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Studies on Methanotrophs from Lonar Lake

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ABSTRACT:

Methanotrophic bacteria were isolated and characterized from sediment from alkaline Lonar Lake. Four bacterial strains were isolated using minimal salt media to study the methanotrophs of Lonar Lake and selected bacterial strains were further characterized, screened on the basis of the temperature, and salt tolerance. Bacterial isolates were subjected to morphological, biochemical characterization and 16S rRNA sequencing. Isolates were related to the phylum proteobacteria contains different genera such as *Acinetobacter baumannii, Pseudomonas aeruginosa, Achromobactor xylosoxidans* and *Ochromobactrum tritici*. These results clearly showed that the Lonar lake ecosystem harbors unexplored methanotrophs which can be used to control global warming as well methanol remediation.

Keywords:
Lonar Lake, methanotrophs, *Acinetobacter, Pseudomonas, Achromobactor* and *Ochromobactrum*.

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INTRODUCTION

The alkaline Lonar Lake, in Central India, situated in the village at Lonar, Buldhana district, Maharashtra ranks third in the world based on diameter and its high (pH 10.5) alkalinity (Taiwade, 1995). It is a closed system without outlets and regular influents are responsible for its existence. Based on the geological studies, it is postulated that the Lake originated as a meteorite impact crater around 50-53 thousand years ago (Jhingran and Rao, 1954; Nandy and Deo, 1961).

The diameter around the Lake is about 1.75 Km and water enters the Lake through rain, ground water seepage and the springs situated in the cliffs at the edge of the Lake. It does not receive any industrial discharges. Alkalinity of the Lake is attributed to the high content of sodium carbonate and hence was used previously as a source of washing soda (Thakker and Ranade, 2002,). The water in the crater is very salty. It is 10 times saltier than drinking water. Salts and Minerals like sodium, chloride, carbonates, fluorides and bicarbonates (TDS around are found and as this water do not drain away these substances get collected beneath the surface (Nathani, 2010, Shoemaker, 1963; Bhandari, 1984). In such conditions one cannot think of any living organisms, but microorganisms like *Arthrospora,* proteobacteria and algae are found abundant (Malu *et al,* 2002). It revealed that Lake water is alkaline (pH 10.3) and characterized by high concentration of salts (9060 mg/l), chloride (3492 mg/l), salinity (6391 mg/l), alkalinity (3751 mg/l), total hardness (480 mg/l), calcium hardness (118 mg/l), magnesium hardness (361 mg/l), sulphate (21 mg/ l), phosphate (0.44 mg/l), nitrate (3.7 mg/l) and dissolved oxygen (0.0034 mg/l). The Lonar Lake is unique in the world for its alkalinity and salinity of the water but it was seen that chlorides and salinity of the Lake water is decreasing day by day (Tambekar *et al,* 2010).

Methanotrophic bacteria are a group of organisms with the ability to use compounds with no carbon-carbon bonds (C_1 compounds) as single sources of carbon and energy, thus playing a role in global carbon cycling. A wide range of C_1 compounds are consumed by methanotrophs in the environment, including methane, methanol, methylated amines, methylated glycines, halomethanes, and methylated sulfur species. Methanotrophs are a unique group of Methylotrophic bacteria, which utilize methane as sole carbon and energy source (Olivier *et al,* 2005). Oxidation of methane to methanol by methanotrophic bacteria is mediated by the particulate membrane bound form (pMMO) or the soluble cytoplasmic form (sMMO) of methane monooxygenase. The conversion of methanol to formaldehyde is catalyzed in extant methanotrophs/ methylotrophs by the methanol dehydrogenase (MDH) enzyme. Methanol plays an important role in global warming, and its atmospheric concentration has been increasing over many decades and very much difficult to degrade (Whittenbury *et al,* 1970). The present study planned to isolate methanotrophic bacteria present in Lonar lakes which can degrade the industrial pollutant methanol and utilize methane by which the global warming can be reduced.

MATERIALS AND METHODS:

Four sediments samples were collected from selected sites of Lonar Lake with the help of scooper in sterile polythene bag. They were labeled and transported to the laboratory and stored at 4^0C until further analysis.

Medium composition: The one liter of medium containing NaNO3 2.5 g, KCL 0.1g, KH_2PO_4 3g, K2HPO4 0.01g, $MgSO_4$ 0.5g, $FeSO_4$ 0.116g, H_3BO_3 0.232g, $CuSO_4$ 0.41g, $MnSO_4$ 0.008g, $(NH_4)_6 Mo_7O_{24}$, 0.008g, and $ZnSO_4$ 0.174g, 20 ml methanol and pH 10 was prepared for isolation of methanotrophs (Haddad *et al*, 2009).

Isolation of bacterial strains: Sediment samples were inoculated in minimal salt media containing 2% methanol as carbon source. All flasks were incubated at 37^0C in Rotary shaker (100rpm) for 3 days and repeated subculturing was made in the same medium. After repeated subculturing, the bacterial growth was subcultured on same solid minimal salt medium for isolation of methanotrophs. Well isolated and differentiated colonies from these minimal media were transferred on Nutrient agar slant and maintained for further study.

Morphological, biochemical identification of isolates: Bacterial strains were examined for colony and cell morphology, motility, Gram staining and standard biochemical test (catalase, oxidase, IMViC and fermentation of sugar such as lactose, dextrose, mannitol, xylose and arabinose, nitrate reduction, urease activity, methyl, hydrolysis of starch etc).

Phylogenetic analysis: 16S rRNA sequencing was performed at NCCS, Pune. Nearly-full-length 16S rRNA gene sequences were submitted to CHECK-CHIMERA, available on the Ribosomal Database

Project release 10.26, in order to identify chimeras. Phylogenetic analyses were performed using the ARB software package. The 16S rRNA gene phylogenetic analyses were performed by the maximum-likelihood method, using 1,285 to 1,392 nucleotide positions. The functional genes were translated into amino acid sequences, and these were included in phylogenetic analyses using the neighbor-joining method (Dayhoff PAM model).

RESULTS AND DISCUSSION:

Methanotrophic bacteria are a group of organisms with the ability to use compounds with no carbon-carbon bonds (C_1 compounds) as single sources of carbon and energy, thus playing a role in global carbon cycling. Traditional microbial techniques such as enrichment and isolation on defined culture media have revealed that methanotrophic bacteria occur in a variety of environments, such as freshwater, marine, and terrestrial habitats, including habitats characterized by extreme conditions of temperature, salinity, or pH. Active members of the bacterial community in the sediment of Lonar Lake with special emphasis on C_1 utilizers (Methanotrophs) were identified by employing two complementary culture-dependent and independent approaches: morphological and standard biochemical identification and 16S rRNA analysis probing C_1 substrates methanol. The objectives of this study were to isolate, identify methanol utilizing bacterial from Lonar Lake and study further to evaluate their potential as methanotrophs. A preliminary study was carried out to isolate the bacterial strains from sediment using Minimal salt broth. Data of four sediment sample as per the characterization are conformed up to generic the level.

Four different isolated strains were identified by using standard procedures. The experimental outcome of morphological and biochemical characterization proved that all four stains are Gram negative and identified as *Acinetobacter, Pseudomonas, Achromobactor, Ochromobactrum* which prove to survive in extremophilic condition of high salt concentration and high pH (10.5). Present study reported that 4 methanotrophic gram negative, bacterial isolates from the sediments of the Lonar Lake. Among four isolates *Acinetobacter baumannii* was oxidase negative and other three *Pseudomonas aeruginosa, Achromobacter xylosoxidans* and *Ochromobactrum tritici* were positive. Catalase, indol, methyl red, voges-proskauer were negative and citrate utilization was positive in all isolates. On the basis of sugar fermentation, *Acinetobacter baumannii* fermented dextrose the other isolates can not ferment the sugars; nitrate reductase test was positive for *Achromobacter xylosoxidans* and negative for all other. Urease production was absent for all isolates. Among four bacterial strains *Acinetobacter baumannii* showed clear zone of starch hydrolysis indicate amylase production. The present studies on optimization of various growth parameters such as temperature, salt concentration

Table 1: Morphological, cultural, biochemical characteristics and 16S rRNA identification of bacteria isolated from sediments of Lonar Lake

Culture no.	Colony Characteristic		Morphology				Catalase test	Oxidase test	Sugar fermentation					IMViC test				Nitrate reductase	Urease	Starch hydrolisis Zone	Growth in 8.5 % NaCl	Growth at 4^0 c	Growth at 42^0 c	Organism identified based on 16S rRNA
	Shape	Color of Colony	Gm Reaction	Shape	Arrangement	Motility			Lactose	Dextrose	Mannitol	Xylose	Arabinose	Indole	MR	VP	Citrate utilization							
A	Cir	W	-	CB	G	-	-	-	-	A	-	-	-	-	-	-	+	-	-	12 mm	+	+	+	*Acinetobacter baumannii*
B	Cir	G	-	SR	S	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	*Pseudomonas aeruginosa*
C	Cir	W	-	SR	S	+	+	+	-	+	-	+	-	-	-	-	+	+	-	-	+	-	+	*Achromobacter xylosoxidans*
D	Cir	W	-	CB	P	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	*Ochromobactrum tritici*

Source – Sediment from Lonar lake, Medium - Minimal salt agar , Cir-Circular, W-White, G-Green, SR-Short rod, CB-Coccobacilli, P-Paired/ Group and G-Group

and the strains showed better growth at 42°C and 6.5% salt concentration (**Table 1**). All the isolates were analyzed by 16S rRNA analysis and found, *Acinetobacter baumannii* (Lonar–A), *Pseudomonas aeruginosa* (Lonar–B), *Achromobacter xylosoxidans* (Lonar–C) and *Ochromobactrum tritici* (Lonar–D) (**Table 2**). A related work was carried out by Jurjen Heyer (2005). It was evidently

Table 2a: 16S rRNA analysis (Blast analysis), Dendrogram showing phylogenic relationship of methanotrophs isolated from Lonar Lake

Lonar – A:

GATGACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGGGGGAAAGGTAGCTTGCTACTGGACCTAGCGGCGGACGGGTGAGTAATGCTTAGGAAT
CTGCCTATTAGTGGGGGACAACATCTCGAAAGGGATGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATG
AGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTATGGTTG
TAAAGCACTTTAAGCGAGGAGGAGGCTACTCTAGTTAATACCTAGGGATAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCC
GCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTATTAAGTCGGATGTGAAATCCCCGAGCTTAACTT
GGGAATTGCATTCGATACTGGTGAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGAT
GGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGT
CTACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATACTAGAAACTTTCCAGAGATGGA
TTGGTGCCTTCGGGAATCTAGATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCC
TTACTTGCCAGCATTTCGGATGGGAACTTTAAGGATACTGCCAGTGACAAACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGGCCA
GGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCTACACAGCGATGTGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCA
ACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGG
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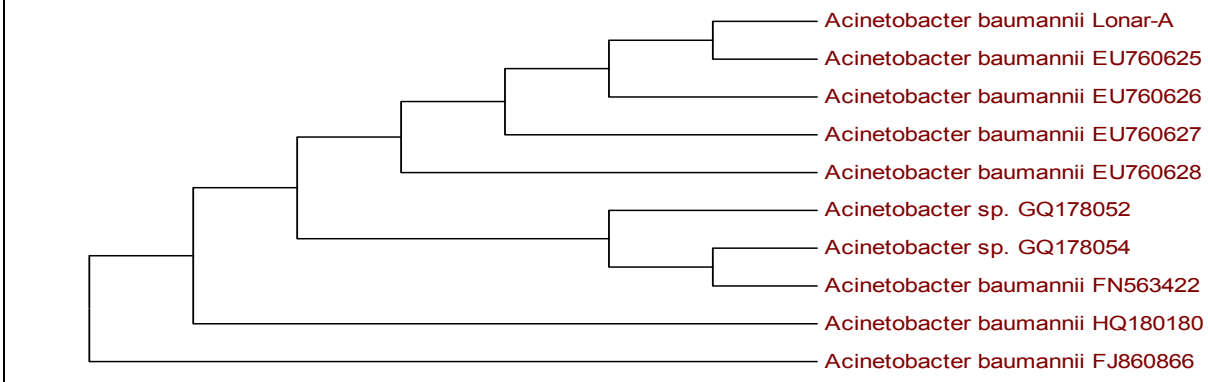
GAA



Lonar B:

TCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGG
AATCTGCCTGGTAGTGGGGGATAACGTCCGGGAAACGGGCGCTAATACCGCATACGTCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAG
ATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAG
ACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGG
ATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAG
CCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAAC
CTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAG
TGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
GTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAA
TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATG
GATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGT
CCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGC
CAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTG
CAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGG
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GAACCTGC

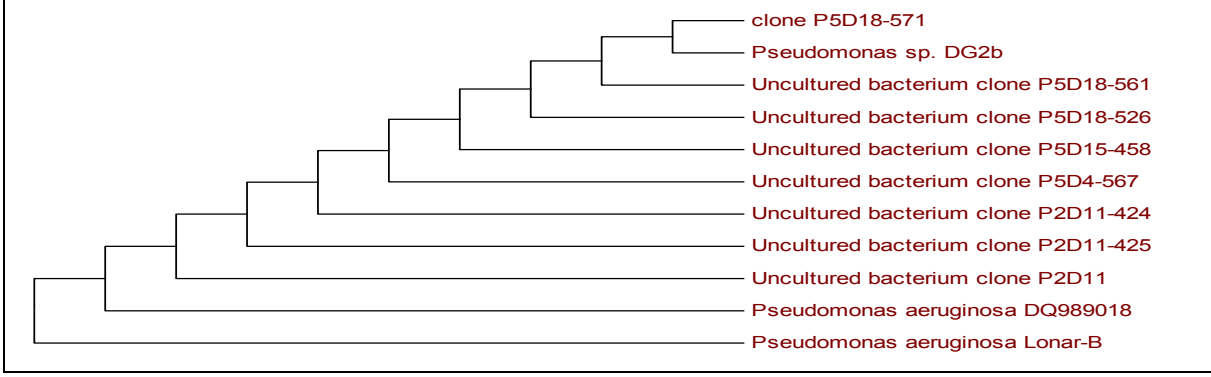

Table 2b: 16S rRNA analysis (Blast analysis), Dendrogram showing phylogenic relationship of methanotrophs isolated from Lonar Lake

Lonar – C

TCAGATGAACGCTAGCGGGATGCTTACACATGCAAGTCGAGCGCAGCACGGACTTCGGTCTGGTGGCGAGTGGCGAACGGGTGAGTAATGTATCGGAAC
GTGCCCAGTAGCGGGGGATAACTACGCGAAAGCGTAGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCGCAAGACCTTGCACTATTGGAG
CGGCCGATATCGGATTAGCTAGTTGGTGGGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCGGGTT
GTAAAGCACTTTTGGCAGGAAAGAAACGTCGCGGGTTAATACCTCGCGAAACTGACGGTACCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGAGCTTAACTT
TGGAACTGCATTTTTAACTACCGGGCTAGAGTGTGTCAGAGGGAGGTGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATG
GCGAAGGCAGCCTCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTC
AACTAGCTGTTGGGGCCTTCGGGCCTTGGTAGCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGA
CGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGTCTGGAATGCCGAAGAGATTTGGC
AGTGCTCGCAAGAGAACCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTC
ATTAGTTGCTACGAAAGGGCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCT
TCACACGTCATACAATGGTCGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCCAATCCCAGAAACCCGATCGTAGTCCGGATCGCAGTCTGCAACTCG
ACTGCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTGG
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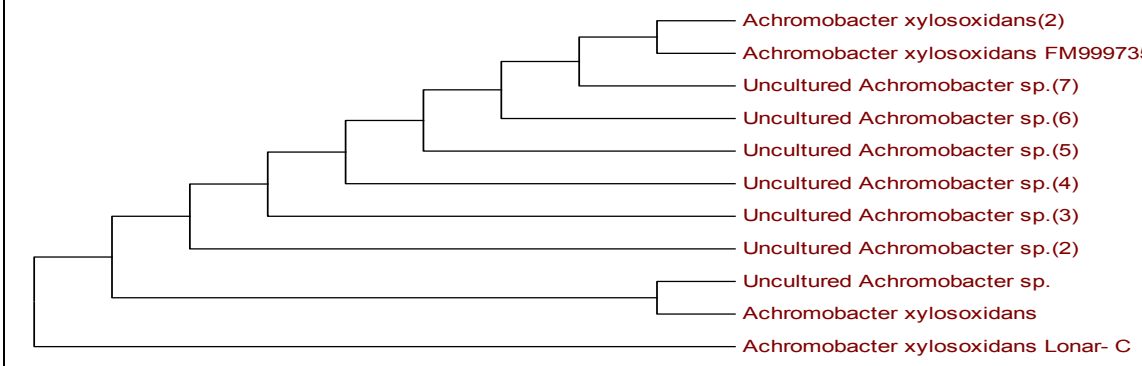

Lonar – D:

AACGAACGCTGCGGCAGGCTTAACACATGCAAGTCGAGCGCCCTTTTCGGAGGGAGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGCTAC
GGAATAACTCAGGGAAACTTGTGCTAATACGTATGTGCCCCCCTTTAAAATTCCAAGAAACATAAAATGCCTTGGCATTTTATGGGGGGGAAAGATTTAT
CGGCAAAGGATCGGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAA
GGCCCTAGGGTTGTAAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAA
GGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAGCGCACGTAGGCGGACTTTTAAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAACTGCCTTT
GATACTGGAAGTCTTGAGTATGGTAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTC
ACTGGACCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGTTAGCCGTTGG
GGAGTTTACTCTTCGGTGGCGCAGCTAACGCATTAAACATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCCCTTGACATCCCGATCGCGGTTAGTGGAGACACTATCCTTCAGTTCGG
CTGGATCGGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGC
ATTGAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACG
TGCTACAATGGTGGTGACAGTGGGCAGCGAGCACGCGAGTGTGAGCTAATCTCCAAAAGCCATCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATG
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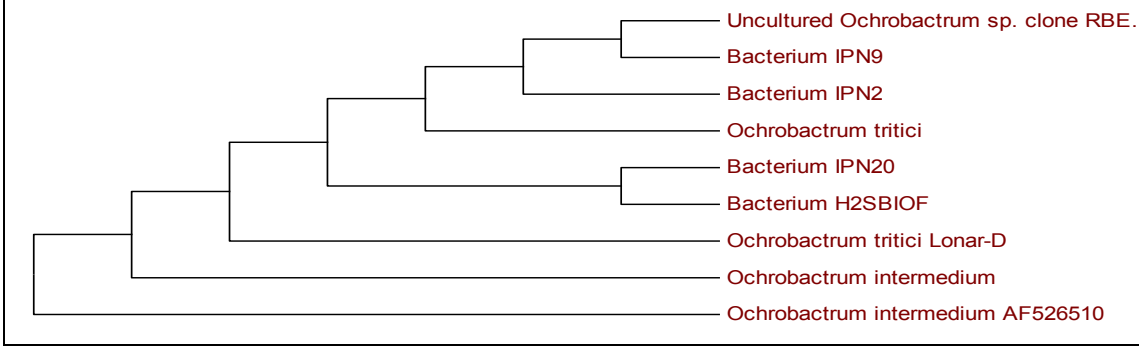

found that methanotrophic bacteria grow at 30^{0}C and 8.7% salt concentrations. Surakasi *et al, (*2010) also reported the phylogenFetic diversity of bacterial communities in microbial mats of two different seasons from saline and hyperalkaline Lonar Lake using 16S rRNA gene library analysis and demonstrated the presence of *Arthrospira* (*Cyanobacteria*), *Fusibacter* (LAI-1 and LAI-59)

and *Tindallia magadiensis* (LAI-27) in post-monsoon and *Planococcus rifietensis* (LAII-67), *Bordetella hinzii* (LAII-2) and *Methylobacterium variabile* (LAII-25) in pre-monsoon. They claimed the first time detection of these putative methanotrophs in surface mats of Lonar Lake

Methanol was primarily assimilated by *Acinetobacter baumannii, Pseudomonas aeruginosa, Achromobacter xylosoxidans* and *Ochromobactrum tritici* species from the family *Methylophilaceae isolated from Lonar Lake.* The majority of known aerobic methane-oxidizing bacteria are members of either *Gammaproteobacteria* (type I) or *Alphaproteobacteria* (type II), though several strains of highly acidophilic methanotrophic *Verrucomicrobia* have also been recently isolated. Most methanotrophs are capable of growth only on methane or other one-carbon compounds, using a methane mono-oxygenase (MMO) enzyme to oxidize methane to methanol (Molly *et al*, 2010). The isolated bacteria *Acinetobacter baumannii, Pseudomonas aeruginosa, Achromobacter xylosoxidans* and *Ochromobactrum tritici* species, are new species and not previously recorded bacterial species from Lonar Lake to utilize methanol as carbon source. Previous records indicated that *Pseudomonas aeruginosa, Acinetobacter baumannii, Achromobacter xylosoxidans,* are opportunistic pathogens found in soil and water, cause nosocomial infections in immunocompromised patients and also involved in biremidiation. The *Ochromobactrum tritici* was found to be resists to high Cr (VI) concentrations, making it a valuable tool in bioremediation. The findings of this study provide a window into the diversity of bacterial community members which are methane degrading from the Lonar Lake. These isolated bacterial species may be used to combat industrial pollution of methanol or to control global warming which may found better choice for further studies like methane, methanol or toxic chemical degradation to combat Global warming.

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