CHAPTER IV: RESULT

Chapter 4.1.
The microbial diversity in the leachate using 16S rDNA sequences

Methane is generated by the process called anaerobic digestion, which comprises of four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Each step is catalyzed by a different set of microbes. So, in order to understand the complete process of methane formation it is necessary to understand the microbial community structure present in the MSW leachate. In order to understand the type of microbial consortium present in MSW leachate and for their enrichment a lab-scale anaerobic reactor was set up.

4.1.1. Anaerobic Digestion of Municipal Solid Waste (MSW)

For enrichment and evaluation of microbial diversity present in the leachate especially designed lab scale glass bioreactor was set up. It contains three ports 1. Inlet Port: for purging of N_2 gas, 2. Outlet Port: for removal of dissolved oxygen and 3: Sampling Port: for sampling of leachate (Figure 4.1).

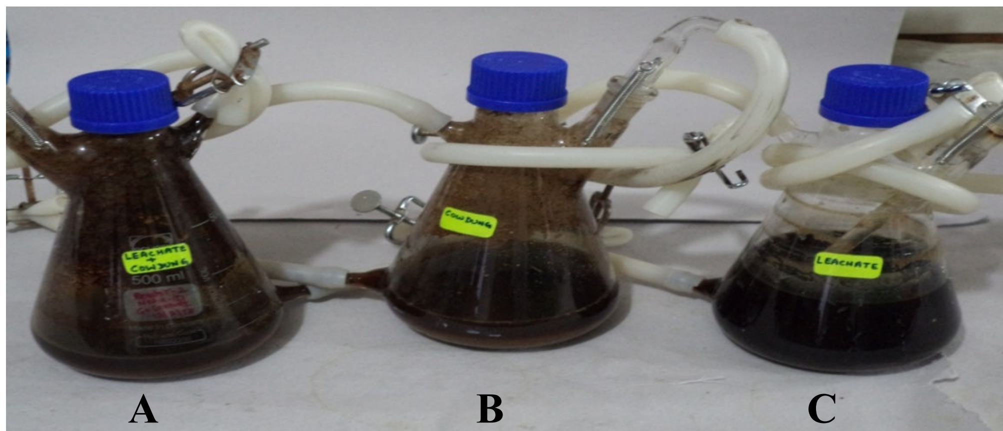


Figure 4.1. Showing specially designed glass bioreactor. A. contains Leachate, B. contains Cowdung+Leachate, C. contains Cowdung.

N_2 gas purging and pH was adjusted to 7 after every three days. Sampling was done at every fifth day. At time of sampling pH was adjusted to 7 and nitrogen gas was purged for 15 minutes. Then genomic DNA from these samples were extracted, PCR amplified, cloned and sequenced for identifying the microbes.

The degradation of organic matter to produce methane relies on the complex interaction of several different groups of bacteria. Because of the variety in substrate utilization exhibited by SRB, they compete with several different types of microorganisms involved in anaerobic digestion. SRB may compete with methanogens, acetogens, or fermentative microorganisms for available acetate, H_2, propionate, and butyrate in anaerobic systems. The outcome of the competition between SRB and other anaerobic microorganisms and physiochemical parameters like pH, heavy metal contents determines the quantity of methane production. Therefore in present section of this study we tried to find the microbial diversity specifically lactic acid bacteria presence and accumulation of lactic acid in anaerobic digestion of MSW leachate. In general lactic acid accumulation does not occur in nature because it is consumed by other microbial consortia which may result in higher rate of methane production.

For elucidation of this anaerobic digestion of MSW leachate experiment analysis of volatile fatty acids was carried out. Lactic acid production and accumulation was observed with N_2 gas purging for near anoxic conditions and contnious adjustment of pH to seven. For determination of lactic acid LDH based enzymatic assay was used.

4.1.2. Genomic DNA extraction

Total microbial community DNA was extracted from the leachate samples of Okhla and Ghazipur landfill site using a Lysozyme/Proteinase K/Sodium Dodecyl Sulphate (SDS) treatment followed by standard phenol/chloroform extractions protocol (Huang et al., 2004). The genomic DNA isolated is shown in Figure 4.2.

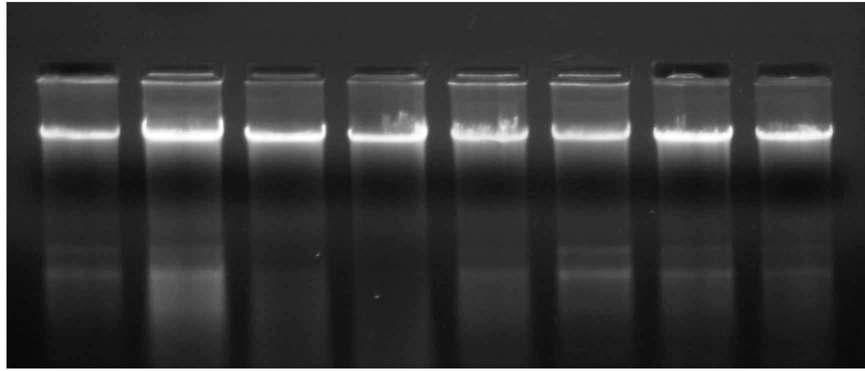


Figure 4.2. Showing metagenomic DNA isolated from Okhla leachate.

4.1.2.1. PCR amplification of Bacterial 16S rDNA gene

For evaluation of bacterial diversity, genomic DNA isolated from landfill leachate samples was amplified using primer specific for bacterial16S rDNA. The sequence of primer and PCR thermal profile has been mentioned in materials and method section. The amplification of the PCR product and the size of the amplicons is approx 1200 bp shown in Figure 4.3.

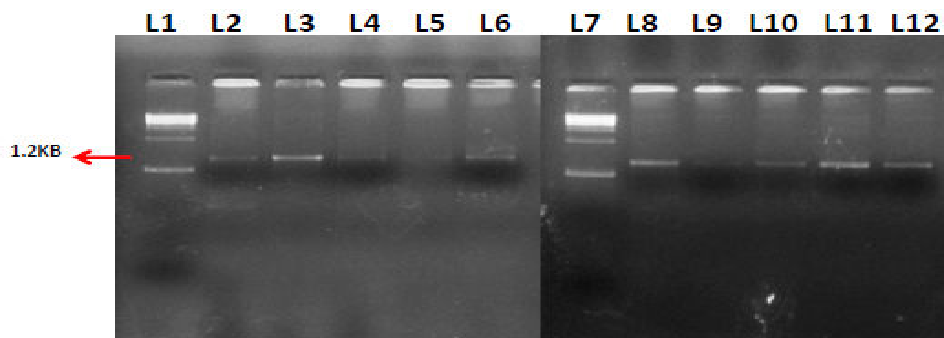


Figure 4.3. Showing bacterial 16S rDNA amplicons with universal 27F and 1492R bacterial 16S rDNA primer. Lane L1 and L7 contain 1KB DNA Ladder. Lane L2-L6 and Lane L8-L12 contain bacterial 16S rDNA amplicons obtained from both crude and filtered leachate reactors.

4.1.3. Cloning and Screening:

Purified PCR product of Bacterial 16S rDNA gene were cloned inside PTZ57R/T vector using the Insta-T/A cloning kit (Fermentas, UK) and transformed into *Escherichia coli* DH5α. The blue and white colonies were screened on Luria-Bertani plates with Ampicillin (100mg/mL), X-gal (20mg/mL), and IPTG (100 mM). The 16S rDNA gene amplicons from landfill and marshlands were examined by colony PCR using vector specific primer M13FWd and M13REV.

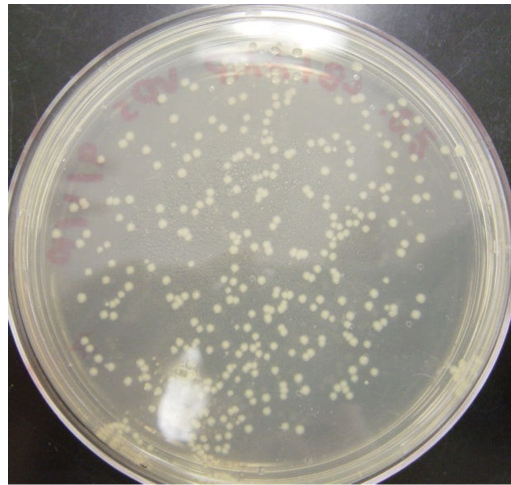


Figure 4.4. Showing screening of clones on LB-AMP^r plate containing Xgal-IPTG for blue-white screening.

4.1.4. Estimation of lactic acid accumulation

Three reactor flasks were set up. These reactors contained samples of 1) MSW leachate 2) Cow-dung only 3) Cow-dung laced with MSW leachate. The reactors had three different outlets: (1) inlet port, (2) outlet port, (3) sampling port. Nitrogen gas was bubbled through the reactors for 15 minutes through the inlet port while the outlet port was kept dipped in water. Nitrogen scrubs out oxygen from media. This was done to provide an anaerobic environment for the microorganisms present in leachate. This was done after a period of every three days.

Estimation of lactic acid assay was done by Lactic acid assay as explained in method section. Little accumulation of lactic acid was observed in reactor containing cowdung and maximum in the third reactor containing cowdung+leachate. The pattern of lactic acid production and accumulation in the three reactors is shown in Figure 4.5 and Figure 4.6.

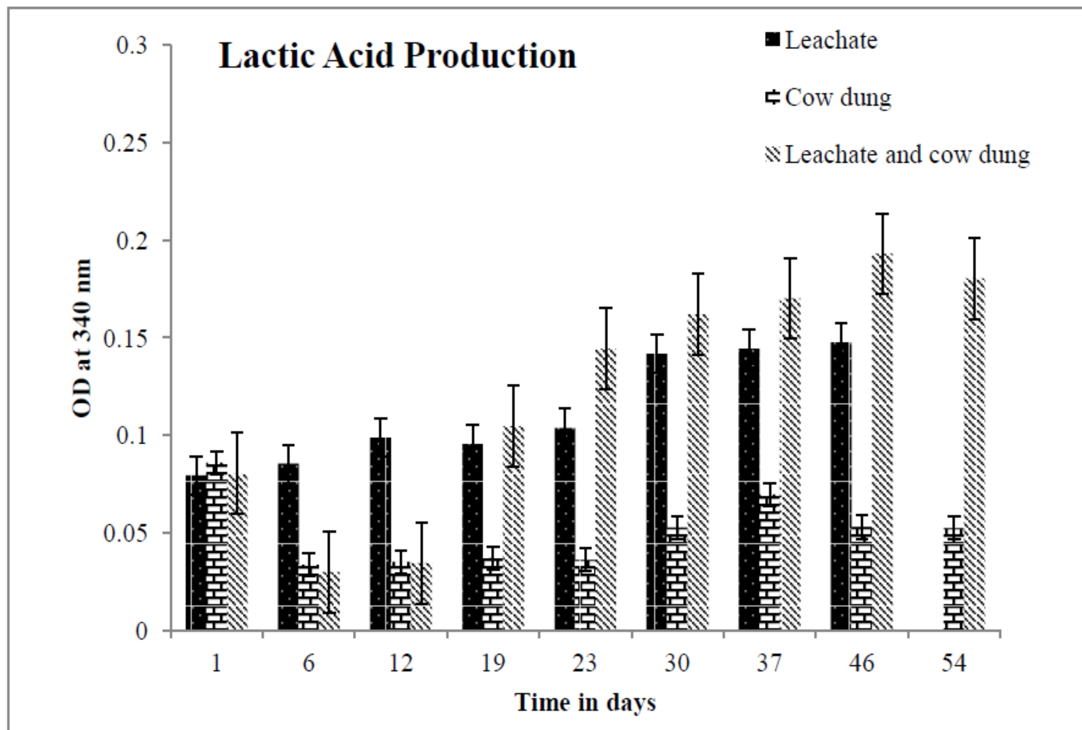


Figure 4.5. Bar graph showing the lactic acid production and accumulation in 1) leachate only 2) cow-dung only 3) cow-dung+leachate bioreactors. O.D. is proportional to lactic acid concentrations.

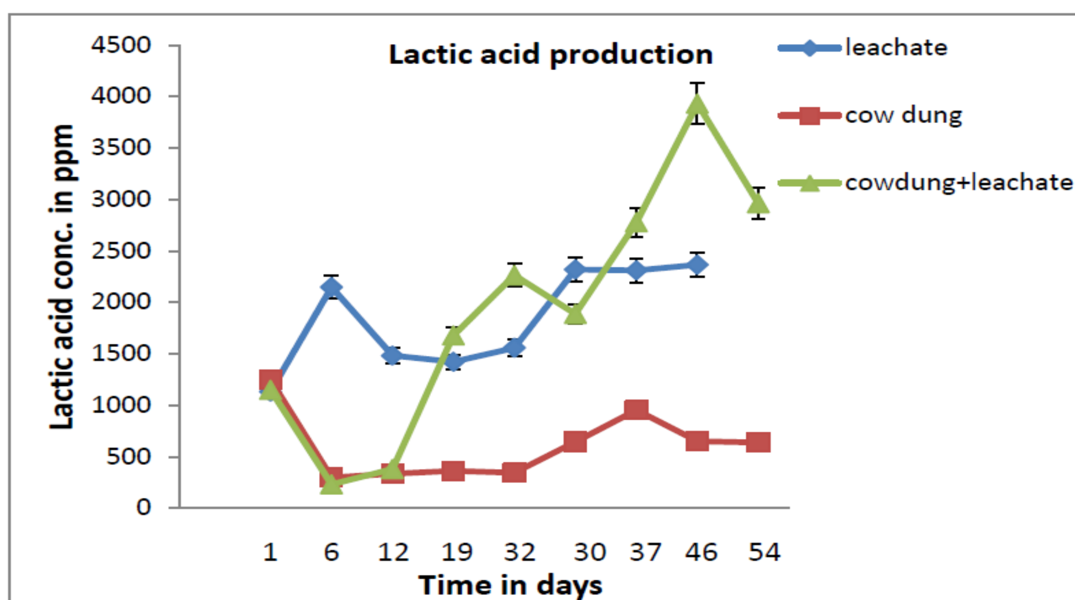


Figure 4.6. Line graph showing the lactic acid production during anaerobic digestion of 1) leachate 2) cow-dung only 3) cow-dung +leachate samples.

The results obtained from above experiments indicate the production and accumulation of lactic acid during anaerobic digestion of MSW leachate. For MSW leachate only reactor, lactic acid was produced but its concentration remained constant as there was no decay. In the reactor with cow-dung only, some lactic acid was produced which decayed. In the reactor with cow-dung and leachate accumulation of lactic acid was significant.

4.1.5. Identification of lactic acid bacteria from leachate

For the enrichment of lactic acid bacteria (indigenous in leachate) deMan, Rogosa Sharpe broth (MRS) was used. In one reactor leachate pH was adjusted to 3.4 to precipitate out the contaminants like humic acid and fulvic acid. It was filtered using 0.45µm PVDF filter membrane (Millipore) to remove the precipitate. Then 100 ml of filtrate was taken and 125 ml of MRS broth and 25ml of leachate was added as inoculum to it. In the second reactor, 100 ml crude leachate plus 150 ml of MRS broth was taken. Initially, both reactors were run for seven days in aerobic condition for the

process of hydrolysis to occur. Then both reactors were subjected to anaerobic condition by nitrogen gas purging at regular three day intervals.

Growth in the first reactor having filtered leachate was more turbid and rapid. At every alternate fifth day sampling was done from both reactor and from these samples total DNA was extracted.

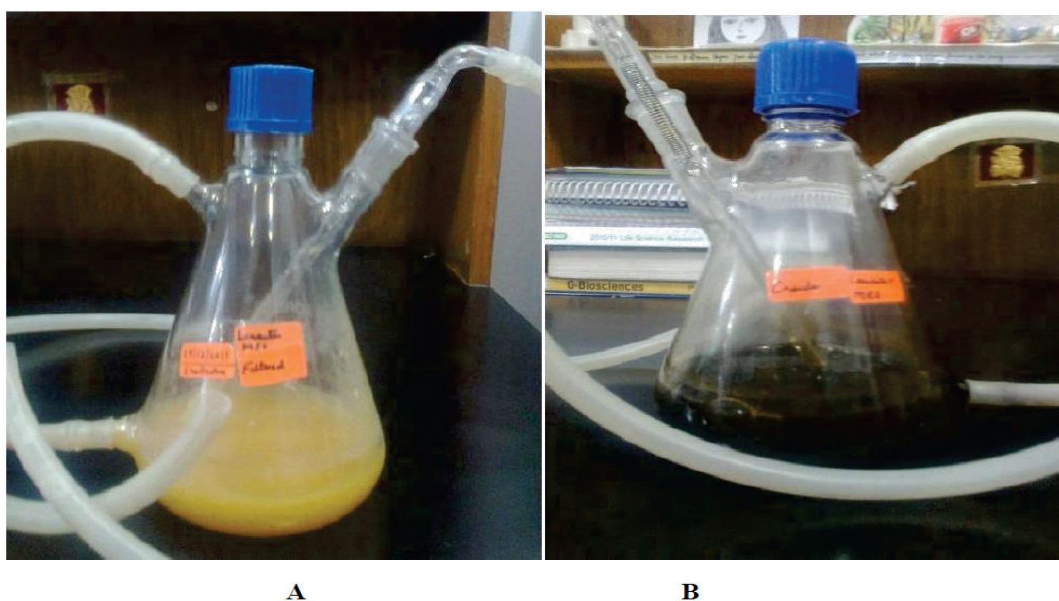


Figure 4.7. Growth in crude and clarified leachate reactor.

A. Reactor containing filtered leachate with MRS broth.
B. Reacor containing crude leachate with MRS broth.

4.1.6. PCR for detection of lactic acid bacteria

Metagenomic DNA isolated from the bioreactors samples were amplified using Lactobacillus specific forward primer and reverse primer. The sequence of primer and PCR thermal profile has been mentioned in materials and method section. The PCR amplicons obtained were cloned inside pTZ57R/T using protocol as mentioned in method section.

4.1.7. Construction of Lactobacillus Identification Ladder (LIL)

For construction of Lactobacillus Identification Ladder (LIL) seven pure cultures of lactobacillus bacteria were obtained from the repository of National Dairy Research Institute (NDRI), Karnal, India.

S.no.	Catalogue no.	Strain name
1	NCDC 11	*Lactobacillus acidophilus*
2	NCDC 17	*Lactobacillus casei*
3	NCDC 20	*Lactobacillus plantarum*
4	NCDC74	*Streptococcus thermophillus*.
5	NCDC 141	*Lactobacillus fermentum*
6	NCDC 194	*Lactobacillus helveticus*
7	NCDC 231	*Bifidobacterium bifidium*

Table 4.1. List of Lactobacillus pure culture obtained from NDRI, Karnal and used for construction of Lactobacillus identification Ladder (LIL).

For construction of LAB identification ladder genomic DNA extracted from these seven pure cultures were subjected for PCR using S-G-Lab-0677-a-A-17 REV and S-D- Bact-0011-a-S-20 FWD primers. PCR products obtained from these pure cultures were in range of 700bp. These PCR products were re-amplified with GC clamp containing primers using S-D- Bact-0011-a-S -20 FWD primer with GC clamp and Lab-0677-a-A-17 REV for DGGE experiments . The master-mix composition and amplification temperature profile was same as described in method section except annealing temperature was 55 ^{o} C for 1 minute. Then the PCR products obtained from all pure cultures were mixed to construct Lactobacillus Identification ladder (LIL).

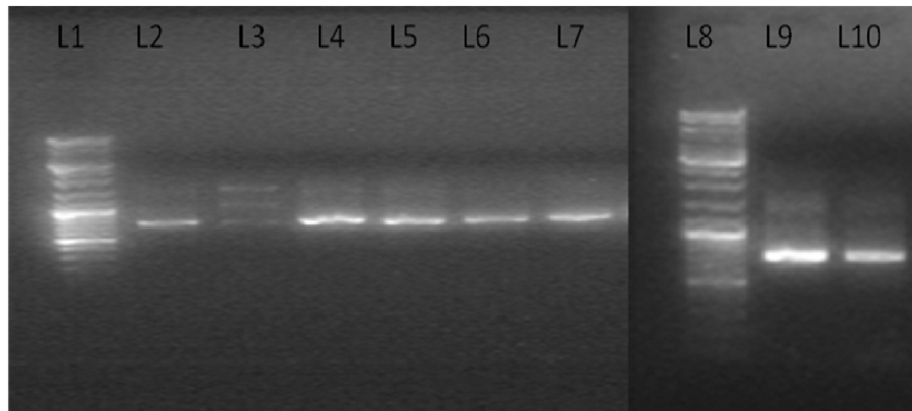


Figure 4.8. Shows PCR amplification of *Lactobacillus* pure cultures using S-D- Bact-0011-a-S -20 FWD and Lab-0677-a-A-17 REV primer. L2 contain L. *acidophillus*, L3 contain L. *casei*, L4 contain L. *fermentum*, L5 contain L. *helveticus*, L6 contain L. *plantarum*, L7 contain B. *bifidium*, L8 contain S. *thermophillus*.

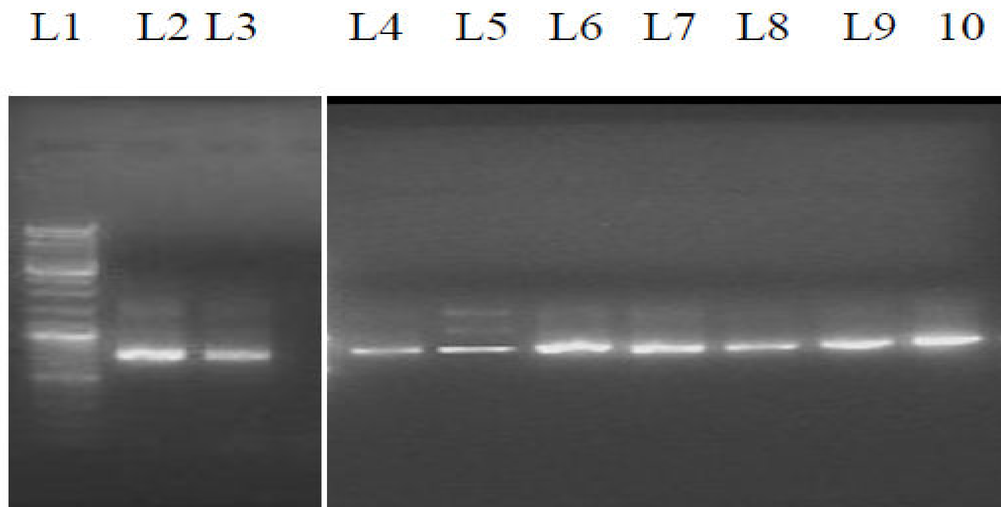


Figure 4.9. Reamplification of 16S rDNA amplicon with S-D- Bact-0011-a-S -20 FWD primer with GC clamp and Lab-0677-a-A-17 REV for DGGE in the following order L2 contain L. *acidophillus*, L3 contain L. *casei*, L4 contain L. *fermentum*, L5 contain L. *helveticus*, L6 contain L. *plantarum*, L7 contain B. *bifidium*, L8 contain S. *thermophillus*.

4.1.8. Denaturing gradient gel electrophoresis (DGGE) - DGGE was performed using D Code universal mutation detection system (Biorad, Hercules, California.) using 16cm by 16cm by 1mm gels. Eight percent polyacrylamide gels were prepared and run with 1X Tris Acetate EDTA (TAE) buffer diluted from 50X TAE buffer supplied by Biorad. The gel had 40% to 60% gradient of urea in the direction of electrophoresis. The electrophoresis was conducted at constant voltage of 140V at 55°C for three 3.30 hours. Following electrophoresis the gel was silver stained and scanned under an HP Scanjet 3110 scanner (An et al., 2009).

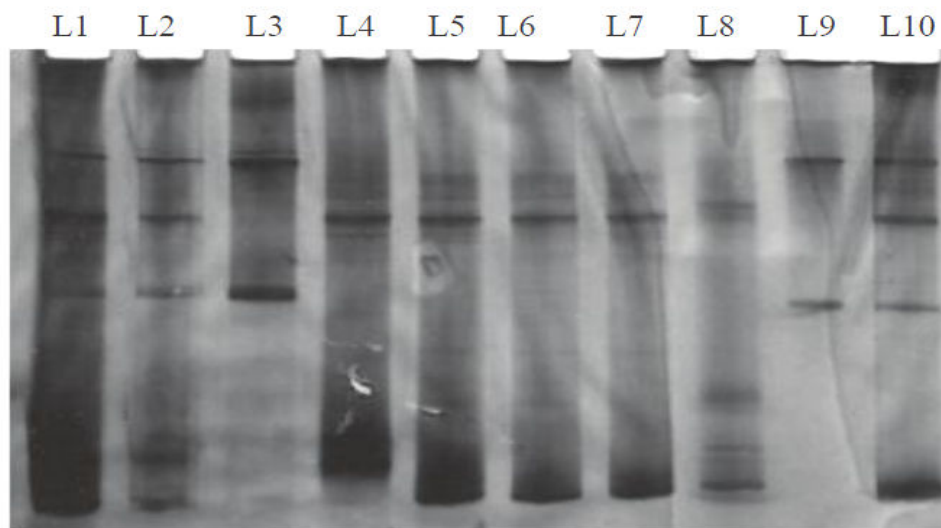


Figure 4.10. Use of lactobacillus identification ladder (LIL) for identification of Lactic Acid Bacteria (LAB) present in MSW leachate.

DGGE gel is showing *Lactobacillus* identification ladder in L1 , L2 –L8 contain 16S rDNA amplicon obtained after amplifying genomic DNA of respective pure culture using S-D- Bact-0011-a-S -20 FWD primer with GC clamp and Lab-0677-a-A-17 REV primer. L2 contain *L. acidophillus*, L3 contain *L. casei*, L4 contain *L. fermentum*, L5 contain *L. helveticus*, L6 contain *L. plantarum*, L7 contain *B. bifidium*, L8 contain *S. thermophillus*, L9 contain PCR amplicon from crude leachate, L10 contain PCR amplicon from filtered leachate reactor.

Migration distances of 16S rDNA amplicons obtained from the unidentified isolates of lactobacilli was compared to those of reference strains in the identification ladder. After running DGGE we found the bands obtained in Lane 9 (crude leachate) were corresponding to the banding pattern as observed in the pure cultures of *L. acidophilus* and *L. casei* in and Lane 10 (filtered leachate) it was corresponding to banding patterns of *L. acidophilus, L. casei , L. fermentum, L. helveticus and L. plantarum.*

Heavy Metals	Conc. in mg/ml
Zn	2.10
Cd	1.26
Cr	1.32
Fe	6.51
Pb	0.11
Ni	0.53
Mo	0.84

Table 4.2. Establishment of presence of heavy metals in MSW leachate (Ghazipur) by Atomic Emission spectroscopy.

Figure 4.11. Toxicity (coagulation) test with SRB *Desulfovibrio vulgaris-MTCC 115.*

1. Tube 1 contains pure culture of *Desulfovibrio vulgaris and crude leachate.*
2. Tube 2 contains pure culture of *Desulfovibrio vulgaris* and 0.2 molar concentration of ammonium molybdate.
3. Tube 3 contains pure culture of *Desulfovibrio vulgaris* and *2* molar concentration of ammonium molybdate.
4. Tube 3 contains only pure culture of *Desulfovibrio vulgaris.*

Results indicated that there was lactic acid accumulation during anaerobic digestion of MSW leachate. When MSW leachate was added in the cow dung good amount of lactic acid accumulation occurred. According to Touzel (Touzel et al., 1981) one group of SRB partially oxidizes the lactic acid into acetic acid while another group completely oxidizes lactic acid/acetic acid. If the accumulation of lactic acid is an indirect evidence of SRB suppression, this may lead to very interesting application in bio-mass (manure) gasification plants.

We know that SRB creates the nuisance of bad odour of H_2S, corrosion of the metallic lead of the anaerobic digesters and poorer productivity of biogas (methane). To remedy these, all we need is to collect MSW leachate and sprinkle it in manure bio-digesters. The suppression of SRB can be due to presence of Molybdate (Nemati et al., 2001) or other heavy metals present in the leachate. Preliminary toxicity tests was performed with one SRB (strain *Desulfovibrio vulgaris* MTCC #1115) obtained from Microbial Type Culture Collection (MTCC), Chandigarh in India. It was grown inside sealed serum bottles under anoxic condition using media as prescribed by MTCC. It grows on suspension culture but on injection of leachate or ammonium molybdate solution (0.2 millimolar and 2 millimolar) (Neimati *et.al.,* 2005), it coagulated and precipitated to the bottom of the bottles. Most probably this was due to cell inactivation (Figure 4.11).

However, cell death assays like Trypan blue exclusion was not possible as no protocol for this is available for anaerobic SRBs. Lactobacillus identification ladder (LIL) indicated the presence of various *Lactobacillus* species in crude leachate and it was found that they grow well in filtered leachate (humic and fulvic acid removed). Presence of heavy metals in MSW leachate was established using atomic ionisation spectroscopy. It indicated presence of heavy metals like nickel, molybdenum etc.

4.1.9. Molecular detection of Methanotrophs

Samples were collected from the Ghazipur landfill site of Delhi. It is situated in east Delhi and is the oldest and largest landfill of Delhi. It was established in the year 1984 and spans 29.6 hectares in area. It has received about 2200 TPD waste till now. The GPS location of sampling point was 28°37’22.4”N-77°19”25.7”E and the pH of the sample was in between 7.4-8.4.

4.1.9.1. Nucleic acid extraction, PCR amplification and cloning

DNA from both landfill leachate samples was extracted on the same day of sampling using Fast DNA Spin Kit for Soil (MP Biomedicals, CA, USA). DNA from leachate was amplified using the universal primer set 27FWD and 1492REV. The amplification profile was 94°C for 5min, 94°C for 30s for 30 cycles, 55°C for 1minute, elongation at 72°C for 2 minute and final extension at 72°C for 10 minutes followed by a cooling step down to 4°C. Obtained 16S rDNA PCR products were purified by PCR purification kit (Fermentas, UK) as recommended by manufacturer protocol. PCR amplicons of 16S rDNA gene were cloned inside PTZ57R/T vector using the Insta-T/A cloning kit (Fermentas, UK) and transformed into *Escherichia coli* DH5α. The positive clones were selected using blue-white screening on Luria-Bertani plates containing Ampicillin (100mg/ml), X-gal (20mg/ml), and IPTG (100 mM). Then positive clones were sequenced using M13 FWD primer.

4.1.9.2. Denaturing Gradient Gel Electrophoresis

For denaturing gradient gel electrophoresis genomic DNA extracted from the landfill and marshland was amplified using primer 27FWD and GC 968 which gave a product length of about 900 bp. DGGE was performed with a D-Code universal mutation detection system (Biorad, Hercules, CA, USA) using 16 cm by 16 cm and one mm gels. PCR products were loaded onto 7% (w/v) polyacrylamide gel.

The polyacrylamide gels (Bis-Acrylamide, 37.5:1) were made with denaturing gradients ranging from 40 to 60%. 100% denaturant contained 7 M urea and 40% Formamide. Electrophoresis was initially run at 200 V for 10 min at 60°C, and afterwards at 130 V for 4 h. After electrophoresis, the gel was silver stained and scanned under white light using Gel doc (Biorad) **Figure 4.12.** DGGE gel was further analyzed using Gel2K software (Svein Norland, Department of Biology-University of Bergen, Norway).

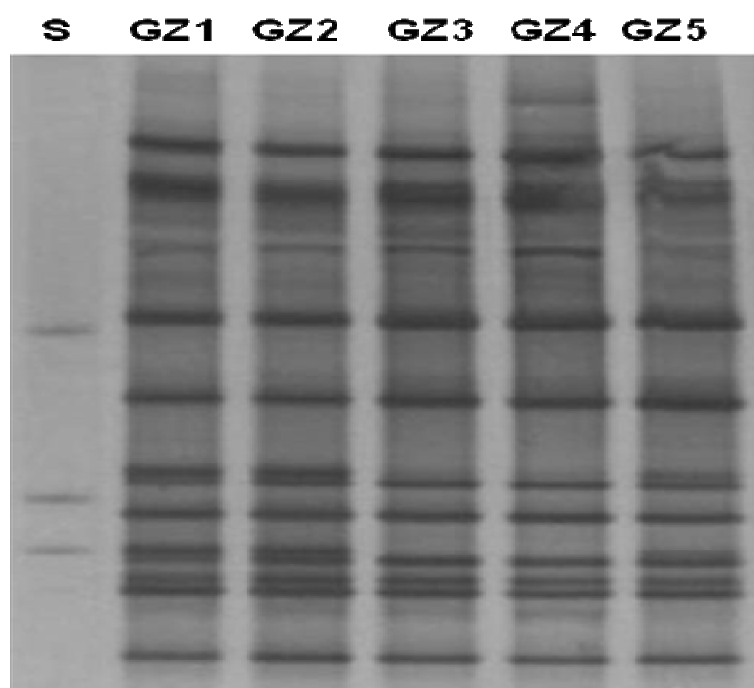


Figure 4.12. Molecular diversity analysis of methylotrophic community present in leachate samples of Ghazipur landfill site. S is 100 bp DNA ladder.GZ1-GZ5 are leachate samples collected from five different locations in Ghazipur landfill site.

Abundance and diversity of methanotrophic bacteria were studied using denaturing gradient gel electrophoresis in leachate samples collected at five different locations at Ghazipur landfill site, Delhi. 16S rDNA amplicons used for cloning were

analyzed on the DGGE gel for estimation of their richness in respective samples. Banding patterns of 16S rDNA amplicons obtained from all five leachate samples of the Ghazipur landfill site were compared using Gel 2K software and the analysis of DGGE image revealed the presence of total 23 bands Figure 4.13.

There are some unique bands in each lane, which indicates the variation of methanotrophic community residing in those particular samples. Cluster analysis of bands using Jaccard analysis indicated the presence of two main clusters on the basis of number of similarity and DGGE bands Figure 4.13. In first cluster samples GZ3, GZ4 and GZ5 sample clustered together showing similar community structure and diversity. In second cluster samples GZ1 and GZ2 of leachate clustered together. It indicates that methanotrophic community structure inhabiting in all five leachate samples were similar. In terms of richness regarding no. of bands, GZ4 samples has maximum 13 bands followed by GZ2, GZ1, GZ2 and GZ5 having 12,11 and 10 bands respectively.

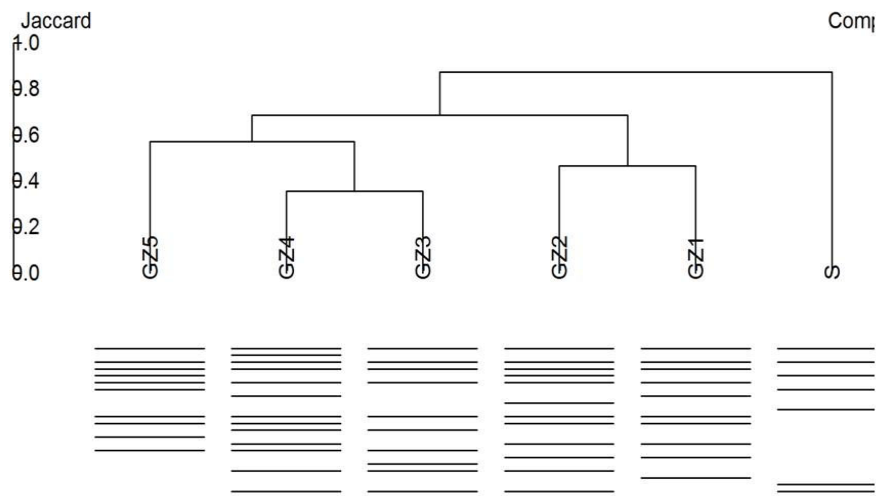


Figure 4.13. Showing Jaccard cluster analysis of methanotrophic community based on 16S rDNA obtained from five location of Ghazipur landfill site Delhi.

Chapter 4.2:

Comparative analysis of methanogenic diversity associated with marshlands samples in and leachate samples of landfill

4.2.1. DNA extraction, PCR amplification and cloning

Leachate samples were collected from the Okhla, Bhalsawa and Ghazipur landfill site New Delhi India. Soil samples were collected from marshlands (Silicoorie Lake) in sterile falcon tubes from Silchar, Assam, India.

DNA from both landfill leachate and marshland sediment samples was extracted on the same day of sampling using Fast DNA Spin Kit for Soil (MP Biomedicals, CA, USA). After isolation DNA was dissolved in 25 µl Mili Q water and 3 µl of it was visualized on 1% agarose gel.

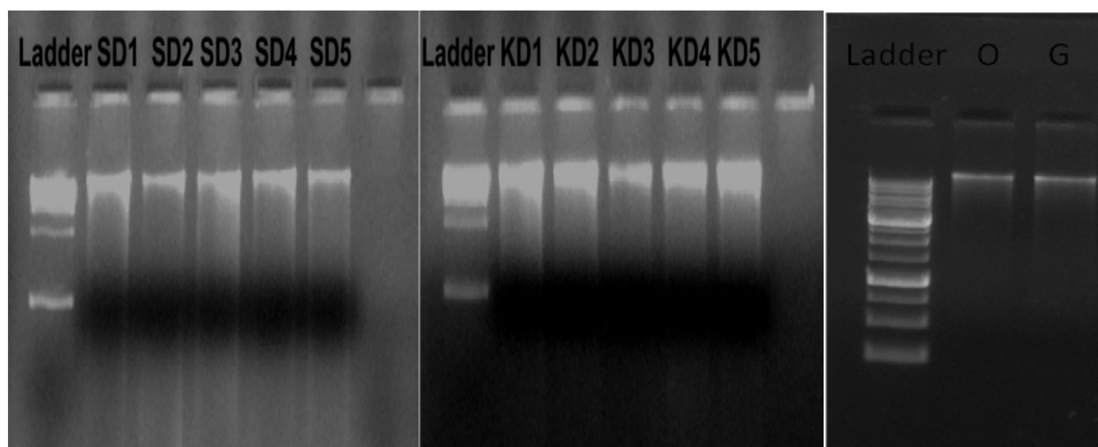


Figure 4.14. Showing genomic DNA isolated from the ten samples of Assam marshland samples and leachate samples of Ghazipur and Okhla.

For identification of methanogens specifically, genomic DNA extracted from the landfill and marshland samples was amplified using the primer set MLFWD and MLREV designed by Luton (Luton et. al.2002).

PCR was performed in a thermo-cycler (Applied Biosystem: Gene amp 9700). A typical reaction mixture of (50µl) for PCR of mcrA gene consisted of Taq buffer 5µl, *Taq* polymerase 1µl,dNTP 5µl, fwd primer 2 µl rev primer 2 µl, $MgCl_2$ 2µl and 32 µl of nuclease free water and template DNA 1 µl (10m-25 ng). The amplification profile was 94^oC for 5min, 94^oC for 30s for 30 cycles, 52^oC for 1 minute and elongation at 72^oC for 2 minute and final extension at 72^oC for 10 minutes followed by a cooling step down to 4^oC.

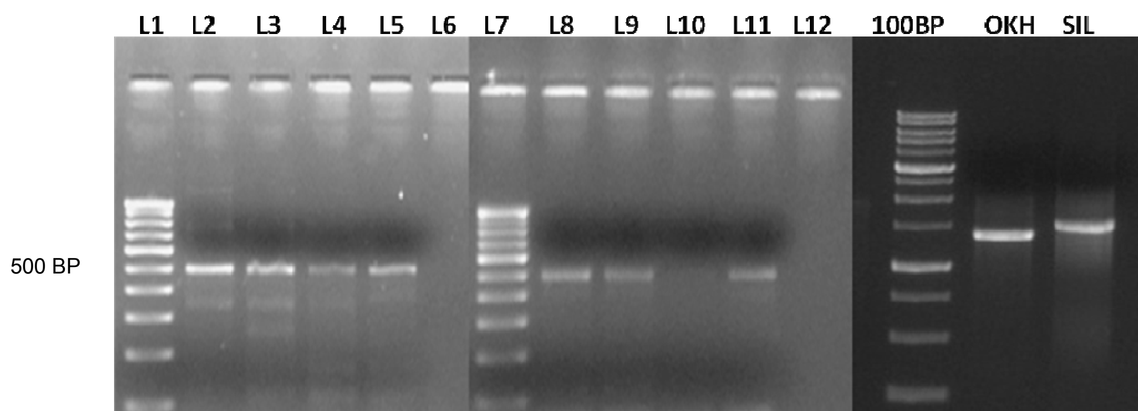


Figure 4.15. Showing mcrA gene amplicons obtained from the genomic DNA isolated from the leachate sample of Ghazipur samples using primer designed by Luton specific for mcrA gene (Luton et al. 2002). Lane 1 and 7 contain 100bp DNA ladder.L2,L3 L4,L5 contain mcrA gene amplicons from Ghazipur leachate samples G1,G2,G3,G4 and G5.Lane 8,910 and 11 contain mcrA gene amplicons from Ghazipur leachate samples G5,G6,G9 and G10. O and SIL are mcrA gene amplicons obtained from Okhla Landfill site Delhi (OKH) and Silicoorie lake (SIL) Silchar, Southern Assam.

After PCR, amplicons were purified using PCR purification kit (Fermentas) as per manufacturer protocol and visualized on 1.5% agarose gel for intensity. After purification the *mcrA* amplicons were cloned using TA cloning (K1214 Fermentas). Positive clones were screened by blue-white screening using Xgal-IPTG and Amp^rplate. Plasmids were isolated from the white colonies and were sent for sequencing. Sequences obtained were curated manually and were submitted to NCBI which is explained in details in phylogenetic analyses which is shown in chapter 4.4.

4.2.2. Community profiling of methanogens from landfill site Delhi and marshland of Silchar using mcrA genes:

For community profiling of methanogens mcrA gene amplicons obtained from both landfill and marshland were subjected to PCR with GC clamp containing primers, MLFWD and MLGC REV for DGGE. The amplification profile was 94°C for 5 min, 94°C for 30s for 30 cycles, 52°C for 1minute, 72°C for 2 min and final extension at 72°C for 10 minutes followed by a cooling step down to 4°C.

DGGE was performed with a DCode universal mutation detection system (Biorad, Hercules, California) using 16cm by 16cm by 1mm gels. Eight percent polyacrylamide gels were prepared and run with 1XTAE buffer diluted from 50X TAE buffer (Biorad). The gel has 40% to 60% gradient of urea in the direction of electrophoresis. The electrophoresis was conducted at constant voltage of 140 V at 55°C for three 4.30 hours. Following electrophoresis the gel was silver stained and scanned. Silver staining of DGGE gel involves incubation of gel in staining solution (5% ethanol + 1% nitric acid+0.1% $AgNO_3$) for five minutes, washing in H_2O for 10 seconds, incubation in developing solution (1.3% NaOH+0.65% $NaCO_3$, 0.4% HCOH) for 5- 10 minutes, then gel was soaked in stop solution (5% ethanol,1% nitric acid) for 1 minute. Gels were scanned after removal of solution and washing with ddH_2O, shown in Figure 4.16.

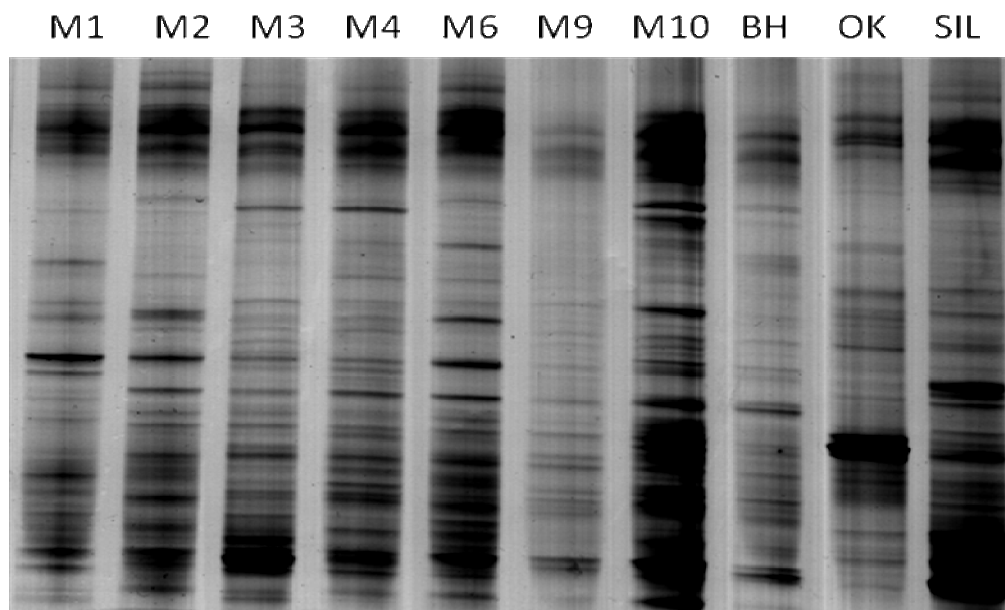


Figure 4.16. Commuity profiling of methanogens present in the landfill site and marshlands usin mcrA gene. M1-M10 contains mcrA gene amplicons amplified from genomic DNA isolated from leachate samples of Ghazipur landfill. BH, OK and SIL are mcrA gene amplicons amplified from genomic DNA isolated from the leachate sample of Bhalsawa, Okhla landfill marshland of Silicoorie lake Silchar Assam.

Band patterns of mcrA amplicons obtained from the landfill site (OK, BH and GZ) of Delhi and Silicoorie Lake samples (SIL) of southern Assam were compared for methanogens richness and diversity analysis using Gel 2K software. Analysis of DGGE image revealed the presence of total unique 50 bands Figure 4.17. There are some unique bands in each lane, which indicates the variation of methanogens community residing in those particular samples. Cluster analysis of bands using Jaccard analysis indicated the presence of three main clusters consisting of localities that differed in the number and similarity of DGGE bands Figure 4.18.

In first cluster, Silicoorie Lake of Southern Assam clustered together with Okhla landfill site and M1 sample of Ghazipur landfill site showing similar band pattern. In second cluster, sample of Ghazipur landfill sites Delhi, i.e. M6, M4, M2 and M3 clustered with each other. In third cluster, the two landfill site of Delhi, i.e. Bhalsawa and Ghazipur sample M9 and M10 clustered together showing similar band pattern. In terms of richness regarding no. of bands, M2 sample of Ghazipur landfill site

has maximum 19 bands followed by M3, M9 and Silicoorie lake having 15 bands each and sample from Bhalsawa and M6 of Ghazipur having 13 bands. Sample M1 of Ghazipur has least 11 bands.

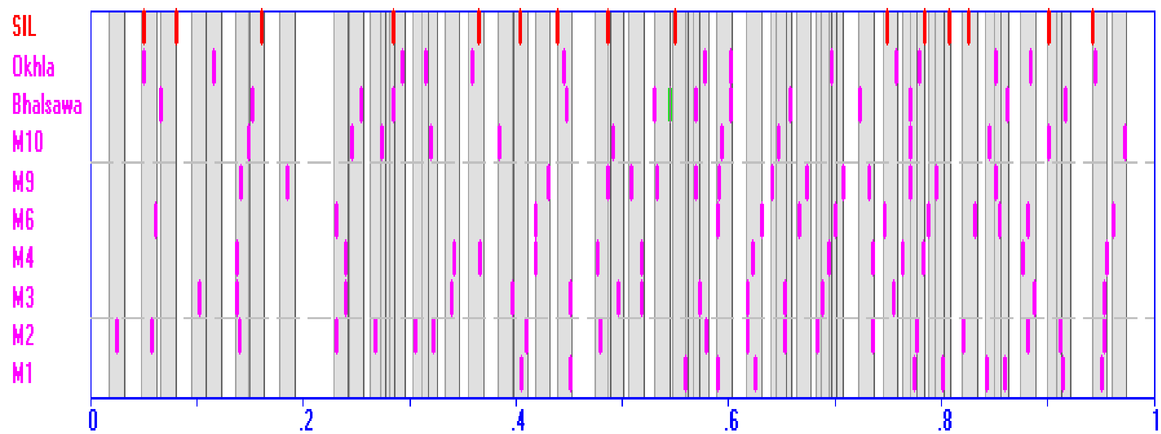


Figure 4.17. Band profile of methanogens from Bhalsawa, Ghazipur and Okhla landfill sites and Silicoorie Lake of southern Assam using Gel 2K software.

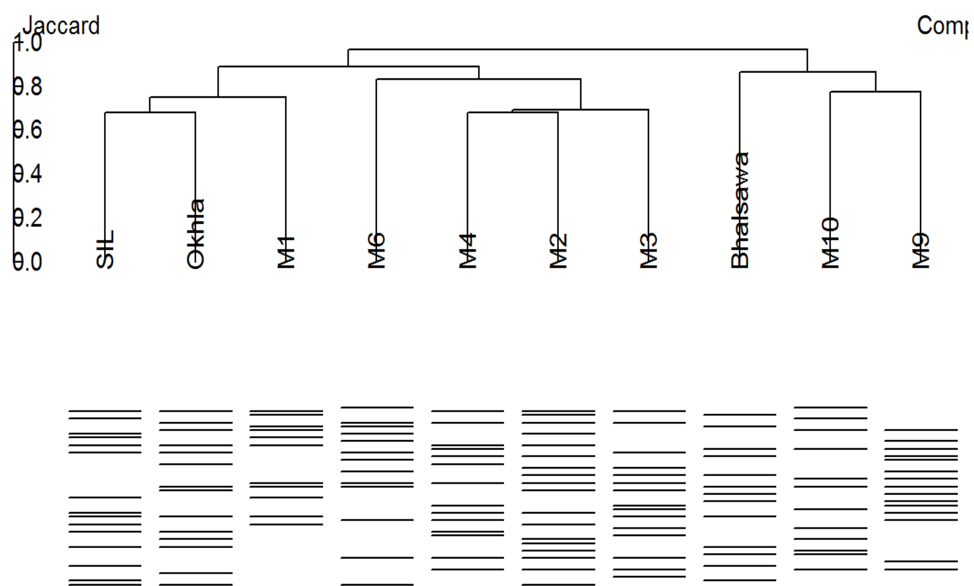


Figure 4.18. Showing Jaccard cluster analysis of methanogenic archaea from Bhalsawa, Ghazipur and Okhla landfill and Silicoorie Lake of southern Assam using Gel 2K software.

4.2.3. Comparative analysis of methanogenic diversity present in the leachate of Delhi landfill sites Ghazipur, Bhalsawa and marshland samples of Silchar using 16S rDNA sequences as a marker

The 16S rDNA serves as a gold standard for identification and diversity analysis of microbes, in present study it was used for methanogenic diversity analysis from both landfill site of Delhi and marshland of Silchar, Assam.

Leachate samples were collected from three landfill sites (Okhla (OK), Bhalsawa (BH) and Ghazipur (GZ)) in the area of New Delhi, India. These sites are active landfill sites and are still in use. They don't have the leachate collection facility or landfill liner to avoid percolation of leachate to the ground water table (aquifer). Soil, sediment sample was collected from marshlands (Sonbill (SON), Silicoorie Lake (SIL), Badarpur (BDR) and Karimganj (KRM)) of Southern Assam, India in sterile falcon tubes.

DNA from both landfill leachate and marshland sediment samples was extracted on the same day of sampling using Fast DNA Spin Kit for Soil (MP Biomedicals, CA, USA). DNA from the marshlands and landfill leachate was amplified using the primer set 86FWD and 1340REV. The amplification profile was 94°C for 5min, 94°C for 30s for 30 cycles, 58°C for 1minute, elongation at 72°C for 2 minute and final extension at 72°C for 10 minutes followed by a cooling step down to 4°C. Obtained 16S rDNA PCR products were purified by PCR purification kit (Fermentas, UK) as recommended by manufacturer protocol.

PCR amplicons of 16S rDNA gene were cloned inside PTZ57R/T vector using the Insta-T/A cloning kit (Fermentas, UK) and transformed into *Escherichia coli* DH5α. The positive clones were selected using blue-white screening on Luria-Bertani plates containing Ampicillin (100mg/ml), X-gal (20mg/ml), and IPTG (100 mM). Then positive clones were sequenced using M13 FWD primer. Sequences obtained were curated manually and were submitted to NCBI which is explained in detail in objective 4.4 i.e. phylogenetic analyses.

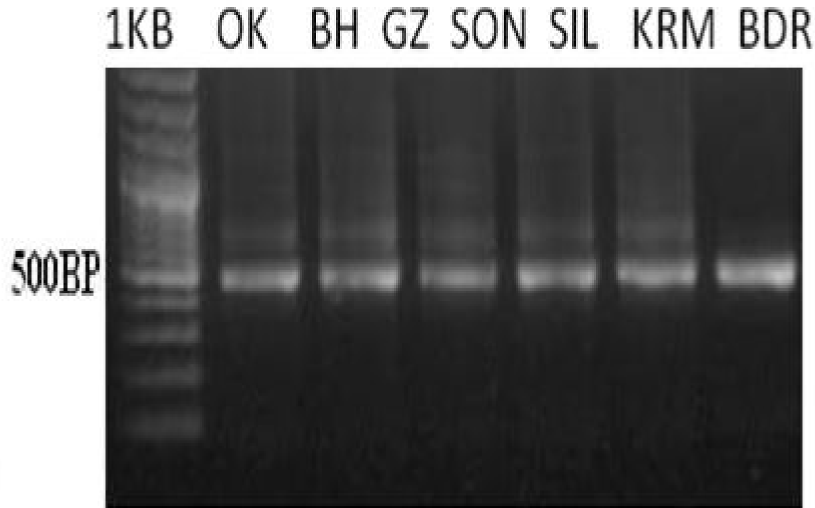


Figure 4.19. Shows GC clamp PCR amplicons of landfill site, Delhi and marshland samples of Southern Assam using primer set 519Fwd and GC915 Rev. L1 contains 1KB DNA Ladder, Fermentas. L2-L7 contains 16S rDNA amplicons derived from genomic DNA isolated from Okhla, Bhalsawa and Ghazipur (OK,BH and GZ) landfill site leachate and Sonbill, Silicoorie lake, Karimganj rice paddy fields and Badarpur beetle nut ponds as SON,SIL,KRM and BDR respectively.

4.2.3.1. Diversity analysis of methanogenic arc1haea based on 16S rDNA gene by denaturing gradient gel electrophoresis

For denaturing gradient gel electrophoresis genomic DNA extracted from landfill and marshland were amplified using primer 519FWD and 915GC which gave a product length of about 500 bp. DGGE was performed with a D-Code universal mutation detection system (Biorad, Hercules, CA, USA) using 16 cm by 16 cm and one mm gels. PCR products were loaded onto 7% (w/v) polyacrylamide gel.

The polyacrylamide gels (Bis-Acrylamide, 37.5:1) were made with denaturing gradients ranging from 30 to 70%. 100% denaturant contained 7 M urea and 40% Formamide. Electrophoresis was initially run at 200 V for 10 min at 60°C, and afterwards for 15 h at 85 V. After electrophoresis, the gel was silver stained and scanned under white light using Gel doc (Biorad). DGGE gel was further analyzed

using Gel2K software (Svein Norland, Department of Biology-University of Bergen, Norway).

Abundance and diversity of methanogenic archaea were studied in three landfill site and four marshlands situated at different location in Delhi and southern Assam, India. 16S rDNA amplicons used for cloning were also analyzed on the DGGE gel for estimation of their richness in respective samples Figure 4.20.

Band patterns of 16S rDNA amplicons obtained from the landfill site (OK, BH and GZ) of Delhi and marshland samples (SON, SIL, KRM and BDR) of southern Assam were compared for methanogens richness and diversity analysis using Gel 2K software. Analysis of DGGE image revealed the presence of total 38 bands Figure 4.21.

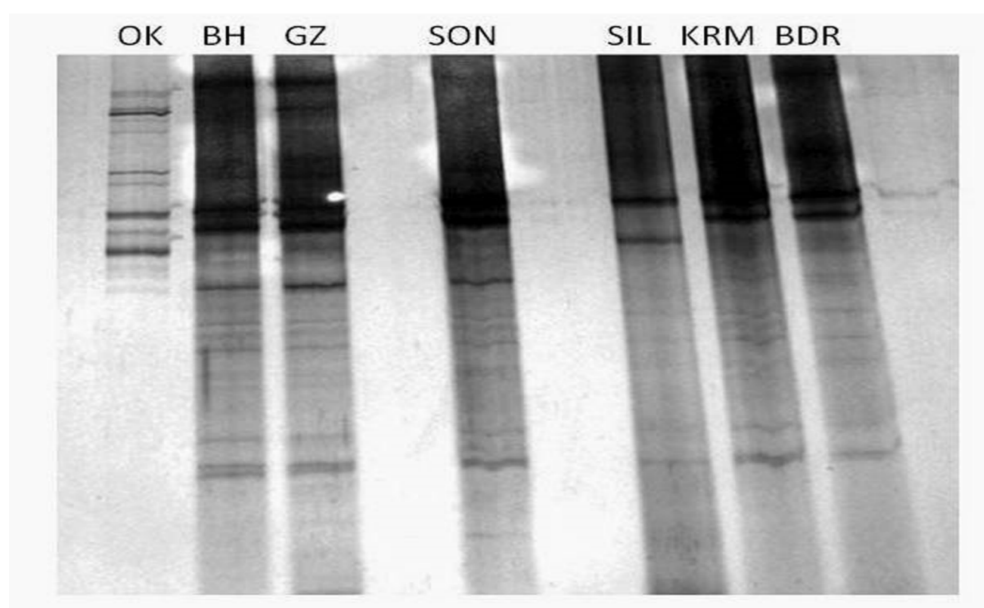


Figure 4.20. Community profiling of methanogens using 16sDNA based DGGE.

The community profiling of methanogenic diversity present in the leachate sample of Delhi landfill site (OK, BH and GZ) and marshland sample of Southern Assam (SON, SIL, KRM and BDR).

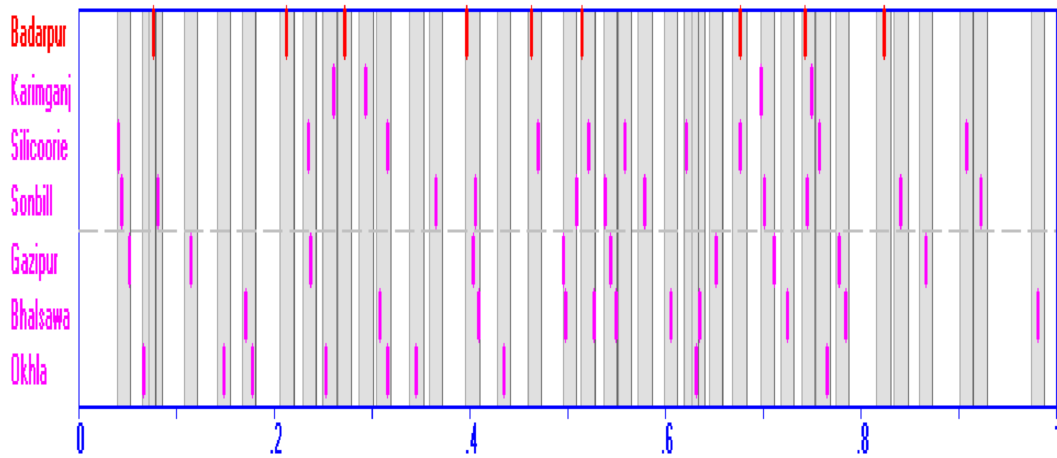


Figure 4.21. Band profile of methanogenic 16S rDNA obtained from landfill Delhi and marshland of southern Assam using Gel 2K software.

There are some unique bands in each lane, which indicates the variation of methanogens community residing in those particular samples. Cluster analysis of bands using Jaccard analysis indicated the presence of three main clusters consisting of localities that differed in the number of similarity and DGGE bands Figure 4.22.

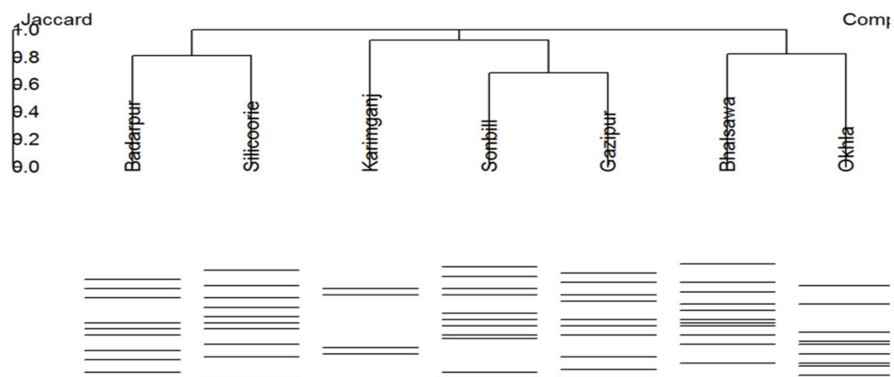


Figure 4.22. Jaccard cluster analysis of methanogenic 16S rDNA bands obtained from landfill Delhi and marshland of southern Assam using Gel 2K software.

In first cluster, Badarpur beetle nut pond and Silicoorie Lake of Southern Assam clustered together showing similar band pattern. In second cluster interestingly, despite being two different ecosystems, Ghazipur landfill site of Delhi clustered with of wetland of Sonbill, southern Assam, India.

In third cluster, the two landfill site of Delhi, i.e. Okhla and Bhalsawa clustered together showing similar band pattern. In terms of richness regarding no. of bands samples from Bhalsawa landfill and Sonbill wetland has maximum 11 bands followed by Ghazipur landfill site and Silicoorie lake having 10 bands each, followed by Okhla landfill and Badarpur beetle nut pond having nine bands each. In the Karimganj rice paddy field sample, only four bands were observed showing least diversity.

Chapter 4.3

Quantification of Methanogens from the samples of marshland and leachate sample using Quantitative *Real Time* PCR

4.3.1. Growing Methanogens pure cultures:

For absolute quantification of methanogens, five pure cultures obtained from DSMZ were grown inside the anaerobic chamber using SAB medium. All solutions were prepared under continuous N_2:CO_2 (80:20) flushing to replace the traces of oxygen. Resazurin dye (redox sensitive) was included in media to monitor the redox potential because it is generally non-toxic to bacteria and is effective at very low concentrations of 0.5 to 1mg/l. Reducing agents like L-cysteine hydrochloride monohydrate, $Na_2S.9H_2O$ were included to depress and poise the redox potential at optimum levels.

The medium was autoclaved and then allowed to cool inside anaerobic chamber under N_2 (100%) flushing until the medium became transparent (anoxic condition). Then SAB media was transferred to the serum bottles preflushed with N_2. Pure cultures were inoculated using 1ml syringe under complete aseptic and anoxic condition. After inoculation tubes were again flushed with N_2 and sealed with aluminium crimp. Then these tubes were incubated at 39°C inside the incubator.

The cultures obtained from DSMZ were *Methanomicrobium mobile, Methanosarcina mazei, Methanobrevibacter ruminantium, Methanobrevibacter arboriphilus* and *Methanobacterium bryantii* respectively.

DNA samples from Delhi landfill site Okhla, Bhalsawa landfill site and Silicoorie Lake, Silchar, Southern Assam were screened for quantification of methanogenic archaea by using real-time PCR. Sybr green based absolute quantification of methanogens was performed using Agilent Mx-Pro 3000 RT-PCR instrument based on SYBR green dissociation curve.

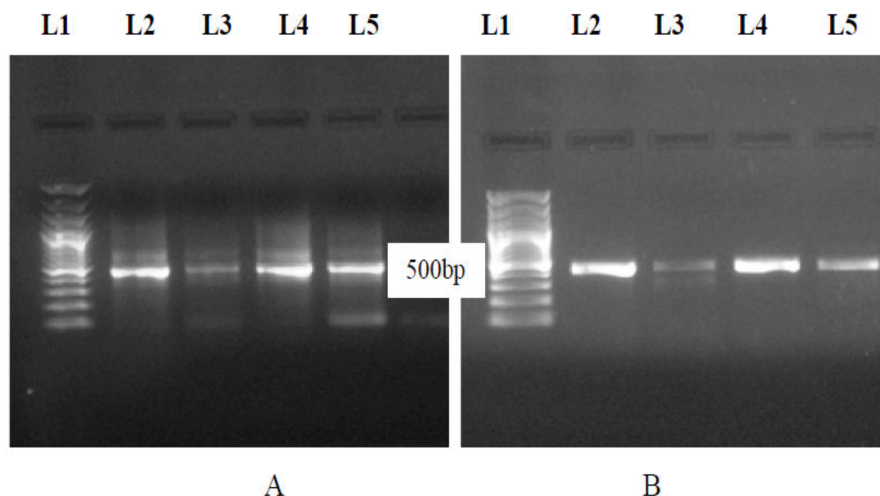


Figure 4.23. Shows 16S rDNA amplicons **(A)** and size of insert **(B)** obtained from pure culture of methanogens. L1 contains 100BP DNA Ladder, L2- L5 contains 16S rDNA amplicons obtained from pure cultures *Methanobrevibacter arboriphilus, Methanobacterium bryantii, Methanomicrobium mobile and Methanosarcina mazei* respectively.

Formula for creation of standard curve

For creation of standard curve, first plasmid were diluted to 50ng/µl and were then diluted serially 10 fold for ten times. These dilution were then checked by doing PCR, whether dilution are correct. Dilutions 3-6 i.e. from 0.125ng/µl to 0.000125ng/µl were used for real time PCR experiment.

Calculation of concentration of insert in total plasmid DNA

Conversion of insert to copy number =

$$\frac{6.023 \times 10^{23}\ (\text{copies/mol}) \times \text{conc}^{n}\ \text{of Plasmid}\ (\text{g}/\mu\text{l})}{\text{Relative molecular mass}}$$

Relative molecular mass = Size of Vector + insert

Conversion of Molecular Wt. of insert = no. of bases*660 in dalton.

Size of insert for all pure cultures was 421BP.

Size of vector was 277860BP.

4.3.1.1. Creation of standard curve for absolute quantification:

For absolute quantification, first plasmid containing 16S rDNA amplicons obtained from pure culture of *Methanobrevibacter arboriphilus* was diluted serially on tenfold basis starting 12.5 ng/µl. The size of the insert was checked by vector specific PCR using agarose gel electrophoresis. Then the ranges from 0.00125ng/µl to 0.000000125 ng/µl was used for plotting standard curve for absolute quantification by Quantitative Real-time PCR analysis.

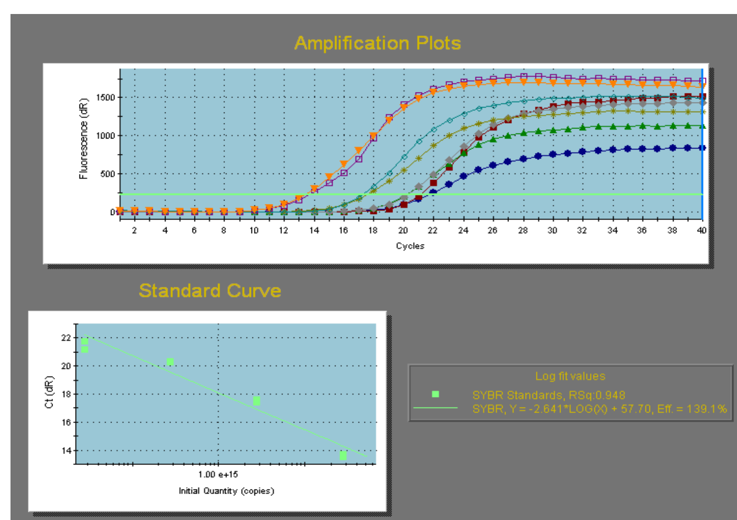


Figure 4.24. Shows amplification plots and standard curve for the *Methanobervibacter arboriphilus.*

Sample	Copy no.	ct value	Threshold(dR)	Slope(dR)	Rsq(dR)
Standard1	2.77E+13	21.21	231.501	-2.641	0.948
Standard1	2.77E+13	21.78	231.501	-2.641	0.948
Standard2	2.77E+14	20.26	231.501	-2.641	0.948
Standard2	2.77E+14	20.35	231.501	-2.641	0.948
Standard3	2.77E+15	17.39	231.501	-2.641	0.948
Standard3	2.77E+15	17.62	231.501	-2.641	0.948
Standard4	2.77E+16	13.51	231.501	-2.641	0.948
Standard4	2.77E+16	13.74	231.501	-2.641	0.948

Table 4.3. Showing copy number and CT value for each standard dilution in duplicate.

After confirmation of standard dilution, for quantification of each pure culture, standard curve was prepared using standard dilutions and run in triplicate along with the samples of landfill site and marshland.

4.3.2. Absolute Quantification of *Methanobrevibacter arboriphilus* using Quantitative Real-time PCR:

Methanobrevibacter arboriphilus belongs to order Methanobacteriales, Family Methanobacteriacea *and Genus Methanobrevibacter.* They are strictly anaerobic and commonly found in anaerobic habitats such as sediments, sewage water, soil and rumen. They utilize hydrogen, formate or CO substrate as energy source for methanogenesis. After cloning 16S rDNA amplicons obtained from *Methanobrevibacter arboriphilus* were diluted serially on two fold basis to 12.5 ng/µl. Then the 12.5 ng/µl initial concentration of plasmid was used for creation of standard curve by diluting it on tenfold basis.

After dilution PCR was run to check whether dilution was correct or not. The primer used for amplification was 519FWD and 915REV. The primer sequence and the amplification profile were mentioned in materials and methods section. After PCR the amplicons were checked by running on 1.5% Agarose gel and visualizing it under UV light. After visualization of gel and being sure that dilutions are correct, dilution ranges from 0.00125ng/µl to 0.000000125 ng/µl was used for Quantitative Real-time PCR analysis. For creation of standard curve each dilution and unknown sample reaction were run in triplicate.

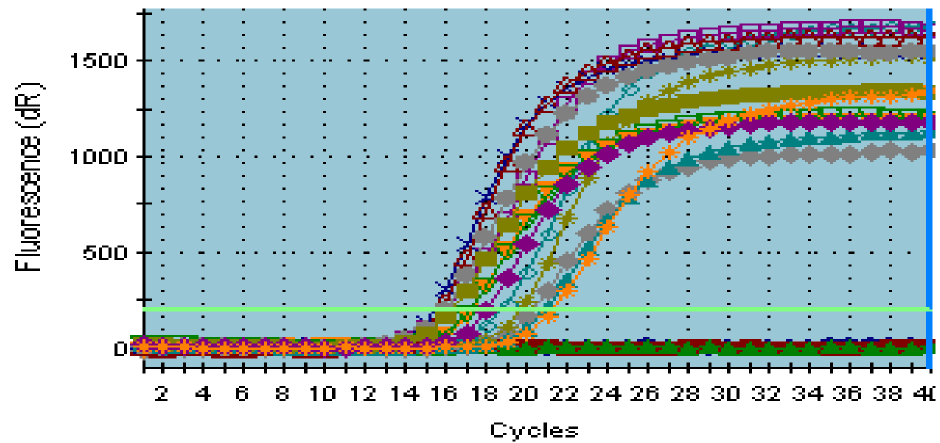


Figure 4.25. Showing amplification plots for standard dilution of *Methanobrevibacter arboriphilus* and samples from landfill and marshland.

Sample	Copy no.	ct value	Threshold(dR)	Slope(dR)	Rsq(dR)
Standard 1	2.77E+13	18.19	142.989	-2.128	0.903
Standard 1	2.77E+13	17.84	142.989	-2.128	0.903
Standard 2	2.77E+14	18	142.989	-2.128	0.903
Standard 2	2.77E+14	18.11	142.989	-2.128	0.903
Standard 3	2.77E+15	15.28	142.989	-2.128	0.903
Standard 3	2.77E+15	15.28	142.989	-2.128	0.903
Standard 4	2.77E+16	12.53	142.989	-2.128	0.903
Standard 4	2.77E+16	12.56	142.989	-2.128	0.903
Okhla	3.23E+14	16.65	142.989	-2.128	0.903
Okhla	5.08E+14	16.19	142.989	-2.128	0.903
Bhalsawa	7.63E+14	15.89	142.989	-2.128	0.903
Bhalsawa	4.47E+14	16.4	142.989	-2.128	0.903
S.Lake	6.89E+11	22.71	142.989	-2.128	0.903
S.Lake	1.45E+12	21.86	142.989	-2.128	0.903

Table 4.4. Showing copy number and ct value for each standard dilution of Methanobrevibacter *arboriphilus* and landfill and marshland samples.

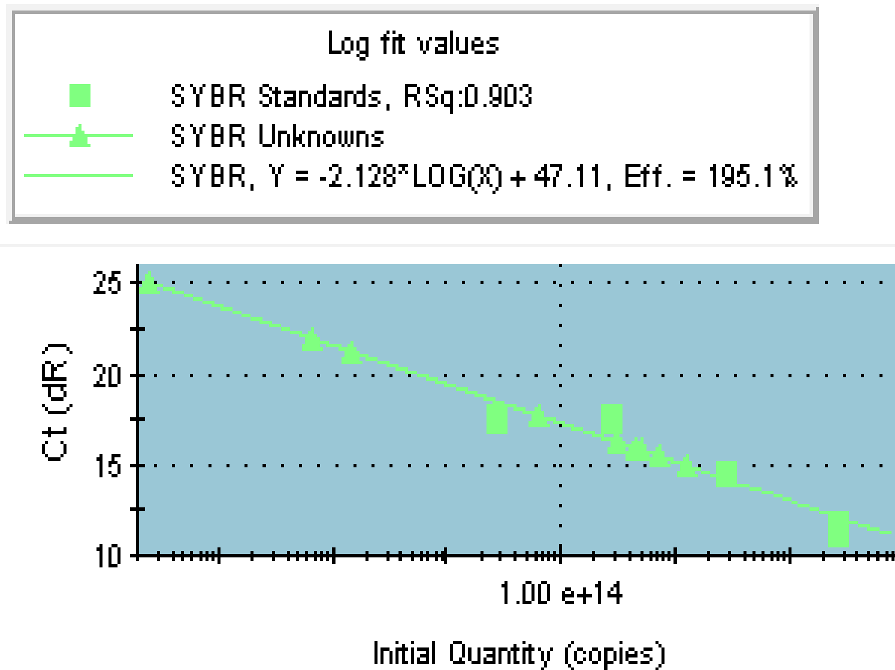


Figure 4.26. Shows linear graph for copy number vs ct value of standard dilutions of *Methanobrevibacter arboriphilus* plasmids and samples from landfill and marshland.

4.3.3. Absolute Quantification of *Methanobacterium bryantii* using Quantitative Real-time PCR:

Methanobacterium bryantii belongs to order Methanobacteriales, Family-Methanobacteriacea *and Genus* Methanobacterium. They are strictly anaerobic and commonly found in anaerobic habitats such as sediments, landfill and marshes. They utilize substrates like hydrogen-CO_2 as energy source i.e. hydrogenotrophic pathway for methanogenesis. After cloning 16S rDNA amplicons obtained from Methanobacterium *bryantii* were diluted serially to12.5ng/µl.

Then the 12.5ng/µl initial concentration of plasmid was used for creation of standard curve by diluting it on tenfold basis. After dilution PCR was run to check whether dilution was correct or not. The primer used for amplification was 519FWD and 915REV. The primer sequence and the amplification profile were mentioned in materials and methods section. After PCR the amplicons were checked by running on 1.5% agarose gel and visualizing it under UV light.

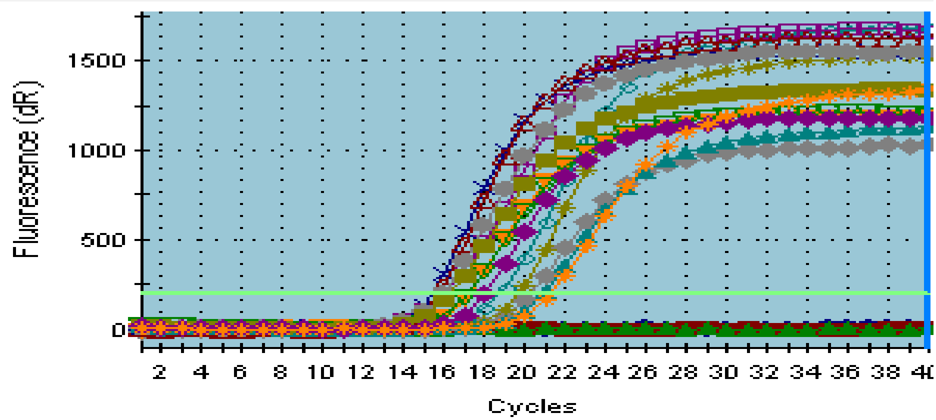


Figure 4.27. Showing amplification plots for standard dilution of Methanobacterium *bryantii* plasmids and samples from landfill and marshland.

Sample	Copy no.	Ct value	Threshold(dR)	Slope(dR)	Rsq(dR)
Standard 1	2.77E+13	20.25	192.143	-1.384	0.877
Standard 1	2.77E+13	19.73	192.143	-1.384	0.877
Standard 2	2.77E+14	18.74	192.143	-1.384	0.877
Standard 2	2.77E+14	18	192.143	-1.384	0.877
Standard 3	2.77E+15	17.25	192.143	-1.384	0.877
Standard 3	2.77E+15	17.62	192.143	-1.384	0.877
Standard 4	2.77E+16	13.74	192.143	-1.384	0.877
Standard 4	2.77E+16	13.51	192.143	-1.384	0.877
Okhla	1.81E+17	17.14	192.143	-1.384	0.877
Okhla	1.14E+17	15.94	192.143	-1.384	0.877
Bhalsawa	1.11E+16	16.28	192.143	-1.384	0.877
Bhalsawa	4.66E+16	20.89	192.143	-1.384	0.877
S.Lake	2.18E+13	18.01	192.143	-1.384	0.877
S.Lake	1.22E+13	21.24	192.143	-1.384	0.877

Table 4.5. Showing copy number and Ct value for each standard dilution of *Methanobacterium bryantii* and landfill and marshland samples.

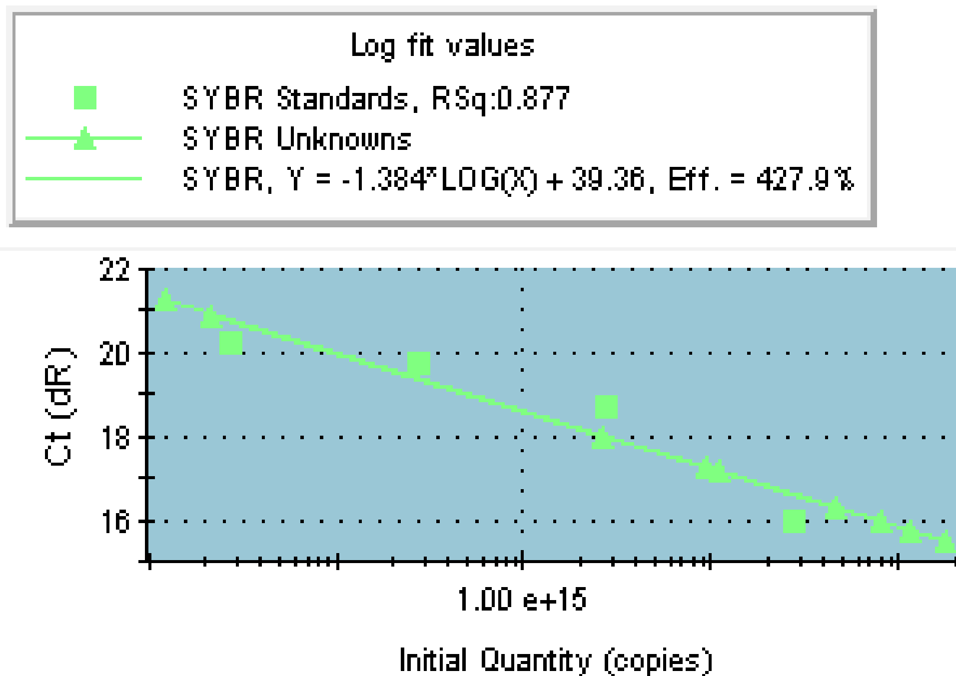


Figure 4.28. Linear graph shows copy number vs Ct value of *Methanobacterium bryantii* plasmids samples from landfill and marshland.

4.3.4. Absolute quantification of *Methanosarcina mazei* using Quantitative Real-time PCR:

Methanosarcina mazei belongs to order Methanosarcinales, Family-Methanosarcinaceae and Genus Methanosarcina. They are strictly anaerobic and commonly found in anaerobic habitats such as sediments, landfill and marshes. They utilize substrates like acetate, formate etc as energy source i.e. utilize acetotrophic/ methylotrophic pathway for methanogenesis. After cloning 16S rDNA amplicons obtained from *Methanosarcina mazei* were diluted serially to12.5 ng/µl. Then the 12.5 ng/µl initial concentration of plasmid was used for creation of standard curve by diluting it on tenfold basis. After dilution PCR was run to check whether dilution was correct or not. The primer used for amplification was 519FWD and 915REV. The primer sequence and the amplification profile were mentioned in materials and methods section. After PCR the amplicons were checked by running on 1.5% agarose gel and visualizing it under UV light.

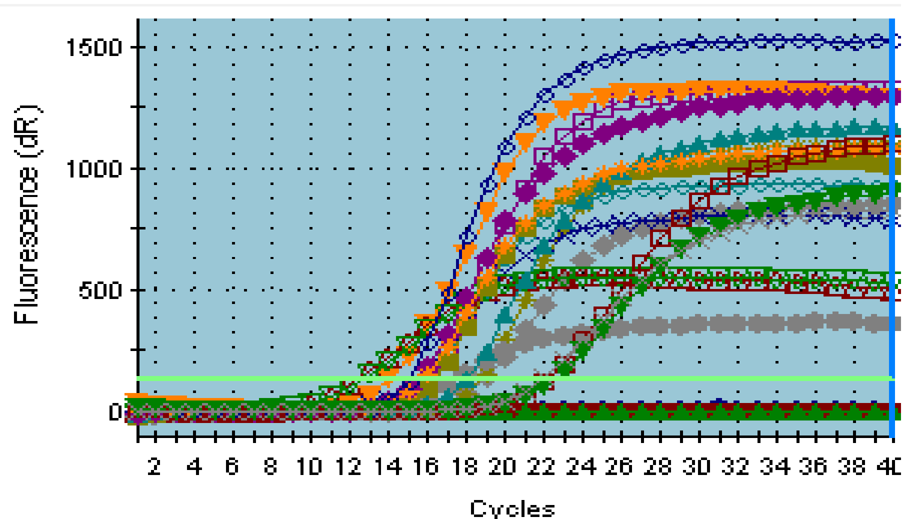
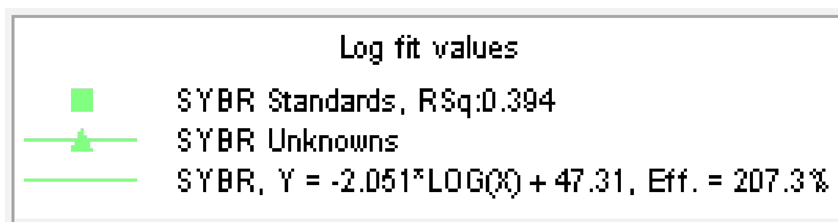


Figure 4.29. Showing amplification plots for standard dilution of *Methanosarcina mazei* and samples from landfill and marshland.

Sample	Copy no.	Ct value	Threshold(dR)	Slope(dR)	Rsq(dR)
Standard 1	2.77E+13	19.0	135.27	-2.051	0.394
Standard 1	2.77E+13	18.89	135.27	-2.051	0.394
Standard 2	2.77E+14	16.09	135.27	-2.051	0.394
Standard 2	2.77E+14	16.13	135.27	-2.051	0.394
Standard 3	2.77E+15	14.13	135.27	-2.051	0.394
Standard 3	2.77E+15	15.55	135.27	-2.051	0.394
Standard 4	2.77E+16	12.47	135.27	-2.051	0.394
Standard 4	2.77E+16	12.8	135.27	-2.051	0.394
Okhla	2.50E+14	17.77	135.27	-2.051	0.394
Okhla	1.80E+14	16.5	135.27	-2.051	0.394
Bhalsawa	2.80E+15	15.63	135.27	-2.051	0.394
Bhalsawa	2.20E+15	15.83	135.27	-2.051	0.394
Silicoorie	1.54E+12	22.31	135.27	-2.051	0.394
Silicoorie	1.13E+12	22.58	135.27	-2.051	0.394

Table 4.6. Showing copy number and Ct value for each standard dilution of *Methanosarcina mazei* and landfill and marshland samples.



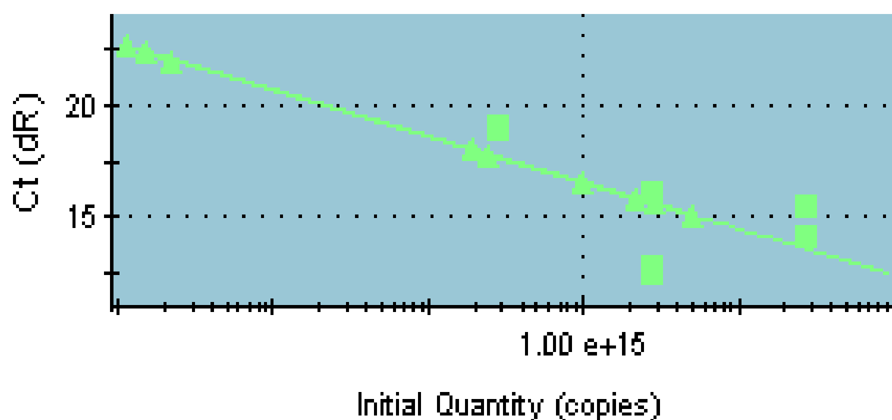


Figure 4.30. Linear graph for copy number vs Ct value of standard dilutions of *Methanosarcina mazei* plasmids and samples from landfill and marshland.

4.3.5. Absolute quantification of *Methanomicrobium mobile* using Quantitative Real-time PCR:

Methanomicrobium mobile belongs to order Methanomicrobiales, Family-Methanomicrobia and genus Methanomicrobium. They are strictly anaerobic and commonly found in anaerobic habitats such as sediments, landfill and marshes. They utilize substrates like H_2.CO_2 etc as energy source i.e. utilize hydrogenotrophic pathway for methanogenesis. After cloning 16S rDNA amplicons obtained from *Methanomicrobium mobile* were diluted serially to12.5 ng/µl. Then the 12.5 ng/µl initial concentration of plasmid was used for plotting of standard curve by diluting it on tenfold basis. After dilution PCR was run to check whether dilution was correct or not. The primer used for amplification was 519FWD and 915REV. The primer sequence and the amplification profile were mentioned in materials and methods section. After PCR the amplicons were checked by running on 1.5% agarose gel and visualizing it under UV light.

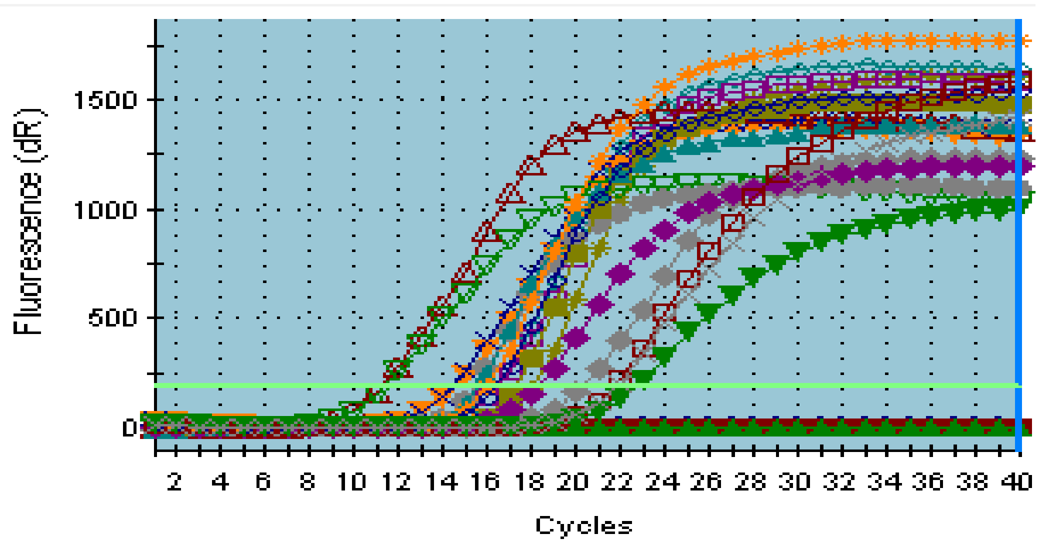


Figure 4.31. Showing amplification plots for *Methanomicrobium mobile* standard dilution and samples from landfill and marshland.

Sample	Copy no.	Ct value	Threshold(dR)	Slope(dR)	Rsq(dR)
Standard 1	2.77E+13	20.32	187.853	-2.563	0.956
Standard 1	2.77E+13	18.89	187.853	-2.563	0.956
Standard 2	2.77E+14	16.49	187.853	-2.563	0.956
Standard 2	2.77E+14	16.13	187.853	-2.563	0.956
Standard 3	2.77E+15	14.49	187.853	-2.563	0.956
Standard 3	2.77E+15	14.63	187.853	-2.563	0.956
Standard 4	2.77E+16	11.22	187.853	-2.563	0.956
Standard 4	2.77E+16	11.41	187.853	-2.563	0.956
Okhla	7.50E+15	17.37	187.853	-2.563	0.956
Okhla	6.60E+15	15.56	187.853	-2.563	0.956
Bhalsawa	6.20E+14	16.05	187.853	-2.563	0.956
Bhalsawa	4.80E+14	18.33	187.853	-2.563	0.956
S.Lake	3.40E+14	16.44	187.853	-2.563	0.956
S.Lake	2.9E+13	21.72	187.853	-2.563	0.956

Table 4.7. Showing copy number and ct value for each standard dilution of *Methanomicrobium mobile* and landfill and marshland samples.

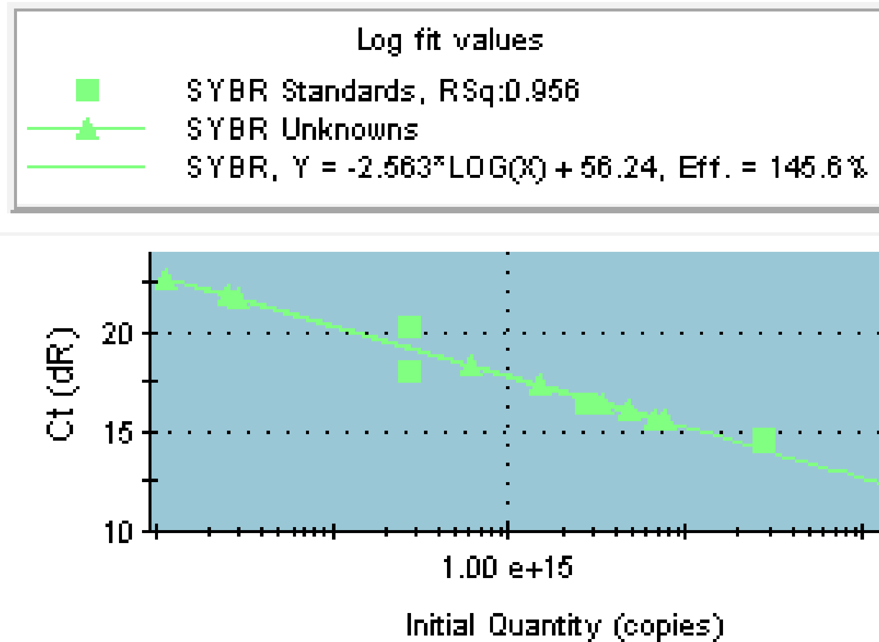


Figure 4.32. Shows linear graph for copy number vs Ct value of standard dilutions of *Methanomicrobium mobile* plasmids and samples from landfill and marshland.

Pure culture	Okhla	Bhalsawa	Silicoorie lake
M. arbophilicus	3.98e+014-6.8e+014	5.38e+014-9.7e+014	2.78e+011-7. 8e+011
M. bryantii	1.14 e+017-1.8 e+017	4.6 e+016-8.2 e+016	1.2 e+013-2.1 e+013
M. mobile	6.67e+015-7.5e+015	3.42e+015-4.8 e+015	3.97e+012-4.1 e+012
M. mazei	1.89 e+014-2.5 e+014	2.21e+015-2.8 e+015	1.13e+012-1.5 e+012

Table 4.8. Shows copy number of methanogens present in the per gram samples of Okhla, Bhalsawa landfill site Delhi and Silcoorie lake, Assam, India.

Domain- Archaea, Kingdome – Archaebacteria, Phylum – Euryarchaeota		
Methanogenic pathway	**Orders**	**Reaction**
Acetoclastic	Methanosarcinales	$CH_3COOH \rightarrow CH_4+CO_2$
Hydrogenotrophic	Methanosarcinales	$4H_2+CO_2 \rightarrow CH_4+2H_2O$
	Methanobacteriales	$4HCOOH \rightarrow CH_4+3CO_2+2H_2O$
	Methanococcales	
	Methanomicrobiales	
	Methanopyrales	
Methylotrophic	Methanosarcinales	$4CH_3OH \rightarrow 3CH_4+CO_2+2H_2O$

Table 4.9. Methanogenic pathways and microorganisms that is associated**.**

DNA extracted from the three sampling point i.e. two landfill site Okhla and Bhalsawa, Delhi and Silicoorie lake of Southern Assam were screened for absolute quantification of methanogens. The copy numbers of all methanogens were higher in the two landfill site than marshland of Southern Assam.

Copy numbers of *Methanomicrobium mobile* belonging to order Methanomicrobiales and *Methanobacterium bryantii* belonging to order Methanobacteriales-1 were highest in both landfill sites than Silicoorie Lake of southern Assam. Copy number of *Methanobrevibacter arboriphilus* belonging to order Methanobacteriales-1 and *Methanosarcina mazei* belonging to order Methanosarcinales were found to be highest in Bhalsawa landfill site than Okhla landfill site and Silicoorie Lake, Southern Assam.

The RSq and slope dR value for standard curve were 0.948 and -2.641 and the efficiency of the reaction were 139.1%. The standard curve and amplification plot have been shown in Figure 4.23. The RSq and slope dR values for absolute quantification of *Methanobrevibacter arboriphilus* are 0.903 and -2.128. RSq (dR) and slope dR values for absolute quantification of *Methanobacterium bryantii* are 0.877 and -1.384. RSq (dR) and slope dR values for absolute quantification of *Methanomicrobium mobile* are 0.956 and -2.563. RSq (dR) and slope dR values for absolute quantification of *Methanosarcina mazei* are 0.394 and -2.051.

In the current study, it was found that methanogenic community present in the two landfill site of Delhi were more similar to each other and quite distinct from marshlands of Silchar, Southern Assam. Copy number of hydrogenotrophic methanogen *Methanobacterium bryantii* were found to be highest in Okhla landfill than Bhalsawa landfill of Delhi and Silicoorie Lake of Southern Assam indicating its dominance.

Although, methanogens performing all three pathways of methane production have been found to be associated from these systems, it can be attributed from copy number, that hydrogenotrophic methanogenesis serves as major source of methane emission from these sites.

Chapter 4.4

Molecular Phylogenetic relationship

4.4.1 Phylogenetic analysis of Bacterial 16S rDNA obtained from Landfill site Delhi using ML approach:

In this objective, the phylogenetic association of bacterial 16S rDNA sequences obtained from landfill site of Delhi was investigated. The phylogenetic analysis of bacterial 16S rDNA will provide the knowledge about the type of bacterial diversity present in the landfill.

19 Bacterial 16S rDNA clone sequences obtained in this study were used for phylogenetic analysis. The similarity searches for sequences were carried out by BLAST. Sequence showing homology between 92-99% was then used for multiple sequence alignment using Clustal X 2.0.

The phylogenetic relatedness among clones was estimated using the Maximum Likelihood Tree using K2+G model with 1000 bootstrap value (Kimura, 1980). For model selection Bayesian analysis was performed and the model with lowest BIC value (i.e.3130.914) was chosen for tree construction. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). The Phylogenetic analysis was carried out using MEGA software version 6.0 (Tamura et al., 2013).

Anaerobic degradation in landfills involves several coordinated groups of microorganisms and follows a process that is typical of waste degradation in anaerobic environments, such as soils, sediment, and sludge. As the primary stage of waste degradation, polysaccharide breakdown is an important limiting factor in anaerobic treatment of waste, which in municipal landfills primarily involves the decomposition of complex polymers, including cellulose, hemicellulose, and lignin. Bacterial 16S rDNA sequences obtained under this study were mostly affiliated to aerobic bacteria, firmicutes performing hydrolysis reaction. Bacterial 16S rDNA clones showed four clusters in the tree Figure 4.33.

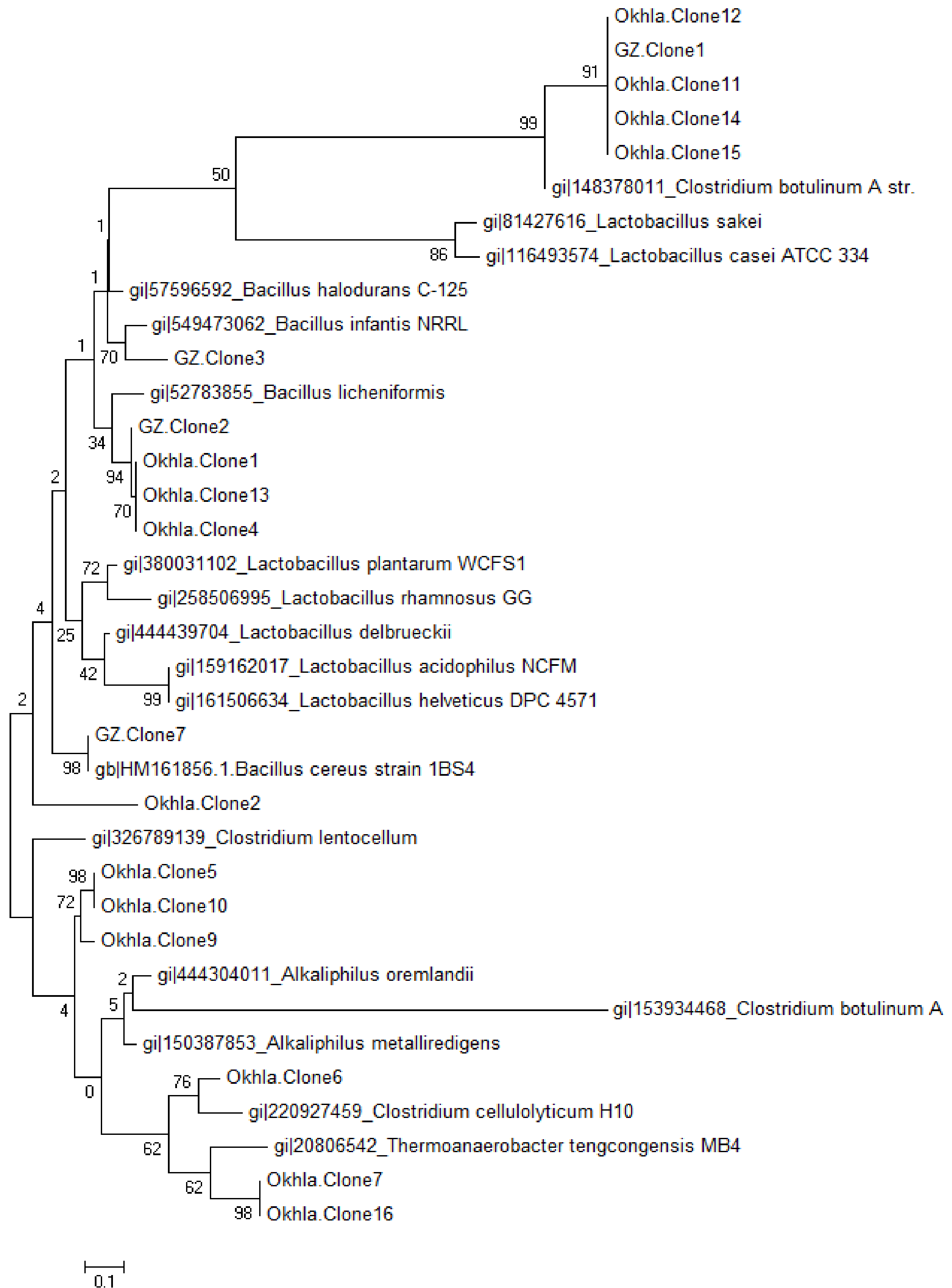


Figure 4.33. Shows maximum likelihood tree of bacterial 16S rDNA sequences obtained from MSW leachate samples of Delhi landfill site Okhla and Ghazipur using Mega6.0.

In first cluster, clones derived from Okhla landfill site i.e. Okhla clone 11,12,14 and 15 and GZclone.1 clustered with *Clostridium botulinum*. Presence of *Clostridium botulinum* in landfill site has not been previously reported from the landfill site of Delhi, India. In second cluster, Okhla clone 1,4 and 13 and Ghazipur clone i.e. GZ clone 2,3 and 7 sequences clustered with *Bacillus infantis NRRL, Bacillus licheniformis and Bacillus cereus. Bacillus Licheniformis* are utilized in enzyme industry for production of extracellular enzymes. In third cluster, Okhla clone 2, 5,9 and 10 clustered with species like *Clostridium botulinum* and *Clostridium lentocellum*. In fourth cluster, Okhla clone 6 clustered with *Clostridium cellulolyticum* which is a non-ruminal, anaerobic microbes which digest cellulose via extracellular enzymes. In fifth cluster, Okhla clone 7 and 16 clustered with *Thermoanaerobacter tengcongensis* MB4 is an obligately anaerobic, rod-shaped, gram negative, saccharolytic and thermophilic eubacterium. Presence of *Clostridium* and *eubacterium* like *Thermoanaerobacter tengcongensis* MB4 indicates that these anaerobic microbes play crucial role in anaerobic cellulose degradation in landfills.

4.4.2. Phylogenetic analysis of methanotrophs in Ghazipur landfill site Delhi using ML approach

Five 16S rDNA sequences obtained in this study were compared with the available nucleotide database from the NCBI Genbank using the BLAST program (Altschul et al., 1990). The partial nucleotide sequences of 16S rDNA genes were submitted to NCBI under accession number which is given in table below

Sequence ID	Organism name	Accession no.	Location
METG2_16S rDNA	*Methylobacillus flagellates* (97% similarity with **NR_074178.1**)	KM041243	Ghazipur landfill
METG3_16S rDNA	*Methylobacillus arboreus* (99% similarity with **NR_108851.1**)	KM041244	Ghazipur landfill
METG4_16S rDNA	*Methylobacillus flagellates* (99% similarity with **NR_074178.1**)	KM041245	Ghazipur landfill
METG6_16S rDNA	*Methylobacillus flagellates* (98% similarity with **NR_074178.1**)	KM041246	Ghazipur landfill
METG7_16S rDNA	*Methylobacillus arboreus* (99% similarity with **NR_108851.1**)	KM041247	Ghazipur landfill

Table 4.10. Methylotrophic 16S rDNA sequences with accession numbers submitted in NCBI.

After performing BLAST, sequences showing similarity above 90-99% were used and aligned using Clustal X 2.0. The phylogenetic relatedness among clones was estimated using the Maximum Likelihood Tree using K2 model with 1000 bootstrap value (Kimura, 1980). For model selection Bayesian analysis was performed and the model with lowest BIC value (i.e.6154.06) was chosen for tree construction. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). The Phylogenetic analysis and the tree was constructed using MEGA software version 6.0 (Tamura et al., 2013) shown in Figure 4.34.

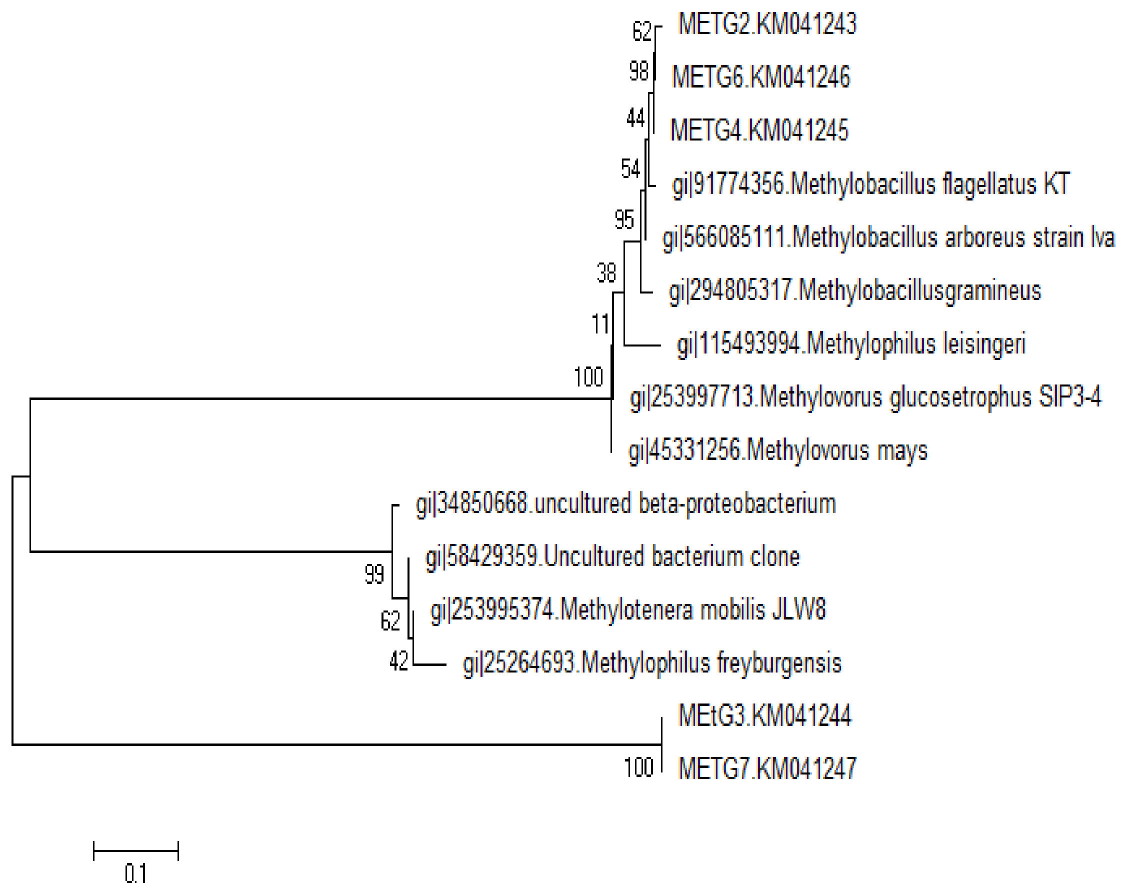


Figure 4.34. Shows maximum likelihood tree of methylotrophic bacterial 16S rDNA sequences obtained from MSW leachate samples of Ghazipur Delhi landfill site using Mega6.0.

The phylogenetic analysis of 16S rDNA clones indicates the presence of type 1 methanotrophs belonging to family *Methylophilaceae* and order *Methylobacter* and *Methylotenera.* Sequence analysis of METG2_16S rDNA, METG3_16S rDNA, METG4_16S rDNA, METG6_16S rDNA and METG7_16S rDNA indicated presence of *Methylobacillus flagellatus KT, Methylotenera mobilis JLW8, Methylobacillus gramineus strain, Methylobacillus arboreus strain and Methylovorus glucosetrophus SIP* in the leachate of Ghazipur landfill site. *Methylobacillus flagellates, Methylobacillus gramineus* and *Methylobacillus arboreus strain* are gram negative*,* asporogenous*, obligate novel methanotrophs* belonging to class *Betaproteobacteria* family Methylophilaceae. *Methylotenera mobilis* JLW8 is a novel species affiliated with family *Methylophilaceae* and utilizes methylamine as energy source. 16S rRNA gene sequence analysis indicated that it is closely related to a broad group of sequences from uncultured *Betaproteobacteria* and distantly related (93–96% similarity) to known methylotrophs of the family Methylophilaceae.

4.4.3. Phylogenetic analysis of methanogens present in landfill site Delhi and marshlands Assam using 16S rDNA sequences using ML approach.

Nine methanogenic and five methanotrophic bacterial 16S rDNA sequences obtained in this study were compared with the available nucleotide database from the NCBI Genbank using the BLAST program (Altschul et al., 1990). The partial nucleotide sequences of 16S rDNA genes were submitted to NCBI under accession number which is given in Table 4.11.

Partial 16S rDNA sequences obtained from this study were used for similarity search in NCBI database using BLAST program. After performing BLAST, sequences showing similarity above 90% were used and aligned in MEGA software version 6.0 (Tamura et al., 2013) using ClustalW. The phylogenetic relatedness among clones was estimated using the Maximum Likelihood Tree using Kimura K2P+G model with 2000 bootstrap value (Kimura, 1980). For model selection Bayesian analysis was performed and the model with lowest BIC value (i.e. 12104.8604) was chosen for tree construction. All positions containing gaps and missing data were eliminated from the

dataset (complete deletion option). The Phylogenetic analysis were carried out using MEGA software version 6.0 (Tamura et al., 2013).

Accession no.	**Sample ID**	**Tentative Organism name**	**Location**
KM041239.1	MET1 LAND	*Methanoculleus thermophiles* (99% similarity with **JF330114.1**)	Bhalsawa landfill
KM041240.1	MET2 LAND	*Methanoculleus thermophiles* (99% similarity with **JF330114.1**)	Bhalsawa landfill
KM041241.1	MET3 LAND	Uncultured archaeon clone (99% similarity with **AB535355.1**)	Bhalsawa landfill
KM041242.1	METG1 LAND	Uncultured archaeon clone (94% similarity with **JF807145.1**)	Ghazipur landfill
KM041248.1	METK2 MARSH	Uncultured euryarchaeote clone (98% similarity with **KF360011.1**)	Karimganj
KM041249.1	METK4 MARSH	Uncultured archaeon clone (100% similarity with **JQ245687.1**)	Karimganj
KM041250.1	SD1 MARSH	Uncultured archaeon clone (97% similarity with **JF304136.1**)	Silicoorie Lake (Silchar)
KM041251.1	SD3 MARSH	Uncultured archaeon clone (97% similarity with **JF708703.1**)	Silicoorie Lake (Silchar)
KM041252.1	SD4 MARSH	Uncultured archaeon clone (91% similarity with **AB364893.1**)	Silicoorie Lake (Silchar)

Table 4.11: Methanogenic 16S rDNA sequences with accession numbers submitted to NCBI.

Sequences of MET1 LAND and MET2 LAND obtained from the Bhalsawa landfill site were clustered with *Methanoculleus thermophilus* methanogens belonging to the order Methanomicrobiales which are hydrogenotrophic in nature. Third sequence of MET3 LAND from the Bhalsawa landfill site clustered with the *Candidatus Methanomethylophilus alvus Mx1201*, which is H_2-dependent methylotrophic methanogens. In the **Figure 4.35**, it is shown that these three sequences from the landfill sites of Delhi are clustered with Euryarchaeota cluster (**Cluster I**). Sequence METG1 LAND obtained from the Ghazipur landfill site, Delhi clustered with *Methanomassiliicoccus luminyensis* (**Cluster II**). Sequences obtained from marshland

sites of Southern Assam were clustered (**Cluster III**) separately with Crenarchaeota (**Cluster IIIa**) and Thaumoarchaeota (**Cluster IIIb**). There are five more sequences from the landfill sites of Delhi. They are related to two different species of methanotrophs (methane oxidizing bacteria) (see Table 4.11), *Methylobacillus arboreus* (marked as ▲) & *Methylobacillus flagellatus* (marked as ●) and were clustered separately, shown in **Figure 4.35**.

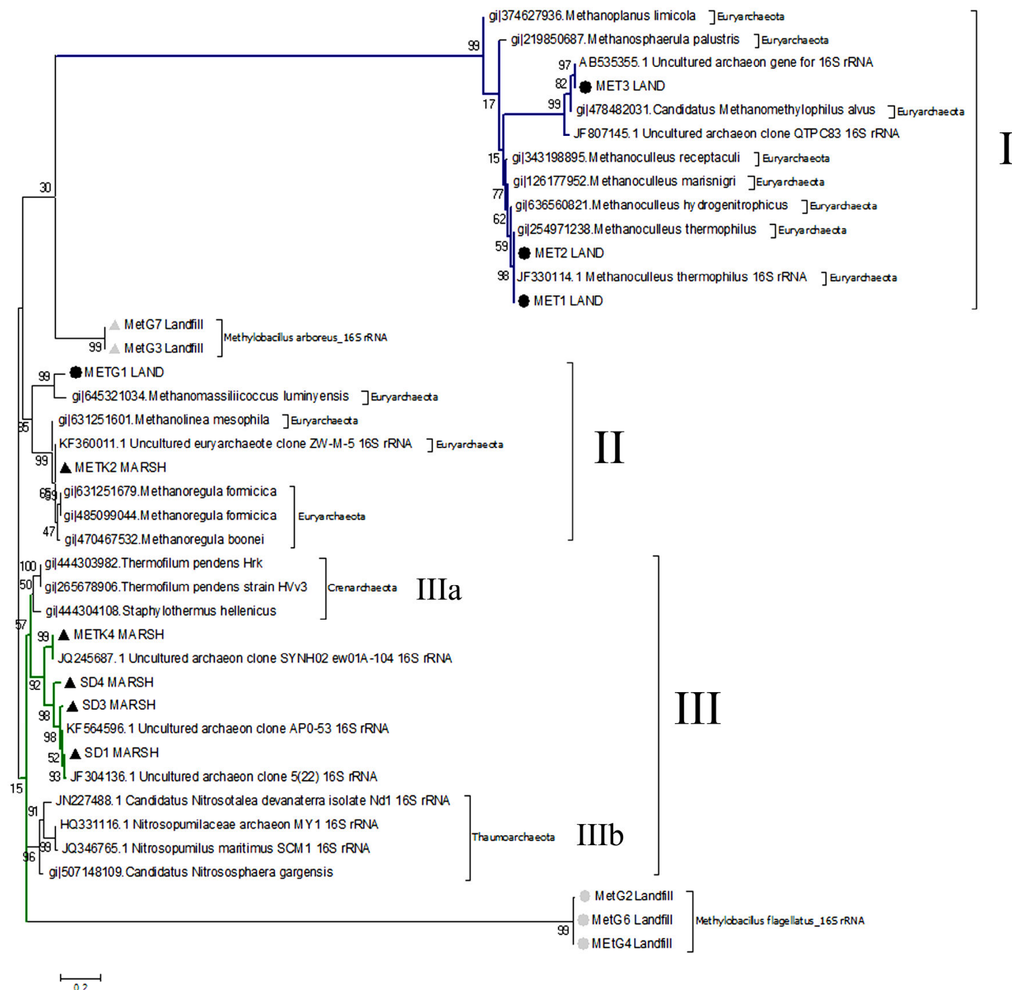


Figure 4.35. The schematic diagram of the Maximum Likelihood (ML) tree based on 40 partial 16S rDNA sequences: The Phylogenetic relationships of partial 16S rDNA sequences of clones (the confirmed 14 sequences generated from this study) recovered from both Delhi landfills (marked with ●, ● & ▲) and Southern Assam marshland sites (marked with ▲). The Phylogenetic analysis and tree was constructed

using K2P+G parameter model with 2000 bootstrap replicates using the MEGA 6 tree building program.

4.4.4. Phylogenetic analysis of methanogens present in landfill site Delhi using mcrA sequences using ML approach.

Nine mcrA gene sequences were then compared with the available nucleotide database from the NCBI Genbank using the BLAST program (Altschul et al., 1990). The partial nucleotide sequences of mcrA genes were submitted to NCBI under accession number KM041253 to KM041260 which is shown in **Table 4.12** below

Sequence ID	Organism name	Accession no.	Location
MCR1	*Methanothermobacter wolfeii*	KM041253	Ghazipur
MCR2	*Methanothermobacter marburgensis*	KM041254	Ghazipur
MCR3	*Methanothermobacter thermautotrophicus*	KM041255	Ghazipur
MCR4	*Uncultured Methanobacteriales archaeon*	KM041256	Ghazipur
MCR7	*Methanofollis liminatans*	KM041257	Ghazipur
BMCR2	*Methanospirillum hungatei JF-1*	KM041258	Bhalsawa
BMCR3	*Methanoculleus marisnigri JR1*	KM041259	Bhalsawa
BMCR4	*Methanosphaerula palustris E1-9c*	KM041260	Bhalsawa

Table 4.12: Methanogens mcrA sequences with accession numbers submitted in NCBI.

After performing BLAST, sequences showing similarity above 85-95% were used and aligned using CLUSTAL X 2.0. The phylogenetic relatedness among clones was estimated using the Maximum Likelihood Tree using Kimura HKY+G model with 1000 bootstrap value (Kimura, 1980). For model selection Bayesian analysis was performed and the model with lowest BIC value (i.e. 10272.56176) was chosen for tree construction. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). The Phylogenetic analysis and tree were constructed using MEGA software version 6.0 (Tamura et al., 2013) shown in **Figure 4.36**.

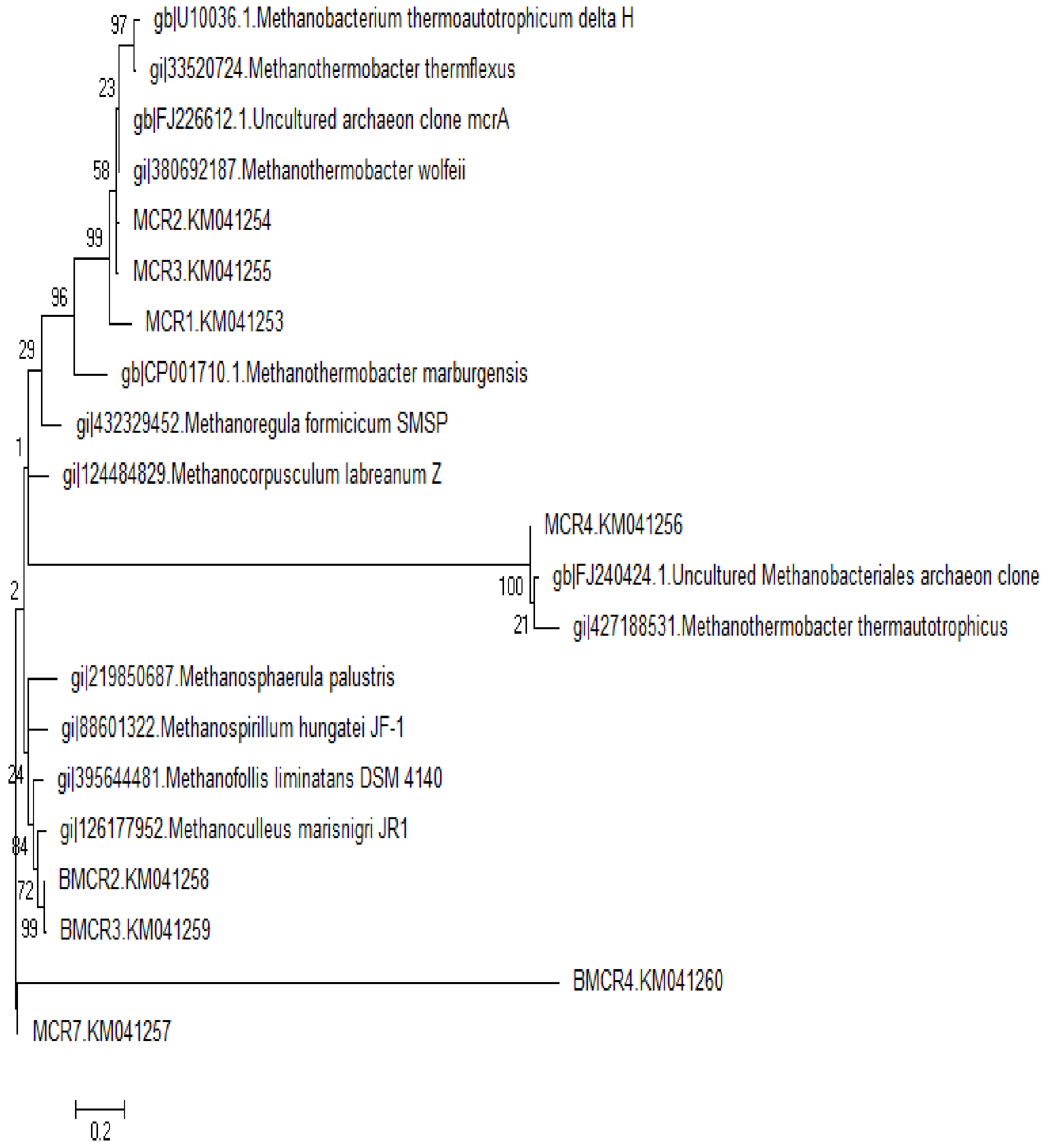


Figure 4.36. Shows maximum likelihood tree of mcrA sequences obtained from MSW leachate samples of Ghazipur and Bhalsawa landfill site, Delhi using Mega6.0.

The mcrA sequences obtained from the landfill leachate samples of both Ghazipur and Bhalsawa landfill site reveals presence of methanogens species belonging to order Methanothermobacter, Methanobacteriales and Methanosarcinales group. Methanogens belonging to group Methanothermobacter and Methanomicrobiales are hydrogenotrophic i.e utilise H_2 and CO_2 for methane production. Methanogens belonging to Methanosarcinales group are aceticlastic i.e. utilise acetate for methane production but can also utilize H_2+CO_2 and formate for methane production.

Phylogenetic tree indicates three main clusters. In first cluster mcrA gene clones derived from Ghazipur landfill site i.e. MCR1.KM041253, MCR2.KM041254 and MCR3.KM041255 clustered with methanogens belonging to order *Methanothermobacter wolfeii* and *Methanothermobacter thermautotrophicus.* In second cluster clone MCR4.KM041256 clustered with Uncultured methanobacteriales clone. In third cluster, MCR7.KM041257 clone from Ghazipur and BMCR2.KM041258, BMCR3.KM041259 and BMCR4.KM041260 of Bhalsawa landfill site clustered with *Methanoculleus marisnigri JR1*belonging to order Methanomicrobiales.