Journal of Applied and Natural Science
16(3), 987 - 997 (2024)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

Research Article

Comparative metagenomic analysis of bacterial diversity in three distantly related soils in India

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Article Info
https://doi.org/10.31018/jans.v16i3.5635
Received: April 12, 2024
Revised: June 27, 2024
Accepted: July 07, 2024

How to Cite
Sankhwar, R. *et al.* (2024). Comparative metagenomic analysis of bacterial diversity in three distantly related soils in India. *Journal of Applied and Natural Science*, 16(3), 987 - 997. https://doi.org/10.31018/jans.v16i3.5635

Abstract
Soil microbial diversity is a vital factor for the progression of vegetation and biogeochemical cycles in an ecosystem. It is affected by the chemical composition, soil microenvironment and anthropogenic activities. The present study investigated the effect of environmental conditions and anthropogenic activities on the bacterial diversity of three distantly related soils in India. The multiple soil samples were collected from Hospital waste sites (BTC2) with extensive anthropogenic activity, Himalayan forest soil (RM1) with low anthropogenic activity and Tso Kar Lake sediment soil samples with negligible anthropogenic activity with environmental factors such as soil pH, temperature and altitude on the bacterial diversity. The soil samples were analyzed for physico-chemical properties that suggest significant variations in pH, electrical conductivity (EC), total dissolved solids (TDS), temperature and altitude. The SEM-EDS (Scanning electron microscopy with energy-dispersive X-ray spectroscopy) analysis revealed the elemental composition of the soil samples. The bacterial diversity of three different soil samples was studied using 16S rRNA sequencing-based metagenomic analysis. The results suggested that the bacterial diversity in Hospital waste site soil samples was higher than in the other two soil samples based on chao1 (richness and evenness) analysis. The Phyla Firmicutes were more abundant in Hospital waste site soil, and Himalayan forest soil showed a higher abundance of phylum Proteobacteria (28.86%) and Actinobacteria (26.70%). Tso Kar Lake sediment soil samples showed the most abundant phylum as Proteobacteria (46.28%). The study suggests that increased anthropogenic activity increases soil bacterial diversity. It may eventually help to develop new approaches for sustainable land-use management practices.

Keywords: Anthropogenic activity, Bacterial diversity, Himalayan forest, Metagenomics, Tso Kar Lake

INTRODUCTION

Soil forms the upper crust of the earth, which is extremely important for a healthy ecosystem. Microorganisms play a critical role through various biogeochemical cycles, the decomposition of waste materials, bioremediation, and enrichment of organic matter that promote plant growth and can anticipate observable changes in soil attributes (Chodak *et al.,* 2013; Sharma and Kaur 2021). Understanding the soil's chemical, physical, and biological components is essential for maintaining soil fertility and sustainable agriculture (Sharma and Kaur 2021; Singh *et al.,* 2023).

Bacteria form the major part of soil microbes and typically represent half of the total microbial biomass of soil (Roesch *et al.,* 2007). Bacteria help to form soil by aggregation of soil microparticles that enhance the water-holding capacity (Ingham, 2009). To preserve the integrity of the plant-soil ecosystem, microorganisms are involved in regulating the ecological processes that

influence plants and soil (Huet *et al.,* 2023).

Traditional culture methods could only identify the culturable bacteria in the laboratory conditions. However, metagenomics helped the researcher identify the non-culturable and culturable bacteria directly from soil samples (Handelsman, 2004). Geographic location environmental factors such as temperature, pH, rainfall and anthropogenic activity mainly affect the soil microbial diversity. Himalaya is one of the geographic locations that holds the highest glacial coverage after the polar regions (Khan *et al.,* 2017). Typically, Himalaya are classified into eastern and western Himalaya. The Ladakh and Uttarakhand forest region that was used in this study belongs to the western Himalayas. Meanwhile, the Varanasi region, situated at the bank of the Ganga River, belongs to the northern mainland of India. Distinct geographic regions in Himalaya have climatic dissimilarities that make them hot spot for plants and microbial biodiversity (Manish and Pandit, 2018). The Himalayan forest samples were collected from Ramnagar (Uttarakhand), a great reservoir of natural antimicrobial agents produced by bacteria and rich in Actinomycetes (Sankhwar *et al.,* 2023). The Tso Kar lake, located in the southern part of Ladakh in the western Himalayan ranges, is a salt lake with large size and depth with negligible anthropogenic activity. The bacterial community is complex and diverse at high altitudes and mainly belongs to psychrophiles, which can survive in extreme weather (Shen *et al.,* 2015).

Hospital waste sites are usually polluted with biomedical and non-biomedical wastes due to dumping of drugs, medical devices, food items and non-biohazard materials (Bottero *et al.,* 2015; Capolongo *et al.,* 2016). All these components may potentially support the survival and growth of various biological agents and pathogens. The hospital environment has high infective risk, making the immunocompromised persons more vulnerable to bacterial, viral, parasitological and fungal infections (D'Alessandro *et al.,* 2016). The bacterial communities that persist and how they change in the soil due to anthropogenic activity are of great concern for public health management. Himalayas are one of the locations with the highest glacial coverage after the polar regions (Khan *et al.,* 2017). Typically, Himalaya are classified into eastern and western Himalaya. Ladakh and Uttarakhand forest regions belong to the western Himalayas. Whereas, the Varanasi region, situated on the bank of the Ganga River, belongs to the northern mainland of India. Distinct geographic regions in the Himalaya have climatic dissimilarities that make them hot spots for plants and microbial biodiversity (Manish and Pandit, 2018). The Himalayan forest samples from Ramnagar (Uttarakhand) have been studied, which are a great reservoir of natural antimicrobial agents produced by bacteria and rich in Actinomycetes (Sankhwar *et al.,* 2023). The Tso Kar lake, located in the southern part of Ladakh in the western Himalayan ranges, is a salt lake of large size and depth with negligible anthropogenic activity. At high altitudes bacterial community is complex and diverse in nature and mainly belongs to psychrophiles which can survive in extreme weather (Shen *et al.,* 2015). There is very little information on the microbial diversity of the sediment soil of Tso Kar Lake of Ladakh. The present study aimed to understand the effect of land use and environmental factors such as soil pH, temperature and altitude on bacterial diversity of three distantly related soils.

MATERIALS AND METHODS

Sampling sites and meteorological parameters

The soil samples were collected from three different locations in India using 6-8 random sites and pooled together to represent that region. Samples were collected from 2- 20 cm depth of the upper crust in sterilized tubes. The sampling sites were Ramnagar, Uttarakhand, India, located in the Himalayan forest range (RM1) (coordinates: 29^{o} 22' 53" N 79^{o} 08'33" E), BTC2 from hospital waste sites of Hospital trauma centre, Varanasi, Uttar Pradesh (coordinates: 25º16'44" N'' 83º0'20" E) and Kar3 sediment soil samples were from Tso Kar lake of Ladakh (coordinates: 33^{o} 18 'N 77^{o} 59'E) (Fig. 1). The parameters like altitude, longitude and latitude were noted by GPS enabled smartphone. The samples were stored in the refrigerator at 4 ^{o}C until further analysis.

Physico-chemical and SEM-EDS analysis of the soil samples

The present study explored the physico-chemical properties of the soil samples of three different geographical locations in India. The physical and chemical properties of the soil samples were determined by the standard protocols using 10 gm of soil sample for each parameter analysis. EC (Electrical conductivity), TDS (Total dissolved solids) and pH were determined by a Water analyzer (371, Systronics, India) (Jackson, 1973). Soil temperature was measured on the sampling sites by digital thermometer at 10-20 cm depth in the soil. Scanning Electron Microscopy (SEM) with Energy Dispersive X-ray spectroscopy (EDS) was performed for RM1, BTC2 and Kar3 samples to analyze the soil's surface morphology and elemental composition. The analysis was done using high-performance and high-resolution scanning electron microscope (JEOL-6490LV, Japan). Chemical composition of the soil samples, such as oxygen, sodium, magnesium, aluminum, silicon, potassium, calcium, titanium, iron, platinum and sulphur was determined as the percent weight of elements using an EDAX spectrometer; the detection limit was 0.01% (Philip and Singh, 2020).

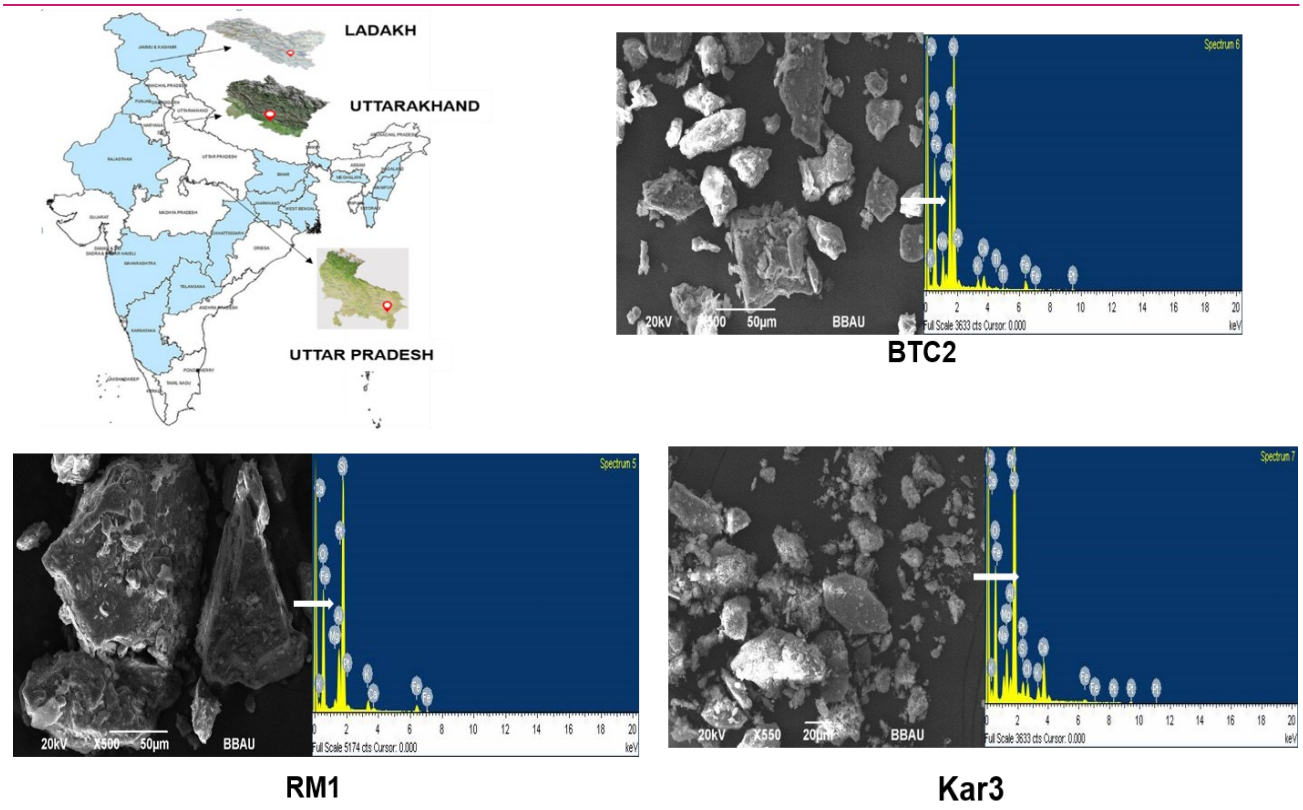

Fig. 1. *Sample collection sites of Hospital waste sites (BTC2), Himalayan forest soil (RM1) and Tso Kar lake sediment (Kar3) of India.*

DNA extraction and metagenomic sequencing

According to the manufacturer's instructions, the DNA of the soil samples RM1, BTC2 and Kar3 were extracted from 5 gm of soil using Xploregen DNA extraction Kit (Xploregen Discoveries, India). DNA was estimated qualitatively and quantitively using agarose gel electrophoresis and Nanodrop (Thermo Fisher Scientific, India) and stored at -20^{0}C till further analysis. 40ng of each DNA sample was amplified using 10 pM of forward and reverse primers (16SF- 5' AGAGTTTGATGMTGGCTCAG 3' and 16SR- 5' TTACCGCGGCMGCSGGCAC 3') in triplicate. Primers were specific for bacterial V3-V4 hypervariable region of 16S rRNA gene. Amplicons were purified by an AMPure XP kit (Beckman Coulter, USA) to remove unused primers. Library preparation was performed with Illumina barcoded adapters with eight additional cycles of PCR. The library was purified by AMPure XP kit and quantified by Qubit dsDNA high sensitivity assay kit (Thermo Fisher Scientific, India). Next-Generation sequencing was performed in Illumina MiSeq using 2x300 PE v3-v4 sequencing kit (Illumina Inc., India) as per the manufacturer instructions and outsourced from BioKart India Pvt. Ltd.

Functional pathway analysis

The present study investigated the functional metabolic pathways present in bacteria using functional annotation of KEGG (Kyoto Encyclopaedia of Genes and Genomes). The OTUs generated in the Illumina sequencing were used to predict metabolic pathways in MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/) based BLAST program using KEGG database. The outcomes were exported to Microsoft Excel (2016) to construct the Venn diagram.

Data processing and taxonomy assignment

The study used NCBI database for 16S V3-V4 sequencing analysis. The data obtained from the sequencer was de-multiplexed into fastq raw data and quality was checked using Fastqc (version 0.11.9) and Multiqc (version 1.10.1) tools. The QC passed data were processed in QIIME 2 (Quantitative Insights Into Microbial Ecology; pipeline ver. 2020.11). Quality control (QC) was typically performed by trimming, filtering and clustering into operational taxonomic units (OTUs). QIIME 2 plugins DADA2 (q2-dada2) and Deblur (q2-deblur) were used for denoising, chimera removal and quality filtering of sequences to generate good quality reads (QC value > 30) and amplicon sequence variants (ASVs). The obtained good-quality reads were analyzed for taxonomic assignment by using QIIME 2 and reference NCBI 16S database (Quast *et al.,* 2013). The alpha diversity and heatmap analysis were performed using MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/). Various alpha diversity

indices such as Chao1, Shannon and Simpson index were calculated using QIIME 2 to determine bacterial diversity and their abundance in RM1, BTC2 and KAR3 samples (Bik *et al.,* 2006; Douglas *et al.,* 2020).

RESULTS

Soil physicochemical and metrological parameters

The Himalayan forest soil samples (RM1) had the highest pH (8.32) compared to the other two soil samples (Table. 1). The results suggest that RM1 soil was more alkaline than BTC2 soil due to the presence of higher amount of magnesium. The EC and TDS were in decreasing order for RM1 >BTC2 >Kar3 (Table 1). The Kar3 sample was collected from the highest altitude of 4530 m and BTC2 sample was from the lowest altitude of 80.71m above sea level. The temperature of the Kar3 soil at the time of sampling was lower than the other two sampling sites (Table 1).

The Scanning electron microscopy (SEM) analysis of three different habitats (Himalayan forest, hospital waste site and Tso Kar Lake of Ladhak) explained the soil microstructure. Results suggest that Himalayan forest soil has bigger particle size than hospital waste soil due to higher amounts of titanium, silicon and sulphur elements (Fig. 1, Table 2). SEM-EDS analysis suggested that Forest soil was more saturated with oxygen, supporting aerobic actinomycetes' growth. Hospital soil was richer in sodium, aluminum and iron elements, which create soil toxicity and more suitable for the growth of firmicutes, which are anaerobic and endospore-forming bacteria (Table 2).

Bacterial diversity analysis

Metagenomic sequencing of Hospital waste site (BTC2), Himalayan forest soil (RM1), and Tso Kar lake sediment soil (Kar3) samples generated 66474, 19403, and 18204 final operational taxonomic units (OTUs), respectively. Taxonomic assignment by using QIIME 2 and reference NCBI 16S database generated 245 Archaea, 66232 Bacteria in BTC2 sample, 196 Archaea and 19210 Bacteria in RM1 and 154 Archaea and 18053 Bacteria Kar3 sample and samples. Results suggested 34 phyla (23 phyla: common), 89 classes (60 classes: common), 228 order (155 order: common), 465 families (302 families: common), 846 genera (439 genera: common) and 924 species (457 species: common) were present among all the three samples.

Phylum diversity

Taxonomic analysis of Hospital waste sites (BTC2) soil samples revealed the abundance at phyla level in decreasing order of Firmicutes (58.37%), Proteobacteria (22.97%), Actinobacteria (8.61%), Acidobacteria (2.80%), Cyanobacteria (2.12%), Bacteroidetes (1.57%), Planctomycetes (1.15), Chloroflexi (0.58%), Fusobacteria (0.52%), Nitrospirae (0.40%) (Table 3, Fig. 2A). Himalayan forest soil (RM1) samples were abundant in phyla in decreasing order of Proteobacteria (28.86%), Actinobacteria (26.70%), Firmicutes (11.100 %), Chloroflexi (10.40%), Acidobacteria (9.27%), Planctomycetes (5.16%), Bacteroidetes (3.7%), Verrucomicrobia (1.2%) and below 1% were Nitrospirae (0.9%) and Euryarchaeota (0.8%) (Table 3, Fig. 2A). The top ten phyla present in Tso Kar lake soil (Kar3) samples were in decreasing order of Proteobacteria (46.28%), Firmicutes (19.63%), Bacteroidetes (15.64%), Chloroflexi (4.22%), Cyanobacteria (3.86%), Planctomycetes (2.15%), Bacteroidetes (1.64%), Verrucomicrobia (1.21%), Nitrospirae (1.17%) and Euryarchaeota (0.73%) (Table 3, Fig. 2A). The most dominant phyla in BTC2 sample was Firmicutes, whereas RM1 and Kar3 soil samples have most abundant phyla Proteobacteria.

Genus and species diversity

The most abundant genera present in Hospital waste soil samples (BTC2) were *Yaniella* (22.67%), *Haloarcula* (14.03%), *Unclassified Hyphomicrobiaceae* (7.14%), *Luteimonas* (4.84%), *Faecalibacterium* (4.13%), *Hyphomicobiome* (3.48%), *Unclssified Ellin6067* (2.71%), *KF-Gitt2-16* (2.32%), *Pilimelia* (2.13%), Propionivibrio (1.97%) (Table 3, Fig. 2B). Himalayan forest soil samples (RM1) have abundance of genus *Alkaliphilus* (7.20%), *Catonella* (7.11%), *Unclassified Gemellaceae* (4.20%), *Proteus* (3.66%), *Actinobacillus* (3.49%), *Unclassified* Acidaminobacteraceae (2.88%), *Unclassified Gomphosphaeriaceae* (2.87%), *Microbacterium* (2.86%), *Unclassified GMD14H09* (2.75%) and *Alcanivorax* (2.67%) (Table 3, Fig. 2B), whereas in TsoKar lake sediment soil samples (Kar3) were *Sulfurimonas* (9.3%), *Unclassified Bacillaceae* (8.15%), *Halomonas* (4.66%), *Thiomicrospira* (4.40%), *Unclassified ML635J-*

Table 1. Physico-chemical and meteorological parameters of the three distantly related soil samples of India

S. No.	Soil samples	pH	EC (dS/cm)	TDS	Altitude	Temperature
1	RM1 (Himalayan forest)	8.32	37.9 µs	17.7ppm	345 m	23ºC
2	BTC2 (Hospital waste sites)	7.44	12.4 µs	5.67ppm	80.71m	25.7ºC
3	Kar3 (Lake sediment)	8.20	1.5 µs	0.71ppm	4530 m	10ºC

Table 2. Energy Dispersive X-ray spectroscopy (EDS) analysis of the three soil samples

S.No.	Element	Soil Samples					
		BTC2 (Hospital waste)		RM1 (Himalayan forest)		Kar3 (Lake sediment)	
		Weight%	Atomic%	Weight%	Atomic%	Weight%	Atomic%
1	O K	51.17	66.05	55.91	70.59	50.91	70.59
2	Na K	4.66	4.19	1.17	0.98	—	—
3	Mg K	1.32	1.12	6.95	5.20	1.17	0.98
4	Al K	9.28	7.10	1.44	1.11	6.95	5.20
5	Si K	25.14	18.48	29.56	21.78	28.90	20.79
6	K K	1.11	0.59	1.71	1.00	1.64	0.85
7	Ca K	1.91	0.99	1.45	0.77	0.53	0.27
8	Ti K	0.32	0.14	4.51	2.33	—	—
9	Fe K	3.07	1.14	0.72	0.27	3.17	1.15
10	Pt M	2.01	0.21	1.27	0.13	1.71	1.15
11	S K	—	—	1.78	1.15	—	—

Abbreviation: O=Oxygen, Na=Sodium, Mg=Magnesium, Al=aluminium, Si=Silicon, K=Potassium, Ca=calcium, Ti=Titanium, Fe=Iron, Pt=Platinum, S=Sulphur

40 (4.12%), *Unclassified Bacteroidales* (4.07%), *Marinobacter Unclassified* (3.56%), *Helicobacteraceae unclassified* (3.35%), *Rhodobacteraceae* (3.26%), *Unclassified Clostridiales* (3.01%) (Table 3, Fig. 2B).

Total 924 common species were present in BTC2, RM1 and Kar3 soil samples. *Unclassified Planococcaceae* sp. (22.67%), *Unclassified Bacillales* sp. (14.03%), *Unclassified Gracilibacteraceae* sp. (7.14 %), *Unclassified Clostridiales* sp. (4.84%), *Comamonadaceae* sp. (4.13%), *Unclassified Actinomycetales* sp. (3.48%), *Paenibacillus* sp. (2.68%), *Unclassified Enterobacteriaceae* sp. (2.32%), *Unclassified Burkholderiales* sp. (2.13%), *Unclassified Streptophyta* sp. (1.97%) were present in Hospital waste soil samples (BTC2) (Table 3, Fig. 2C), whereas Himalayan forest soil samples (RM1) have *Unclassified Actinomycetales* sp. (7.20%), *Unclassified Bacillales* sp. (7.11%), *Unclassified Bacillales* sp. (7.11%), *Unclassified* iii1-15 sp. (3.66%), *Unclassified A4b* sp. (3.49%), *Unclassified Myxococcales* sp. (2.88%), *Unclassified Rhodospirillales* sp. (2.87%), *Unclassified Gaiellaceae* sp. (2.86%), *Unclassified Rhodospirillaceae* sp. (2.75%), *Unclassified Acidimicrobiales* sp. (2.67%) (Table 3, Fig. 2C). Tso Kar lake sediment soil samples (Kar3) have *Sulfurimonas* sp. (9.3%), *Unclassified Bacillaceae* sp. (8.15%), *Halomonas* sp. (4.66%), *Thiomicrospira* sp. (4.40%), *Unclassified ML635J-40* sp. (4.12%), *Unclassified Bacteroidales* sp. (4.07%), *Marinobacter* sp. (3.56%), *Unclassified Helicobacteraceae* sp. (3.35%), *Unclassified Rhodobacteraceae* sp. (3.26%), *Unclassified Clostridiales* sp. (3.01%) (Table 3, Fig. 2C).

Circos map represents the circular visualization, position and relationship between the three distant samples at genus level. The results suggest that the highest bacterial diversity was observed in Hospital waste soil compared to Forest and lake sediment soil samples at the genus level (Fig. 3A). Venn diagram suggests that 135 (27.1%) genera were common among all three samples. RM1 samples consisted of 29 (5.8%), Kar3 of 63 (12.7%) and BTC2 samples consisted of 96 (19.3%) specific genera (Fig. 3B).

Comparative bacterial diversity analysis

Comparative bacterial diversity, richness, and abundance were measured using different statistical analysis tools such as Chao1, Shannon, Simpson index, and Heat map. The heat map analysis results suggest that Hospital waste soil samples (BTC2) were negatively correlated with lake sediment soil samples (Kar3). However forest soil samples (RM1) have no correlation with the other two samples in terms of bacterial diversity at the genus level (Fig. 4D). The dominant genera in Kar3 samples were *Halomonas*, *Hydrogenophaga*, *Unclassified Rhodobacteraceae*, *Brevefilum, Hymenobacter, Thiomicrospira, Hydrogenovibrio, Candidatus Cyclonatronum, Unclassified Campylobacteraceae, Unclassified Piscirickettsiaceae, Candidatus, Gracilibacteria, Geobacillus, Marinobacter* whereas dominant genera in BTC2 samples were *Bacillus, Streptomyces, Unclassified Caulobacteraceae, Unclassified Burkholderiales, Paenibacillus, Rhodococcus, Unclassified Bacillales, Clostridium, Unclassified Comamonadaceae, Unclassified Peptostreptococcaceae*. Alpha diversity, which indicates the bacterial species diversity in a soil sample, was measured using the Chao1, Shannon and Simpson indexes. Results suggested that Hospital waste soil (BTC2) has the highest species richness (379.73) among all three samples (Fig. 4A). Shannon's and Simpson's indexes results suggested that forest soil samples have highest relative abundance and species diversity in comparison to other two samples (Fig. 4B, C).

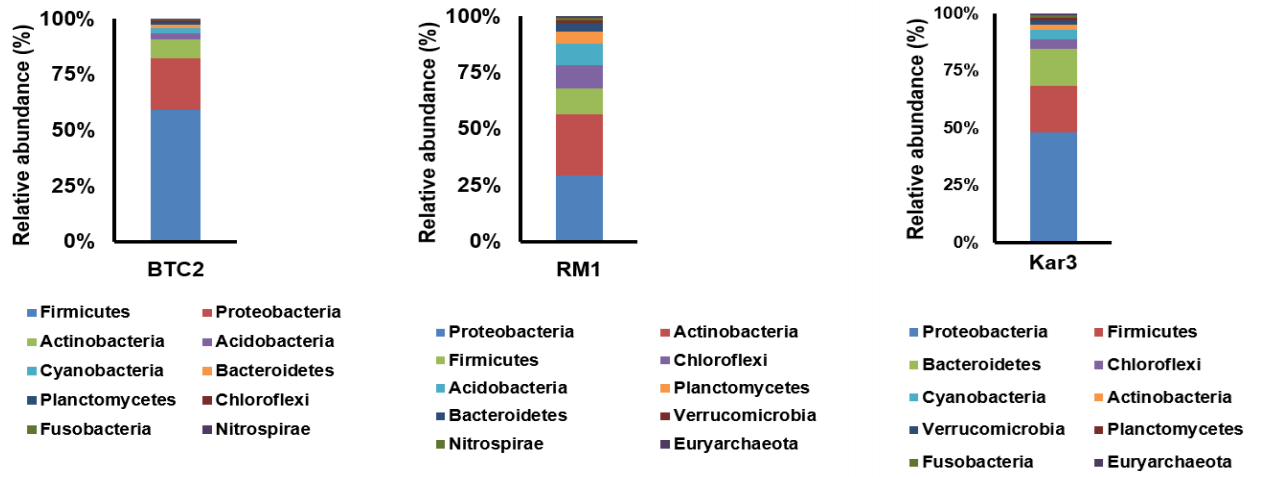

Figure 2A. Top ten relative abundance of phyla in BTC 2 (soil sample of Hospital waste), RM1 (soil sample of Himalayan soil) and Kar3 (Tso Kar lake sediment) samples.

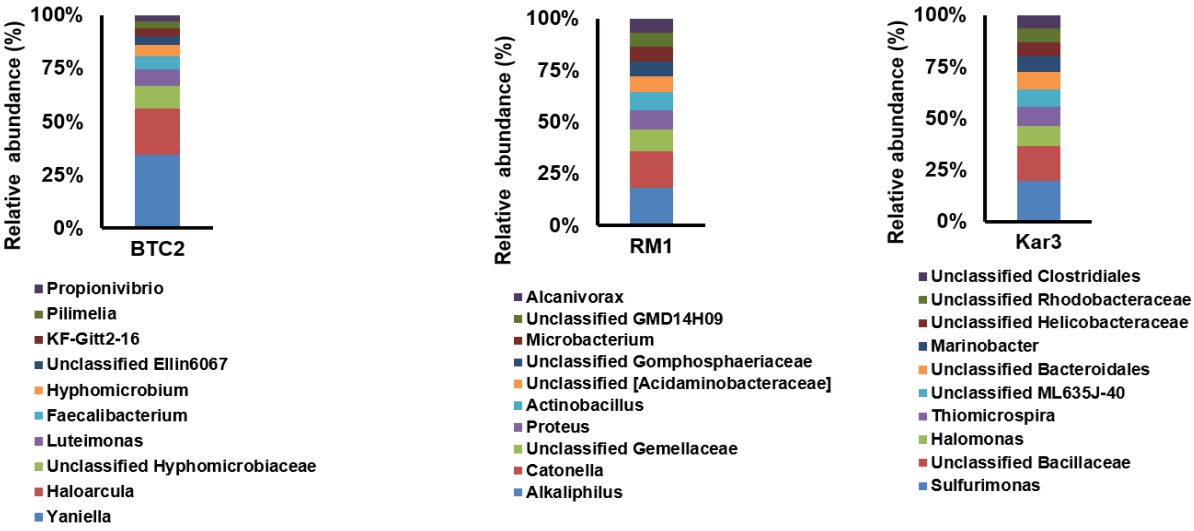

Fig. 2B. *Top ten relative abundance of Genus in BTC 2 (soil sample of Hospital waste), RM1 (soil sample of Himalayan soil) and Kar3 (Tso Kar lake sediment) samples.*

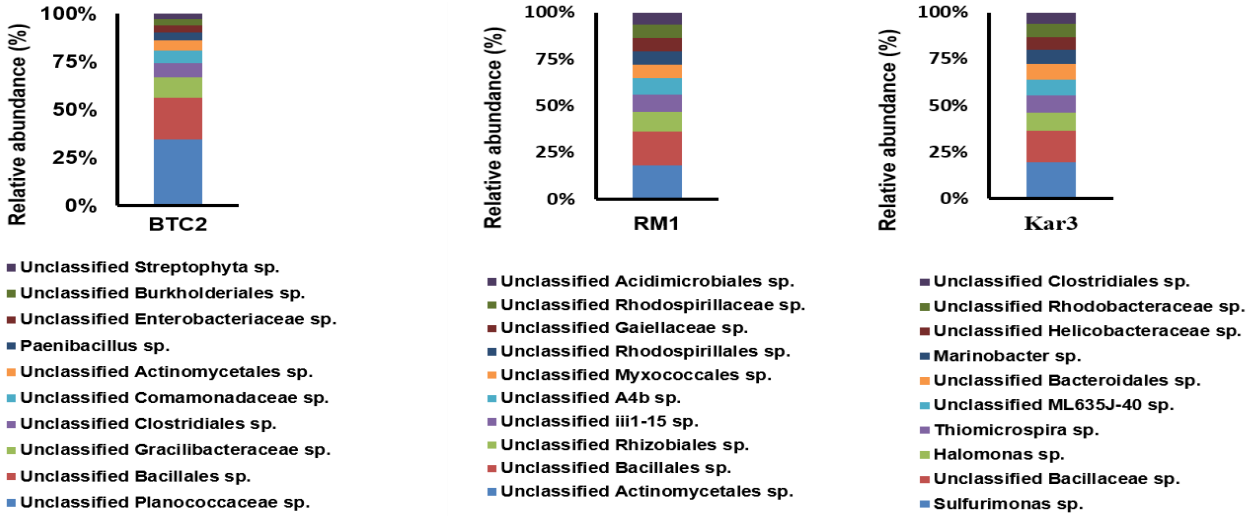

Fig. 2C. *Top ten relative abundance of species in BTC 2 (soil sample of Hospital waste), RM1 (soil sample of Himalayan soil) and Kar3 (Tso Kar lake sediment) samples.*

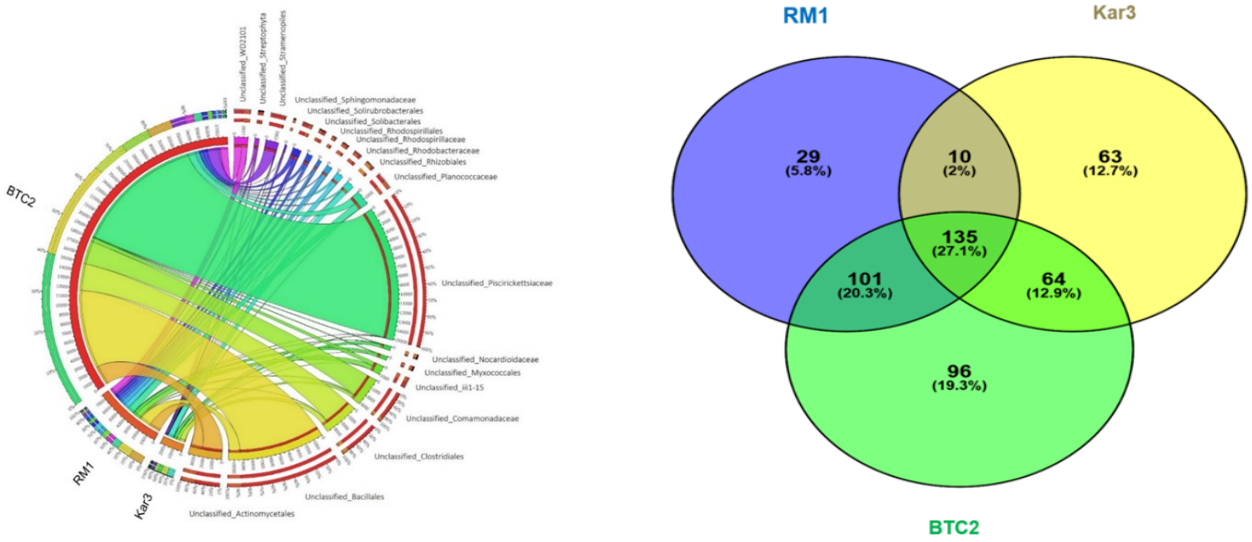

Fig. 3. (A) *Circos map showing the dominant genera present in the soil samples of BTC2, RM1 and Tso Kar lake sediment* ***(B).*** *Venn diagram represent the common and unique genera present in BTC2, RM1 and Kar3 soil samples.*

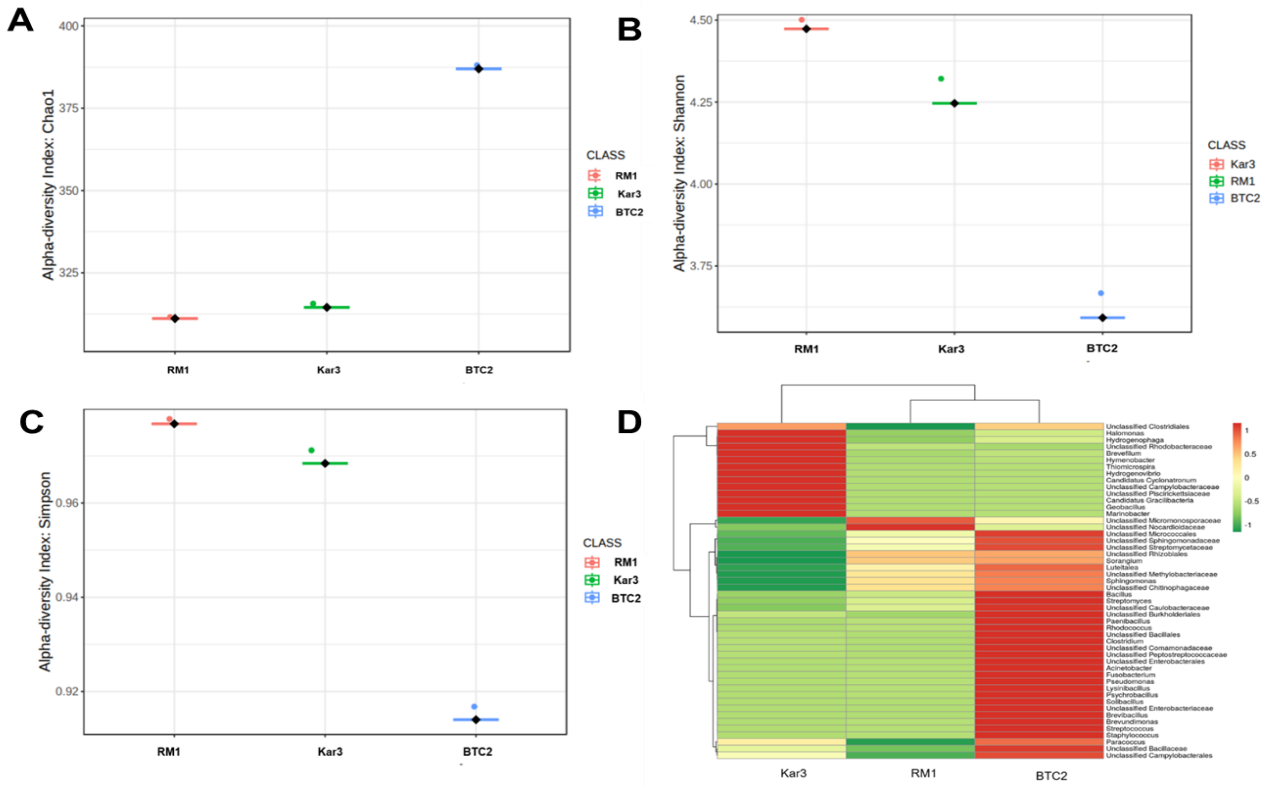

Fig. 4. *Alpha diversity of bacteria in all the three samples by* ***(A).*** *Chao 1,* ***(B).*** *Shannon and* ***(C).*** *Simpson indexes.* ***(D)*** *Heat map showing the abundance of genera in three soil samples and their correlation with each other.*

Metabolic pathway analysis

Functional metabolic pathways were identified by Microbiomeanalyst and KEGG web server using metagenome sequences (OTUs) of all three samples as described in methods. The general metabolic pathways related to carbon metabolism, amino acid biosynthesis and methane metabolism were dominant among all three samples. Total 44 metabolic pathways were predicted in all three samples, of which 33 pathways were common among all the samples. The unique metabolic pathways present in hospital waste sites (BTC2) were related to lipoic acid metabolism, Himalayan forest soil (RM1) consisted limonene and pinene degradation pathway and lake sediment soil (Kar3) samples consisted drug metabolism, propanoate metabolism, starch and sucrose metabolism and purine metabolic path-

Table 3. Bacterial diversity of three soil samples. Top ten Phyla, genus and species are represented in percentage of abundance in their decreasing order

Abundance (Top ten)	BTC2 (Hospital waste)	RM1 (Himalayan forest)	Kar3 (Lake sediment)
Phylum	Firmicutes (58.37%)	Proteobacteria (28.86%)	Proteobacteria (46.28%)
	Proteobacteria (22.97%)	Actinobacteria (26.70%)	Firmicutes (19.63%)
	Actinobacteria (8.6%)	Firmicutes (11.10%)	Bacteroidetes (15.64%)
	Acidobacteria (2.8%)	Chloroflexi (10.40%)	Chloroflexi (4.22%)
	Cyanobacteria (2.1%)	Acidobacteria (9.27%)	Cyanobacteria (3.86%)
	Bacteroidetes (1.57%)	Planctomycetes (5.16%)	Planctomycetes (2.15%)
	Planctomycetes (1.15%)	Bacteroidetes (3.73%)	Bacteroidetes (1.64%)
	Chloroflexi(0.58%)	Verrucomicrobia (1.29%)	Verrucomicrobia (1.21%)
	Fusobacteria (0.52%)	Nitrospirae (0.98%)	Nitrospirae (1.17%)
	Nitrospirae (0.40%)	Euryarchaeota (0.82%)	Euryarchaeota (0.73%)
Genus	Yaniella (22.67%)	Alkaliphilus (7.20%)	Sulfurimonas (9.38%)
	Haloarcula (14.03%)	Catonella (7.11%)	Unclassified Bacillaceae (8.15%)
	Unclassified Hyphomicrobiaceae (7.14%)	Unclassified Gemellaceae (4.20%)	Halomonas (4.66%)
	Luteimonas (4.84%)	Proteus (3.66%)	Thiomicrospira (4.40%)
	Faecalibacterium (4.13%)	Actinobacillus (3.49%)	Unclassified ML635J-40 (4.12%)
	Hyphomicrobium (3.48%)	Unclassified [Acidaminobacteraceae] (2.88%)	Unclassified Bacteroidales(4.07%)
	Unclassified Ellin6067 (2.71%)	Unclassified Gomphosphaeriaceae (2.87%)	Marinobacter (3.56%)
	KF-Gitt2-16(2.32%)	Microbacterium (2.86%)	Helicobacteraceae (3.35%)
	Pilimelia(2.13%)	Unclassified GMD14H09 (2.75%)	Unclassified Rhodobacteraceae (3.26%)
	Propionivibrio (1.97%)	Alcanivorax (2.67%)	Unclassified Clostridiales (3.01%)
Species	Unclassified Planococcaceae sp. (22.67%)	Unclassified Actinomycetales sp. (7.20%)	Sulfurimonas sp. (9.35%)
	Unclassified Bacillales sp. (14.03%)	Unclassified Bacillales sp. (7.11%)	Unclassified Bacillaceae sp. (8.15%)
	Unclassified Gracilibacteraceae sp. (7.14%)	Unclassified Rhizobiales sp. (4.20%)	Halomonas sp. (4.66%)
	Unclassified Clostridiales sp. (4.84%)	Unclassified iii1-15 sp. (3.66%)	Thiomicrospira sp. (4.40%)
	Unclassified Comamonadaceae sp. (4.13%)	Unclassified A4b sp. (3.49%)	Unclassified ML635J-40 sp. (4.12%).
	Unclassified Actinomycetales sp. (3.48%)	Unclassified Myxococcales sp. (2.88%)	Unclassified Bacteroidales sp. (4.07%)
	Paenibacillus sp.(2.68%)	Unclassified Rhodospirillales sp. (2.87%)	Marinobacter sp. (3.56%)
	Enterobacteriaceae sp. (2.32%)	Unclassified Gaiellaceae sp.(2.86%)	Unclassified Helicobacteraceae sp. (3.35%)
	Unclassified Burkholderiales sp.(2.13%)	Unclassified Acidimicrobiales sp. (2.67%)	Unclassified Rhodobacteraceae sp. (3.26%)
	Unclassified Streptophyta sp. (1.97%)	Unclassified Rhodospirillales sp. (2.87%)	Unclassified Clostridiales sp. (3.01%)

ways (Fig. 5A, B).

DISCUSSION

India has a vast array of flora and fauna which is attributed to its diverse environmental and soil microbial diversity. The Himalayas are the world's tallest and recently developed mountains located at high altitudes and mostly have cold climates (Bishop and Chatterjee, 2023). Several metagenomic studies have explored the Himalayan soil's bacterial diversity (Joshi *et al.,* 2017). The present study investigated the soil bacterial diversity present in three distantly related samples of Hospital waste sites (BTC2), Himalayan forest soil (RM1) and Tso Kar lake sediment soil (Kar3) of Ladakh, India. Lake sediment soil samples were unique in their micro-environment underwater with less access to sunlight and oxygen, which creates microbial diversity related to

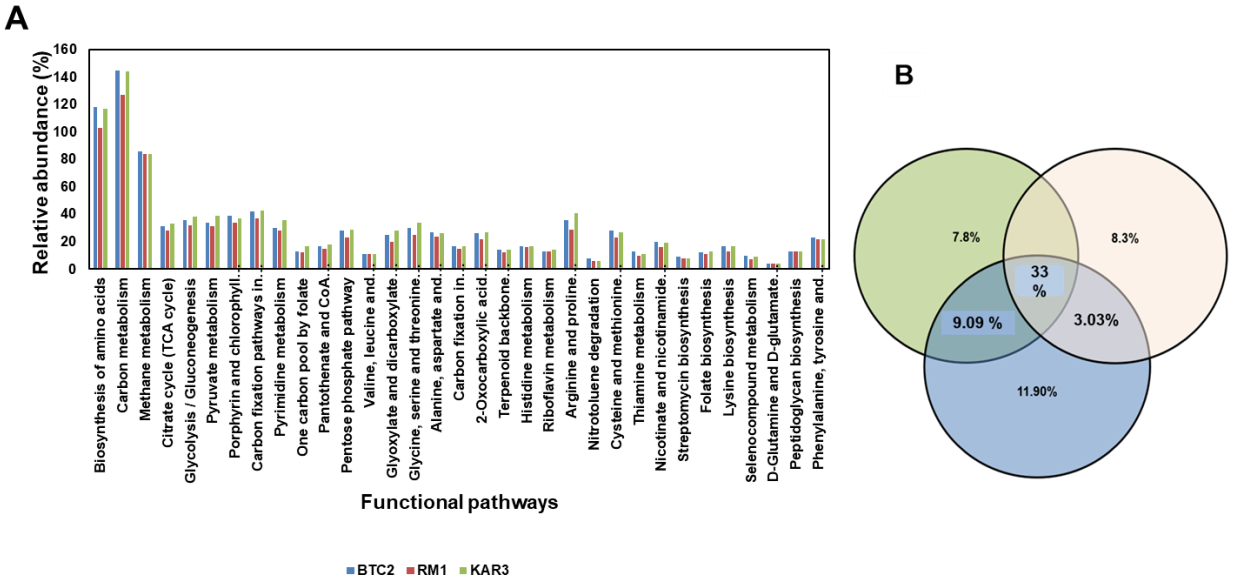

Fig. 5 . *Functional metabolic pathway analyzed by MicrobiomeAnalyst and KEGG web server in all the three samples represented by* ***(A)*** *Bar diagram* ***(B)*** *Venn diagram.*

stagnant habitat (Wang *et al.,* 2012). The hospital waste site soil samples obtained from Varanasi, Uttar Pradesh had a humid, subtropical climate and had more anthropogenic activity which might be responsible for higher bacterial diversity than other samples. Several key environmental factors contribute to soil bacterial diversity, such as altitude, temperature, pH, and anthropogenic activities (Table 1) (Ren *et al.,* 2018). BTC2 soil samples contained higher alpha diversity which was attributed to the vigorous human activity and the hazardous waste at the hospital dumping sites. Results suggest that these sites are rich in diverse bacterial pathogens, a major threat to healthcare providers and the general population (Fig. 2C) (Boyce *et al.,*1997). The Himalayan forest soil samples were mostly rich in Gram-positive and Gram-negative bacteriomes, including Proteobacteria and Actinobacteria (Table 3). Proteobacteria are highly prevalent in various soil habitats and are linked to various processes, including the cycling of sulphur, nitrogen and carbon (Spain *et al.,* 2009). Actinobacteria are involved in the catabolism of soil organic matter breakdown of the complex compounds such as lignocellulose, xylan and lignin into simpler compounds that help plants growth (Olanrewaju and Babalola, 2019). Actinomycetes are also known for producing secondary metabolites and natural antimicrobial agents. Recently, actinomycetes from the Himalayan forest soil, an excellent source of broad-spectrum antimicrobial compound Emycin-E, have been reported (Sankhwar *et al.,* 2023). In the present study, the most abundant phyla identified in the Tso Kar Lake sediment soils were Proteobacteria, Firmicutes and Bacteroidetes (Table 3 and Fig. 2A). The previous study reported a similar bacteriome in Pangong Lake, Ladakh, India (Rathour *et al.,* 2017). This is the first study that explored the bacterial diversity of sediment soil of Tso Kar Lake of Ladakh, India. The metabolically diversified phylum of Proteobacteria have bacterial spp. known for photo-autotrophy, CO_2 fixation, and chemo-autotrophy (Kersters *et al.,* 2006; Badger and Bek, 2008). Firmicutes and Proteobacteria were the most abundant phyla in the Hospital waste sites (BTC2) soil samples (Table 3). Interestingly, similar results were reported previously in the hospital environment samples from several locations of UC San Diego Health System (King *et al.,* 2016).

The bacterial species diversity, richness and abundance estimated by the Chao1, Shanon and Simpson indexes suggest that hospital contaminated soil BTC2 had more alpha diversity and that Himalayan forest soil had more species diversity and abundance (Fig. 4). The major factors which contributed to a diverse bacterial community in all three samples are human activity, deposition of chemicals and xenobiotic compounds, including improper disposal of hospital waste, industrial activity, agricultural activity, heavy metals accumulation in the soil, oil spillage and acid rain which causes soil contamination (Mishra *et al.,* 2016). High altitudes have low human population and extreme environmental conditions that stimulate bacterial population growth in response to new selection pressure. This could be why heat map results of hospital waste sites' soil microbiota in the present study were negatively correlated to the samples of the other two sites (Fig. 4D).

Functional metabolic pathways analysis by KEGG suggested that all three site samples had common energy metabolic pathways related to carbon, amino acids and methane metabolism (Fig. 5). The most abundant

greenhouse gas on the earth is methane and methanogenesis is the major part of the carbon cycle on earth (Sharma *et al.,* 2020). Previous studies also reported the presence of amino acid and carbon metabolism pathways in most of the microbiota (Xie *et al.,* 2020). The lipoic acid metabolic pathway in bacteriome of Hospital waste soil only indicates the presence of pathogenic strains in the samples. Most Gram-negative and Gram-positive pathogens have lipoate metabolism enzymes responsible for virulence and pathogenesis (Spalding and Prigge, 2010). Himalayan forest soil bacteriome is rich in pinene and limonene degradation pathway, suggesting coniferous plants in the sampling area (Fig. 5) (Savithiry *et al.,*1998). Pinene are monoterpenes produced by Pinaceae family plants (Ndao and Adjalle, 2023). Thus, the study demonstrates a better understanding of soil physicochemical properties and bacterial diversity analysis of three distantly related soils in India. These findings uncover a new perspective on land use management for sustainable development and provide in-depth information on the soil bacterial diversity of India.

Conclusion

Sustainable soil management includes the prevention of physical, chemical, and biological degradation of the soil (Hospital waste sites (BTC2), Himalayan forest soil (RM1) and Tso Kar Lake sediment soil). Microbes play an important role in the bioremediation of heavy metals, increasing water-holding capacity and preventing soil erosion. This study provides in-depth knowledge of soil physicochemical properties by SEM-EDS analysis and bacterial diversity in three distantly related soil samples. Results suggest that pathogenic bacterial strains are more prevalent in Hospital waste soil, with the highest bacterial diversity among the three samples having more Firmicutes, prompting immediate land-use management action in these areas. The present study also suggests that Himalayan forest soils could be explored to investigate novel antimicrobial compounds since they are rich in phyla actinobacteria and proteobacteria.

ACKNOWLEDGEMENTS

The authors thank Babasaheb Bhimrao Ambedkar University for providing the space and the instrumentation facility to assist in SEM-EDS analysis. We acknowledge the ESEP program 2022 from Amity Center of Excellence, Amity University Mumbai, for help collecting samples from Tso Kar Lake, Ladhak.

Conflict of interest

The authors declare that they have no conflict of interest.

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