ORIGINAL RESEARCH PAPER
Volume - 12 | Issue - 03 | March - 2023 | PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

CHARACTERIZATION OF METHANOGENIC ARCHAEAL COMPOSITION IN PERMAFROST-AFFECTED SOILS OF THE CHANGTHANG REGION OF LADAKH

Zoology

Ahmad Ali Research Scholar, Department of Zoology, Panjab University, Chandigarh

Archana Chauhan* Assistant Professor, Department of Zoology, Panjab University, Chandigarh
*Corresponding Author

ABSTRACT

Permafrost habitats are characterized by harsh environmental conditions, primarily due to the seasonal freezing and thawing cycle in the top active layer of permafrost that results in a unique temperature and geochemical gradients. Microorganisms need to develop specialized resistance to adapt to harsh conditions. Climate change has an enormous impact on permafrost microorganisms thriving in such environmental conditions as they regulate the soil biogeochemical cycles. understanding the microbial community structure, therefore, is important for assessing greenhouse gas flux and mitigating climate change in permafrost-affected soils. The objective of this study was to investigate the community composition of methanogenic Archaea in the permafrost-affected soil using the 16S rRNA gene on the Illumina MiSeq platform. Results of the study showed that the majority of the sequences belonged to the phylum Crenarchaeota (77%) followed by Euryarchaeota (9%), unclassified sequences (9%), Thaumarchaeota (5%) and Korarchaeota (0.03%). Among the following archaeal phyla, the methanogenic archaea belonged exclusively to the phylum Euryarchaeota.

KEYWORDS

Archaea, Methanogen, Climate change, Methane and Illumina MiSeq

INTRODUCTION

Permafrost is any soil or sediments that have been frozen permanently for at least two consecutive years (Dobiński, 2016). The top surface layer is known as the active layer that seasonally freezes and thaws, along with a unique soil temperature and geochemical gradients (Fiedler et al., 2004). Permafrost soils show a substantial temperature gradient over their depth profiles during summer, and this is one of the primary climatic variables that influence the microbial community structure in such severe environments (Kotsyurbenko et al., 1993; Wagner et al., 2003). Microbial activities are almost responsible for the production of greenhouse gases when the active layer thaws seasonally it enhances the soil water saturation, allowing microorganisms to break down complex organic materials and sequestered organic carbon into simpler chemicals such as acetate, hydrogen (H_2), carbon dioxide (CO_2), methanol and formate. Methanogens are exclusively anaerobes and they use simpler methanogenic precursors as a substrate to produce methane (CH_4) (Zinder, 1993; ASM, 2000; Garcia et al., 2000).

Methanogens are members of the domain Archaea (Woese et al., 1978) and belong to the kingdom Euryarchaeota (Woese et al., 1990; Boone et al., 1993). They are categorized into three main nutritional groups that include hydrogenotrophic (oxidize H_2 and reduce CO_2 to form CH_4), methylotrophs (which utilize methyl compounds to produce CH_4), and acetoclastic methanogens (utilizes the methyl group of acetate to produce CH_4) (Garcia et al., 2000). They can be found in a range of habitats such as freshwater sediments (Chan et al., 2005), saline lakes (Jurgens et al., 2000; Keough et al., 2003), hypersaline habitats (Mathrani & Boone, 1985), permafrost soils, and sediments (Kobabe et al., 2004), peatlands (Sizova et al., 2003) and hydrothermal vents (Jeanthon et al., 1999).

Methanogens have been frequently studied using the 16S rRNA gene (Marchesi et al., 2001; Wilkins et al., 2015; Xavier et al., 2020; Meng, et al., 2022). In this study, we investigated the community composition of methanogens in the permafrost-affected soil of the Changthang region of Ladakh using the 16s rRNA gene.

Site description and soil sampling

The study was conducted in Tsokar (33°18′N 77°59′E) which is about 160km from Leh district in the Changthang region of Ladakh union territory, at an elevation of 4535m above sea level. In this region, the area where Tsokar is situated in a very cold and arid arctic type of climate with temperatures varying from sub-zero at night to 28°C during day time (Pangare et al., 2006; Das, 2008). Permafrost in this region belongs to high altitude permafrost, and its distribution characteristics are different from those of high latitude permafrost (Rastogi and Nawani, 1976; Dimri, et al., 1983; Bhattacharyya, 1989). Permafrost-affected soil samples have been aseptically collected from three different sites of Tsokar using a sterilized soil sampler according to standard protocols (Schoeneberger et al., 2012), with additional adaptations for permafrost-affected soils (Ping et al., 2013). After taking the soil samples, it was collected and wrapped in aluminum foil. The collected samples were then transported by air in a -20°C ice box to the Department of Zoology, Panjab University (PU), Chandigarh and the sampled cores were stored intact and frozen at -80°C until further analysis.

DNA Isolation, Purification and Quantification:

Total community DNA was isolated from 0.5g of soil core from each sample using FastDNA™ SPIN Kit (MP Biomedicals, USA) with a little modification in the given protocol. Multiple DNA elution in triplicates from the same sampling sites were pooled and purified using DNA Clean & Concentrator kit (Zymo Research Corp., USA). The integrity of the purified DNA was confirmed by electrophoresis in 0.8% agarose gel (Figure 1). The purified sample was again estimated spectrophotometrically using Qubit® 2.0 fluorometer with BR (Broad Range) reagents and the Qubit concentration was 45.2 ng/µl in the MET sample. The extracted DNA was immediately stored at -20 °C until further processing.

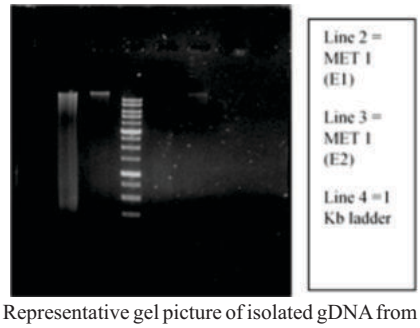

Figure 1. Representative gel picture of isolated gDNA from the Tsokar site

Amplification of targeted sequences

To check if the isolated DNA is suitable for molecular analysis PCR was carried out by amplifying the V3-V4 region of the 16S rRNA using the primer set Arc 21F-516R. The quantity and quality of the PCR products targeted the 16S rRNA V3-V4 region was measured by Nanodrop. The samples passed the QC assessment and proceeded for library preparation that is compatible with the Illumina platform.

NGS library preparation and quantification

Paired-end libraries were prepared from the composite DNA using Herculase II Fusion DNA Polymerase Nextera XT Index V2 Kit. The sequencing library is prepared by random fragmentation of the DNA or cDNA sample, followed by 5' and 3' adapter ligation. The amplicon library so obtained was purified using Ampure XP beads to remove the

unused reagents that can serve as contaminants for downstream processing. To amplify the amplicon libraries 16S rRNA V3-V4 region universal primer with standard Illumina barcodes and adapters was used. The quality and quantity of the barcoded 16S rRNA libraries were validated by Agilent TapeStation 4200. The quantity of the barcoded 16S rRNA libraries was quantified by Picogreen and Agilent TapeStation 4200 (Figure 2). The Picogreen concentration of the library was 12.21 ng/µl and the fragment size was 633 bp. Thus, the concentration and quality of the library were good enough and passed the QC measurement and proceeded to sequence on the MiSeq platform at 2x301PE format.

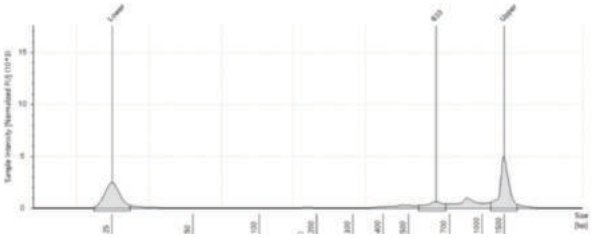

Figure 2. Analysis by Agilent TapeStation system Electropherogram of sample intensity (in fluorescence units, FU) for amplicon analysed by Agilent TapeStation High Sensitivity DNA assay. (Lower: lower marker; Upper: upper marker).

Next generation sequencing

NGS sequencing was carried out on the Illumina MiSeq platform. Since the quality of the reads on the Illumina platform tends to fall in the last 50bp, therefore, 2301 paired-end sequencings were carried out to obtain high-quality and full-length sequences for the V3-V4 amplicons of 16s rRNA.

Data Analyses:

The very first step in the data analyses was to assess the quality of raw reads. The quality of FASTQ files using FASTQC software, a quality control tool for high throughput sequence data (www.bioinformatics.babraham.ac.uk/projects/**fastqc**). From the QC analysis, it was observed that the base quality of the Reads was of good quality as the phred score was more than twenty. The Q20 and Q30 score of the reads is shown in table 1.

Table 1. Raw data Stats

Sample	Total bases (bp)	Total reads	GC (%)	AT (%)	Q20 (%)	Q30 (%)
MET	88,685,436	294,636	56.78	43.22	69.03	58.13

Assembly by FLASH

The paired-end reads were merged into sequence tags using FLASH 2.0 (Magoč and Salzberg, 2011). The total number of bases was 2,12,45,087bp and GC content was 60.94% (table 2). The total read count is shown in figure 3a and the Q20 (%) and Q30 (%) are displayed in figure 3b.

Table 2. Result of assembly by FLASH

Sample Name	Total Bases	Read Count	GC (%)	Q20 (%)	Q30 (%)
MET	2,12,45,087	59,247	60.94	98.26	93.85

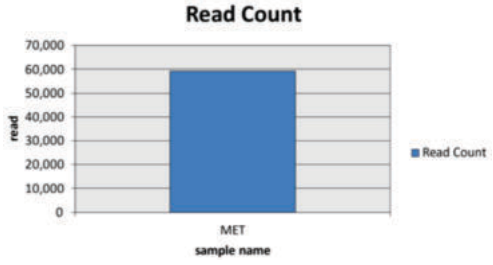

Figure 3. a) The total number of read counts.

Pre-processing

To lessen the impact of sequencing errors and artifacts, the low-quality sequence region, primers, and sequencing index, chimera and adapters were trimmed using BBDuk of the BBTools packages (https://sourceforge.net/projects/bbmap/) before executing downstream processing. The Short reads(<400bp) are filtered out and extra-long tails (>700bp) are trimmed. A total of 573 and 410 sequences were identified as chimers and low-quality reads, respectively and were filtered out from further analyses. The filtered reads are clustered at 97% identity using CD-HIT-DUP. The summary of the 16s rRNA gene sequence pre-processing statistics has been shown in table 3.

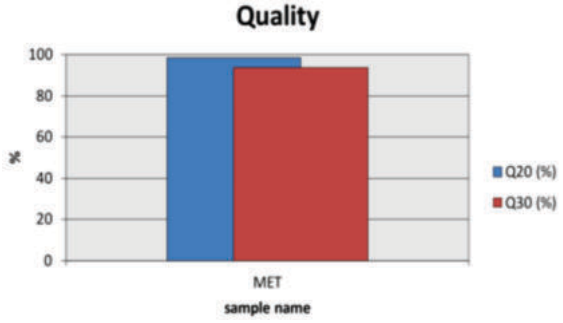

Figure 3. b) Q20 (%) and Q30 (%) of sequence after assembly.

Table 3. Summary of metagenomics sequencing statistics of MET sample

Results of Pre-processing	
Ambiguous	0
Low-Quality	410
Chimera	573
Other	25,128
Read count	6,624

MG-RAST analysis

The raw sequences i.e., forward and reverse read files generated from sequencing were uploaded into the MG-RAST web server, which identifies any problems with the sequencing run, by performing an automated quality control step based on an md5 checksum. There were no problems found in our files. In addition, it offers statistical data on the reads contained in each file, including base pair count, sequence count, sequence length, and GC content which is summarized in table 4.

Table 4. Stats of raw sequence on MG-RAST

Upload: bp Count	82,652,138 bp
Upload: Sequences Count	264,757
Upload: Mean Sequence Length	312 ± 40 bp
Upload: Mean GC percent	56 ± 10 %
Artificial Duplicate Reads: Sequence Count	95,266
Post QC: bp Count	27,774,530 bp
Post QC: Sequences Count	96,246
Post QC: Mean Sequence Length	289 ± 64 bp
Post QC: Mean GC percent	63 ± 7 %
Processed: Predicted Protein Features	65,951
Processed: Predicted rRNA Features	6,128
Alignment: Identified Protein Features	25,696
Alignment: Identified rRNA Features	5,075

OTU identification and feature Annotations

After performing a quality check, MG-RAST extracts rRNA at 70% identity using VSEARCH (Rognes et al., 2016) from a 90% identity clustered reduced version of the SILVA, Greengenes (DeSantis et al., 2006), and RDP (Cole et al., 2003) databases. we opted to use the "RDP" option, which will provide the RDP annotation of the features, based on the M5rna database.

Data Availability

All raw sequences obtained in this study have been submitted to the MG-RAST with metagenomic id 4964542.3

Richness and diversity indices

The α.diversity indices were analysed based on 16s rRNA gene data for the MET sample (table 5). The Good's coverage estimator was 1.0 for the sample which indicates that the sequences obtained represent the dominant phylotypes. In total, 240 OTUs were detected in the samples, which implies that archaeal species are distributed widely in this sampling site. The result of the Shannon indices and Gini Simpson indices were 5.3 and 0.9 respectively which indicated that the archaeal community in the sample was diverse (figure 4).

PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr

Table 5. Alpha diversity indices

Sample Name	OTUs	Chao1	Shannon	Gini-Simpson	Good's Coverage
MET	240.0	240.0	5.3678112169	0.9225591136	1.0

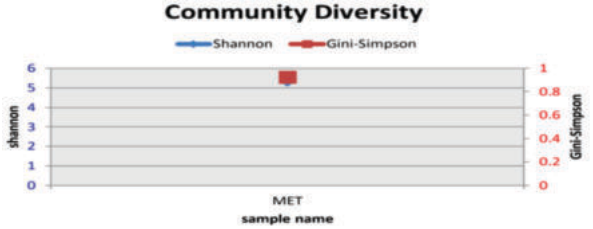

Figure 4. Alpha diversity of the sample

Taxonomic classification, Archaeal abundance and distribution

The community structure of methanogenic archaea in the permafrost-affected soil was investigated through 16S rRNA gene amplicon sequencing. Analysis and annotation of the sequences were carried out using the MG-RAST web server with default parameters (Meyer et al., 2008). The output file contained a total of 96,246 sequences comprised of 27,774,530 bps with 63% G + C content after quality control analysis. The community analysis revealed the predominance of the domain Archaea (98%), followed by bacteria (2%) and Eukaryota (0.43%) (figure 5). The 16S rRNA amplicon sequencing of the Archaeal community in the Tsokar permafrost-affected soil from the Changthang region of Ladakh yielded a total of 240 OTUs encompassing 4 phyla, 9 classes, 16 orders, 25 families, 62 genera and 134 species and unclassified sequences.

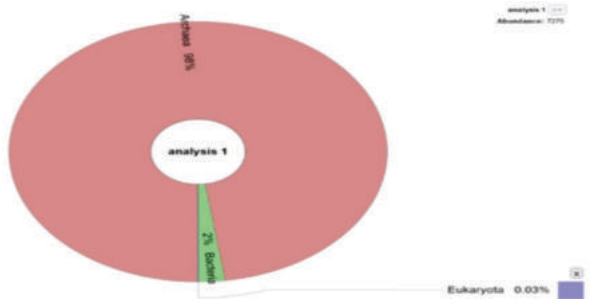

Figure 5. major Domain present in the sample.

Relative Abundance of Phyla

The analysis of the 16s rRNA sequences showed a diverse archaeal community in the sample. We observed several OTUs belonging to both methanogenic and non-methanogenic archaeal communities. The OTU classification on the phylum level showed that the dominant archaeal phyla in the MET sample of the Tsokar site were Crenarchaeota (77%) followed by Euryarchaeota (9%), unclassified sequences (9%), Thaumarchaeota (5%) and Korarchaeota (0.03%) (figure 6). Among the following archaeal phyla, the methanogenic archaea belonged exclusively to the phylum Euryarchaeota (Enzmann et al., 2018).

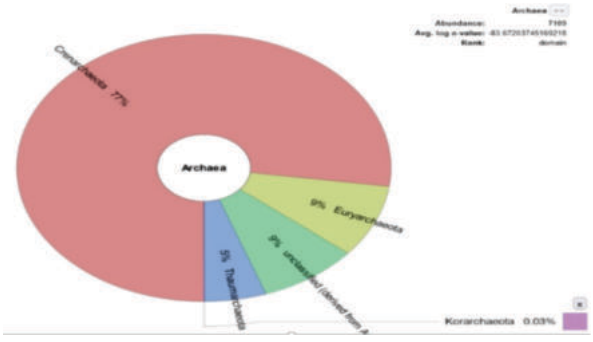

Figure 6. Krona charts showing the phylum-level abundance and diversity of archaeal populations in the MET sample.

Abundant classes of phylum Euryarchaeota

Analysis of the sequences showed various archaeal classes in the sample. However, the dominant methanogenic archaeal classes of the phylum Euryarchaeota present in the MET samples were class Thermococci (27%) followed by Methanococci (26%), Methanomicrobia (24%), Methanobacteria (13%), Archaeoglobi (3%), Thermoplasmata (6%), Halobacteria (1%) and Methanopyri (0.3%) (figure 7).

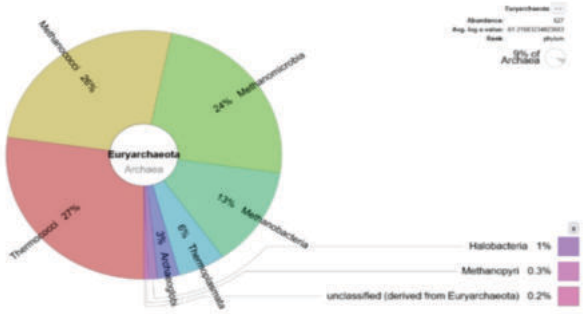

Figure 7. Krona charts showing the Class level abundance and diversity of methanogens in the MET sample.

Relative Abundance of orders

Analysis of the sequence at the deeper level showed that the five well-established methanogenic archaeal orders were present in the sample (figure 8). The dominant methanogenic order detected in the samples were *Methanococcales* (26%) followed by Methanosarcinales (21%), *Methanobacteriales* (13%), Methanomicrobiales (3%) and *Methanopyrales (0.3%).* In addition, the non-methanogenic orders of the phylum Euryarchaeota were also present in the samples were Thermococcales (27%), Archaeoglobales (3%), Halobacteriales (1%) and Thermoplasmatales (6%). This shows that the permafrost-affected soil in the Changthang region of Ladakh has a substantial population of methanogens thriving in the study sites.

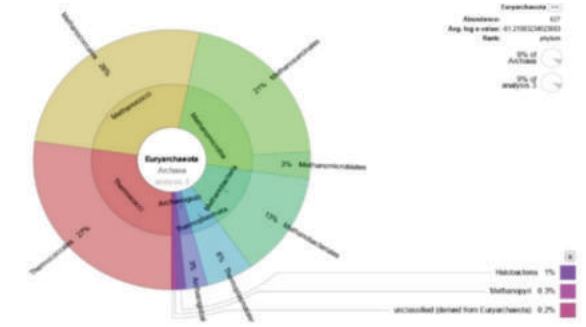

Figure 8. Krona charts showing the order level abundance and diversity of methanogens in the MET sample.

Relative Abundance of methanogenic genera

The 16S rRNA analysis of the permafrost-affected soil revealed a diverse methanogenic community composition in the studied samples. The OTU analysis at deeper taxonomic level shows different methanogenic taxa present in the samples. The predominant methanogenic genera present in the samples included *Methanococcus* (20%) *Methanobacterium (8%), Methanohalophilus (6%), Methanococcoides (6%), Methanosaeta (5%), Methanomethylovorans (5%), Methanocaldococcus (4%), Methanothermobacter (3%) Methanomicrobiales (3%), Methanosaeta (2%), Methanobrevibacter (2%), Methanotorris (2%), Methanolinea (1%)* and *Methanosarcina (1%)* (figure 9).

Figure 9. Krona charts showing the genus-level abundance and diversity of methanogens in the MET sample.

DISCUSSION AND CONCLUSION.

An ecological interpretation of the findings and an understanding of archaeal community abundance and structure in the permafrost-affected soil, particularly concerning temperature, are required to foresee how microbial communities may respond to future climate change. Microorganisms especially the 'archaea' are crucial and a key component of the geochemical cycle of permafrost affected soil. The methanogenic archaea are engaged in the generation of greenhouse gases such as CO_2 and CH_4, both of which contribute to climate change (Welte, 2018). In the final step of organic matter degradation anaerobically when inorganic electron acceptors such as nitrate, ferric iron, or sulfate have been exhausted. In this study, the archaeal community compositions were investigated in a permafrost-affected soil sample from the Changthang region of Ladakh. The rarefaction analysis of the sequences showed that the sequencing depth was sufficient enough to cover the methanogenic diversity of the soil (figure 10).

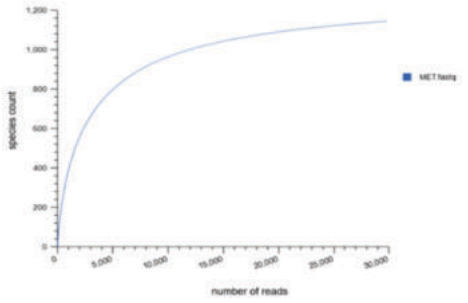

Figure 10. Rarefaction graph

The 16s rRNA gene sequences of the Archaea community in the permafrost-affected soil indicated a diversified archaeal composition, including environmental sequences belonging to the phylum Euryarchaeota, Crenarchaeota, and Thaumarchaeota as well as sequences closely related to methanogens and halophiles. The phylum Euryarchaeota was the dominant phylum(9%) that is mainly involved in methane production in permafrost-affected soil (Steven et al., 2007). Thaumarchaeota was (5%) this phylum is an ammonia-oxidizing archaea in permafrost-affected soils (Ahlgren et al., 2017). Additionally, a significant amount of Crenarchaeota was discovered in this study (77%), which is generally known to be abundant in temperate acidic soil (Lima-Perim et al., 201). The analysis of the sequences revealed a significant population of methanogenic archaea in the studied samples. These microorganisms can release methane through several pathways, including hydrogenotrophic, acetotrophic, and methylotrophic pathways, depending on the temperature, substrate, and geochemistry in which they are found. The result of the study showed diverse microbial populations belonging to the archaeal families including Methanococcaceae, Methanosarcinaceae, Methanobacteriaceae, Methanocaldococcaceae, Methanosaetaceae, Methanocorpusculaceae, and Methanopyraceae which resembled those from prior studies on the diversity of archaea in permafrost affected soils (Wachinger et al., 2000; Hoj et al., 2005; Juottonen et al., 2005). The family Methanosarcinaceae can grow on all methanogenic substrates except formate, while the members of the Methanosaetaceae grow only on acetate as an energy source. Species of the family Methanomicrobiaceae can only thrive on hydrogen, formate and alcohols (excluding methanol; Hedderich & Whitman, 2005). Acetate is most likely to be found in habitats having a considerable amount of polysaccharides (Kotsyurbenko et al., 2004). Acetate can only be used as a substrate by members of the genera *Methanosarcina* and *Methanosaeta*. The genus *Methanosarcina* is capable of producing methane from a variety of precursors. Although the primary process of methane production in most situations is methanogenesis via acetate and hydrogen, some *Methanosarcina* species, in particular, prefer methanol as a carbon and energy source. Even though methanogenesis using acetate and hydrogen is the primary pathway of methane generation in most situations, *Methanosarcina* species, in particular, prefer methanol as a source of carbon and energy (Conrad, 2005). In addition, the permafrost-affected soil samples from the Changthang region contained members of the methanogenic archaeal genera *Methanococcus, Methanobacterium, Methanohalophilus, Methanococcoides, Methanomethylovorans, Methanocaldococcus, Methanothermobacter, Methanobrevibacter, Methanotorris,* and *Methanolinea*. The coccoid methanogenic genus *Methanococcus* belongs to the family Methanococcaceae. This species is strictly anaerobic, mesophilic and heterotrophic, and will produce methane from CO_2/H_2 or formate (Rosenzweig and Ragsdale, 2011; Azizawa, 2014; Enzmann et al., 2018). *Methanobacterium* species are purely anaerobic hydrogenotrophic methanogens that use hydrogen and carbon dioxide and occasionally formate and alcohols for growth and a substrate for methane synthesis (Zhang et al., 2019; Zhou et al., 2021). The member of the genus *Methanohalophilus* belonging to the family Methanosarcinaceae is an anaerobe that uses methyl compounds as substrates to produce methane as its only source of energy (Guan et al., 2019). *The genus Methanococcoides of the Methanosarcinaceae family utilize methylated compounds as a sole source of energy for growth and substrate to produce methane* (Boone, 2001; Guan et al., 2014). The genus *Methanothermobacter* belongs to the family Methanobacteriaceae and grows in extreme temperatures. They are methanogenic and use CO_2/H_2 as substrates to produce methane for growth and energy. The genus *Methanomethylovorans* belonging to the family Methanosarcinaceae utilize Methanol, methylamines, dimethyl sulfide and methanethiol as the source of energy to produce methane (Sowers et al., 1984). The genus *Methanobrevibacter* of the family Methanobacteriaceae is strictly anaerobic. They reduce carbon dioxide via hydrogen to produce methane. The order Methanomicrobiales which includes the genus *Methanolinea* also utilize an H_2/CO_2 to produce methane (Imachi et al., 2009).

In conclusion, we were successful in getting an in-depth profile of the methanogens inhabiting permafrost-affected soil in the Changthang region of Ladakh. Our results show the presence of both aceticlastic and hydrogenotrophic methanogen in the studied samples. To the best of our knowledge, this study is the first of its kind. Owing to the advancement in sequencing technologies, we believe that this study will provide a vital addition to current knowledge about the methanogenic community thriving in the studied area. The methanogenic community linked to greenhouse emissions could increase the microbial breakdown of sequestered soil organic carbon and exacerbate the positive feedback from soil organic carbon to climate change. Thus, the metabolic potential of methanogens linked to methane production in this site should be analyzed with caution and in consideration for future studies.

Funding

The study was funded by the Department of Science and Technology, Science and Engineering Research Board (DST-SERB) project under the grant number SERB/F/8753/2016-17 and Ahmad Ali is grateful to University Grant Commission (UGC) India for the fellowship.

Acknowledgment

Sampling in this region was done with the permission from Wildlife Protection Department, UT Ladakh. The authors would like to thank Chief Wildlife Warden, Wildlife Protection Department, Ladakh and Wildlife Warden, Leh for granting permission to collect samples from this region. Also, thank UGC for providing fellowship to Ahmad Ali during the study.

Declaration of competing interest

The authors declare that they have no known competing interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

1. Ahlgren, N. A., Chen, Y., Needham, D. M., Parada, A. E., Sachdeva, R., Trinh, V. & Fuhrman, J. A. (2017). Genome and epigenome of a novel marine Thaumarchaeota strain suggest viral infection, phosphorothioation DNA modification and multiple restriction systems. *Environmental microbiology*, *19*(6), 2434-2452.
2. ASM. (2000). Environmental Change: microbial contributions, microbial solutions. American Society for Microbiology.
3. Azizawa, S. I. "Methanococcus voltae-Archael Flagella or Archaellum." *Shin-Ichi Aizawa, The Flagellar World* (2014): 94-95.
4. Bhattacharyya, A. (1989). Vegetation and climate during the last 30,000 years in Ladakh. *Palaeogeography, Palaeoclimatology, Palaeoecology, 73*(1-2), 25-38.
5. Boone, D. R. (2001). Order III. Methanosarcinales ord. nov. *Bergey's Manual of Systematic Bacteriology-The Archaea and the Deeply Branching and Phototrophic Bacteria*, *1*, 268.
6. Boone, D. R., Whitman, W. B., & Rouvière, P. (1993). Diversity and taxonomy of methanogens. In *Methanogenesis* (pp. 35-80). Springer, Boston, MA.
7. Chan, O. C., Claus, P., Casper, P., Ulrich, A., Lueders, T., & Conrad, R. (2005). Vertical distribution of structure and function of the methanogenic archaeal community in Lake Dagow sediment. *Environmental Microbiology*, *7*(8), 1139-1149.
8. Cole, James R., Benli Chai, Terry L. Marsh, Ryan J. Farris, Qiong Wang, S. A. Kulam, S. Chandra et al. "The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy." *Nucleic acids research* 31, no. 1 (2003): 442-443.
9. Conrad, R. (2005). Quantification of methanogenic pathways using stable carbon isotopic signatures: a review and a proposal. *Organic geochemistry*, *36*(5), 739-752.

10. Das, B. K. (2008). *Lakes: Water and Sediment Geochemistry*. Satish Serial Publishing House.
11. DeSantis, Todd Z., Philip Hugenholtz, Neils Larsen, Mark Rojas, Eoin L. Brodie, Keith Keller, Thomas Huber, Daniel Dalevi, Ping Hu, and Gary L. Andersen. "Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB." *Applied and environmental microbiology* 72, no. 7 (2006): 5069-5072.
12. Dimri, D. B., Baranwal, M. & Biswas, U. K. (1983). Integrated geophysical studies in Tsokar Basin, District Ladakh, J & K, India. *Indian Minerals*, *37*(2), 39-46.
13. Enzmann, F., Mayer, F., Rother, M., & Holtmann, D. (2018). Methanogens: biochemical background and biotechnological applications. *Amb Express*, *8*(1), 1-22.
14. Fiedler, S. D. W. L. K. E. M. P., Wagner, D., Kutzbach, L., & Pfeiffer, E. M. (2004). Element redistribution along hydraulic and redox gradients of low.centered polygons, Lena Delta, Northern Siberia. *Soil Science Society of America Journal*, *68*(3), 1002 1011.
15. Franzmann, P. D., Liu, Y., Balkwill, D. L., Aldrich, H. C., De Macario, E. C., & Boone, D. R. (1997). Methanogenium frigidum sp. nov., a psychrophilic, H2-using methanogen from Ace Lake, Antarctica. *International Journal of Systematic and Evolutionary Microbiology*, *47*(4), 1068-1072.
16. Garcia, J. L., Patel, B. K., & Ollivier, B. (2000). Taxonomic, phylogenetic, and ecological diversity of methanogenic Archaea. *Anaerobe*, *6*(4), 205-226.
17. Guan, Y., Ngugi, D. K., Blom, J., Ali, S., Ferry, J. G., & Stingl, U. (2014). Draft genome sequence of an obligately methylotrophic methanogen, Methanococcoides methylutens, isolated from marine sediment. *Genome announcements*, *2*(6), e01184-14.
18. Guan, Yue, David K. Ngugi, Manikandan Vinu, Jochen Blom, Intikhab Alam, Sylvain Guillot, James G. Ferry, and Ulrich Stingl. "Comparative genomics of the genus Methanohalophilus, including a newly isolated strain from Kebrit deep in the Red Sea." *Frontiers in microbiology* 10 (2019): 839.
19. Hedderich, R., & Whitman, W. B. (2005). Physiology and Biochemistry of the methane-producing Archaea. The Prokaryotes, An Evolving Electronic Resource for the Microbiological Community, 3rd edn, release 3.20, December 31, 2005 Dworkin M., et al., eds.
20. Høj, L., Olsen, R. A., & Torsvik, V. L. (2005). Archaeal communities in High Arctic wetlands at Spitsbergen, Norway (78 N) as characterized by 16S rRNA gene fingerprinting. *FEMS microbiology ecology*, *53*(1), 89-101.
21. Imachi, H., Sakai, S., Nagai, H., Yamaguchi, T., & Takai, K. (2009). Methanofollis ethanolicus sp. nov., an ethanol-utilizing methanogen isolated from a lotus field. *International journal of systematic and evolutionary microbiology*, *59*(4), 800-805.
22. Jeanthon, C., l'Haridon, S., Pradel, N., & Prieur, D. (1999). Rapid identification of hyperthermophilic methanococci isolated from deep-sea hydrothermal vents. *International Journal of Systematic and Evolutionary Microbiology*, *49*(2), 591-594.
23. Juottonen, H., Galand, P. E., Tuittila, E. S., Laine, J., Fritze, H., & Yrjälä, K. (2005). Methanogen communities and Bacteria along an ecohydrological gradient in a northern raised bog complex. *Environmental Microbiology*, *7*(10), 1547-1557.
24. Jurgens, G., Glöckner, F. O., Amann, R., Saano, A., Montonen, L., Likolammi, M., & Münster, U. (2000). Identification of novel Archaea in bacterioplankton of a boreal forest lake by phylogenetic analysis and fluorescent in situ hybridization. *FEMS Microbiology Ecology*, *34*(1), 45-56.
25. Keough, B. P., Schmidt, T. M., & Hicks, R. E. (2003). Archaeal nucleic acids in picoplankton from great lakes on three continents. *Microbial Ecology*, *46*(2), 238-248.
26. Kobabe, S., Wagner, D., & Pfeiffer, E. M. (2004). Characterisation of microbial community composition of a Siberian tundra soil by fluorescence in situ hybridisation. *FEMS Microbiology Ecology*, *50*(1), 13-23.
27. Kotsyurbenko, O. R., Chin, K. J., Glagolev, M. V., Stubner, S., Simankova, M. V., Nozhevnikova, A. N., & Conrad, R. (2004). Acetoclastic and hydrogenotrophic methane production and methanogenic populations in an acidic West.Siberian peat bog. *Environmental microbiology*, *6*(11), 1159-1173.
28. Kotsyurbenko, O. R., Nozhevnikova, A. N., & Zavarzin, G. A. (1993). Methanogenic degradation of organic matter by anaerobic bacteria at low temperature. *Chemosphere*, *27*(9), 1745-1761.
29. Lima-Perim, J. E., Romagnoli, E. M., Dini-Andreote, F., Durrer, A., Dias, A. C. F., & Andreote, F. D. (2016). Linking the composition of bacterial and archaeal communities to characteristics of soil and flora composition in the Atlantic Rainforest. *PloS one*, *11*(1), e0146566.
30. Magoč, T., & Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, *27*(21), 2957-2963.
31. Marchesi, J. R., Weightman, A. J., Cragg, B. A., Parkes, R. J., & Fry, J. C. (2001). Methanogen and bacterial diversity and distribution in deep gas hydrate sediments from the Cascadia Margin as revealed by 16S rRNA molecular analysis. *FEMS Microbiology Ecology*, *34*(3), 221-228.
32. Mathrani, I. M., & Boone, D. R. (1985). Isolation and characterization of a moderately halophilic methanogen from a solar saltern. *Applied and environmental microbiology*, *50*(1), 140-143.
33. Meng, X., Cao, Q., Sun, Y., Huang, S., Liu, X., & Li, D. (2022). 16S rRNA genes-and metagenome-based confirmation of syntrophic butyrate-oxidizing methanogenesis enriched in high butyrate loading. *Bioresource Technology*, *345*, 126483.
34. Meyer, Folker, Daniel Paarmann, Mark D'Souza, Robert Olson, Elizabeth M. Glass, Michael Kubal, Tobias Paczian et al. "The metagenomics RAST server–a public resource for the automatic phylogenetic and functional analysis of metagenomes." *BMC bioinformatics* 9, no. 1 (2008): 1-8.
35. Pangare, G., Pangare, V., & Das, B. (2006). *Springs of Life: India's Water Resources*. Academic Foundation.
36. Pasalari, H., Gholami, M., Rezaee, A., Esrafili, A., & Farzadkia, M. (2021). Perspectives on microbial community in anaerobic digestion with emphasis on environmental parameters: A systematic review. *Chemosphere*, *270*, 128618.
37. Ping, C. L., Clark, M. H., Kimble, J. M., Michaelson, G. J., Shur, Y., & Stiles, C. A. (2013). Sampling protocols for permafrost.affected soils. *Soil Horizons*, *54*(1), 13-19.
38. Rastogi, S., & Nawani, P. (1976). *Permafrost areas in Tsokar Basin.* Paper presented at the Symposium on the contribution of earth sciences towards research and development activities in the NR.
39. Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open-source tool for metagenomics. *PeerJ*, *4*, e2584.
40. Rosenzweig, A., & Ragsdale, S. W. (Eds.). (2011). *Methods in methane metabolism, part a: methanogenesis*. Academic Press.
41. Schink, B., & Zeikus, J. G. (1982). Microbial ecology of pectin decomposition in anoxic lake sediments. *Microbiology*, *128*(2), 393-404.
42. Schoeneberger, P. J., Wysocki, D. A., & Benham, E. C. (Eds.). (2012). *Field book for describing and sampling soils*. Government Printing Office.
43. Sizova, M. V., Panikov, N. S., Tourova, T. P., & Flanagan, P. W. (2003). Isolation and characterization of oligotrophic acido-tolerant methanogenic consortia from a Sphagnum peat bog. *FEMS microbiology ecology*, *45*(3), 301-315.
44. Sowers, K. R., Johnson, J. L., & Ferry, J. G. (1984). Phylogenetic relationships among the methylotrophic methane-producing bacteria and emendation of the family Methanosarcinaceae. *International Journal of Systematic and Evolutionary Microbiology*, *34*(4), 444-450.
45. Steven, B., Briggs, G., McKay, C. P., Pollard, W. H., Greer, C. W., & Whyte, L. G. (2007). Characterization of the microbial diversity in a permafrost sample from the Canadian high Arctic using culture-dependent and culture-independent methods. *FEMS microbiology ecology*, *59*(2), 513-523.
46. Wachinger, G., Fiedler, S., Zepp, K., Gattinger, A., Sommer, M., & Roth, K. (2000). Variability of soil methane production on the micro-scale: spatial association with hot spots of organic material and Archaeal populations. *Soil Biology and Biochemistry*, *32*(8-9), 1121-1130.
47. Wagner, D., Kobabe, S., Pfeiffer, E. M., & Hubberten, H. W. (2003). Microbial controls on methane fluxes from a polygonal tundra of the Lena Delta, Siberia. *Permafrost and periglacial processes*, *14*(2), 173-185.
48. Welte, C. U. (2018). Revival of archaeal methane microbiology. mSystems 3: e0018117.
49. Wilkins, D., Lu, X. Y., Shen, Z., Chen, J., & Lee, P. K. (2015). Pyrosequencing of mcrA and archaeal 16S rRNA genes reveals diversity and substrate preferences of methanogen communities in anaerobic digesters. *Applied and environmental microbiology*, *81*(2), 604-613.
50. Woese, C. R., Kandler, O., & Wheelis, M. L. (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences*, *87*(12), 4576-4579.
51. Woese, C. R., Magrum, L. J., & Fox, G. E. (1978). Archaebacteria. *Journal of molecular evolution*, *11*(3), 245-252.
52. Xavier, K. V. M., Silva, E., de Aquino Santos, E., & Diniz, M. C. (2020). Genetic diversity of 16S rRNA and mcrA genes from methanogenic archaeas. *Acta Scientiarum. Biological Sciences*, *42*, e49877-e49877.
53. Zhang, S., Chen, X., Zhong, Q., Zhuang, X., & Bai, Z. (2019). Microbial community analyses associated with nine varieties of wine grape carposphere based on high-throughput sequencing. *Microorganisms*, *7*(12), 668.
54. Zhou, H., Xing, D., Xu, M., Su, Y., Ma, J., Angelidaki, I., & Zhang, Y. (2021). Optimization of a newly developed electromethanogenesis for the highest record of methane production. *Journal of Hazardous Materials*, *407*, 124363.
55. Zinder, S. H. (1993). Methanogenesis: ecology, physiology, biochemistry and genetics. *Ed. GJ Ferry, Chapman and Hall, New York*, 128-206.