different portions of microbial communities from the hot springs environments. The occurrence of such differences was also found in previous studies from other types of environments (Stefani et al. 2015). Different factors can account for such differences in the microbial analysis since proper physicochemical sources and nutrients are needed for the growth of a microorganism; these factors possibly facilitated the cultivation of some organisms (Pham and Kim 2012). While in the case of culture-independent studies, bias analyses of predominance give rise to different microbial portions (Poretsky et al. 2015). However, as observed in the present study, most of the bacterial genera detected in culture-independent studies could be cultivated with the help of diversifications of culture conditions. In addition, such comparative analysis may also be helpful for ecological and bioprospecting studies of hot springs environments. In addition, it also makes reorganization of other factors such as sampling size, physicochemical factors important while analyzing microbial diversities or culturing them from such robust environments. Therefore, microbial investigation of such environments should follow in a coordinated way using both methods, where data obtained from advanced metagenomics approaches possibly could help to design diverse cultivation strategies using high throughput in order gain access to previously hidden metabolic diversity. In recent years, such efforts are accomplished through the use of 'culturomics', a high-throughput approach that multiplies culture conditions to increase the culture population of bacterial species (Stewart 2012; Lagier et al. 2015). Use of such techniques apparently has greater advantages, as it allows access to rare population present in the ecosystem, also could provide a large number of strains for newly identified bacterial species for the greater elucidation of metabolism and viability of microbial species (Greub 2012). Furthermore, this facet of microbial ecological studies will change using culturomics as these techniques allow studying the interaction between microbial species of a particular habitat, which helps to increase our knowledge about microbial ecosystem dynamics (Greub 2012; Gibbons and Gilbert 2015). More importantly, these techniques could help to design appropriate culture conditions for newly isolated bacteria which further can be found useful with some variation of parameters for isolation of previously uncultured species. Presently, such an approach is being used for the description of human-associated micro-flora, and 1000 human-associated bacterial species including 247 new species have been isolated and described in a short time (Lagier et al. 2016; Abdallah et al. 2017). In the coming years, such techniques will possibly be used for other types of environments, as different types of these techniques have been employed for marine and aquatic environments (Gao et al. 2013). Furthermore, use of culturomics in the future will possibly add a large number of bacterial species to the databases which can also pose a challenge inevitably for present bacterial taxonomic approaches (Rosselló-Móra et al. 2017; Abdallah et al. 2017). However, use of such complementary approaches of both culturomics and metagenomics in coordination and with in-depth investigations will possibly change the face of microbial ecology studies, which is the need of future microbial research (Ma et al. 2014; Gutleben et al. 2017; Poddar and Das 2017).

Here, we tried a combination of both such methods for functional analysis; tools generally used for predictive functional analysis of microbial communities were instead used for cultured isolates to assess their functional potentials. Such assessment reveals an abundance of genes for metabolic pathways, among which a number of pathways were mapped to the production of secondary metabolites, and xenobiotic compound degradations which could further help to harness the metabolic potential via bioprospecting. For example, the pathways mapped for secondary metabolites synthesis such as polyketides indicates that possibly some of the isolates could have such metabolite production abilities. In accordance, the genera having such biosynthetic potential can have applications in the production of antibiotics or other bioactive compounds. For instance, genera such as *Actinomodura, Micromonospora, Streptomyces* obtained from this hot spring were also isolated previously from hot springs with the presence of such biosynthetic genes (Liu et al. 2016).

The hot spring microbes are usually sought in bioprospecting for hydrolytic enzymes due to their thermo-stability and various studies have reported such enzymes from these environments, still, further research effort is necessary to find suitable enzymes which will meet the industrial demands (Niehaus et al. 1999; Obeng et al. 2017). From the present study, those were previously not reported for such applications from hot springs were also found to produce enzymes. It is also observed that group members of *Firmicutes* and *Actinobacteria* have greater abilities for enzyme production than members of the *Proteobacteria*. This could be informative as recently more attention was given to members of thermophilic *Actinobacteria* for production of such enzymes (Shivlata and Satyanarayana 2015; Liu et al. 2016). Among the genera which seem to be previously underexplored from hot springs for enzymes productions such as *Azospirillum, Ferrovibrio, Novosphingobium, Gordonia, Rhizobium, Nocardioides, Hydrogenophaga, Aeromicrobium, Micromonospora, Brevibacterium* were found positive for at least one enzyme. Although, further biochemical and thermo-stability of enzymes needs to be evaluated to have a possible new microbial source for industrial applications. However, other genera such as *Cellulomonas, Streptomyces*

were reported for cellulase production (Goswami et al. 2016; Liang et al. 2014) and *Bacillus, Exiguobacterium* and *Bhargavaea* for amylase and protease production, among them *Exiguobacterium* was found to be an important member for such enzyme productions (Kasana and Pandey 2017).

In the predictive functional analysis, pathways were also mapped for degradation of xenobiotic compounds which could reveal the potential use of the isolated strains for environmental applications. In general, microbial species from such environments have the ability to survive under various stress conditions including heavy metal stresses. Recently, microbes with the ability to oxidize arsenate were reported from Jinqing hot spring (Yang et al. 2017). From the present hot spring, around 25 bacterial genera were found to tolerate higher concentrations of both the heavy metals employed in the study. However, here we found more such genera for heavy metal tolerance from *Proteobacteria* along with some actinobacterial members. This may be due to differences in the mechanisms used by Gram-positive and Gram-negative bacterial strains for heavy metal stress tolerance. The Gram-negative bacteria can show tolerance to heavy metal tolerance via either by oxidation–reduction of heavy metal or by efflux mechanisms (Silver and Misra 1984). Genera such as *Caulobacter, Hydrogenophaga, Aeromicrobium, Streptomyces, Dietzia, Janibacter, Brevibacterium, Limnobacter, Ramlibacter, Azospirillum* and *Micromonospora* had shown such tolerance properties, and most of them were previously explored for such applications from hot spring environments and further understanding of the metabolic potential of the present isolates possibly will help find more efficient strains for use in environmental applications. However, more studies regarding taxonomy, biochemistry and genetics are necessary to identify and fully exploit these microorganisms.

Conclusions

The present study reports the isolation of diverse types of bacterial taxa from Unkeshwar hot spring of India, which were largely underexplored or missed in previous studies of Indian as well as global hot springs. This study also shares an interesting aspect in basic microbiology that the use of multiple isolation techniques, especially using low nutrient media could be useful for isolating the diverse range of microbial taxa including some rare bacterial genera from hot spring environments and probably from other extremophilic niches too. Additionally, it also sheds a light on consideration of other factors such as sampling size, physicochemical factors while culturing the microbes from such hostile environments. Metabolic potential of observed diversity revealed that many bacterial genera isolated could be potential sources for the production of industrially important enzymes, also in the environmental application for management of heavy metal stresses. In summary, our study promotes the use of larger scale microbial cultivation studies for isolating the unexplored species missed previously, which can be further exploited in both basic and applied microbiology. However, still newer cultivation approaches need to be applied for gaining increased access to a greater part of bacterial communities in hot spring environments.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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