CHAPTER III: MATERIALS AND METHODS

3.1 MATERIALS

3.1.1. General chemicals and Kits

Tris saturated Phenol: Chloroform: Isoamyl alcohol, Chloroform, Isoamyl alcohol, Isopropanol, Tris (hydroxymethyl) aminomethane, Ethylenediamine tetrachloroacetic acid (EDTA), Sodium Dodecyl Sulfate (SDS), Proteinase K, Lysozyme, Ampicillin, X-GAL, IPTG, Luria Broth, Agar, MRS Broth (Himedia),Calcium Chloride, magnesium chloride, Glycerol, Ethanol, Nitric acid, Formaldehyde, Urea,40% Bis-Acrylamide: (37.5: 1, acrylamide: bisacrylamide), 50XTAE, Deionized Formamide, cysteine x HCl, Resazurin,Urea, Ammonium Persulphate, TEMED (N,N,N',N'-tetramethylenediamine), sterile water, Ethidium bromide, Silver nitrate,2X Master Mix (Fermentas), PCR Purification Kit (Fermentas), Fast DNA Spin kit for Soil (MP Biomedical), SYBR green master mix (Agilent).

3.1.2. Buffers

1. TE buffer

1M Tris (pH 8.0)	10mM
0.5M EDTA	1mM

2. 50X TAE buffer: **100ml**

Tris-acetate:	24.2 g
Glacial acetic aciad:	5.7 ml
0.5 M EDTA, pH 8.0:	10 ml

Make volume upto 100 ml using autoclaved MilliQ.

3. **Lysis buffer**

Tris HCL	10mM
EDTA	10mM
SDS	10%

4. Loading Dye

Bromophenol Blue	25mg
Glycerol	9ml
Nuclease free water	7ml

3.1.3. Reagents

1. Tris (hydroxymethyl) aminomethane-HCL or Tris-HCL-10ml (1M, pH 8.0)

Tris	1.211 gm
Nuclease free water	10 ml

Tris base 1.211 gram was added to the 7ml of nuclease free water, pH was adjusted to 8.0 using concentrated HCL. After adjusting pH volume was adjusted to 10 ml using nuclease free water and autoclaved at 15lb for 15 minutes.

2. Ethylenediamine tetrachloroacetic acid (EDTA): 100ml (0.5M, pH8.00)

EDTA	18.62 gm
Nuclease free water	100ml

Add 18.6g in 75ml Mili-Q water; adjust pH 8.0 using 10M NaOH. Then make up volume 100ml using Mili-Q. Autoclave and store at room temperature. EDTA is a chelating agent which chelates metals ions like Mg^{2+} and also inactivates nucleases by binding to metal cations required by these enzymes.

3. Sodium Dodecyl Sulfate (SDS) – (10%), 100ml

SDS	10gm
Nuclease free water	100ml

Add 10 g of SDS in 80ml of Mili-Q water, place on magnetic stirrer for dissolution. Then incubate it at 68^0C for 30 minutes and adjust pH at 7.2 using conc. HCL.ake up volume upto 100ml using Mili-Q. Do not autoclave.

4. 20mg/ml Proteinase K solution – 20ml

Add 400mg of Proteinase K powder in 15ml of 50mM Tris (pH 8.0), add 1.5mM $CaCl_2$. Make up volume upto 20ml using Mili-Q. Aliquot the stock and store in -20^0C.
20mg/ml Lysozyme solution- 20ml

5. 20mg/ml Lysozyme solution- 20ml

Add 400mg of Lysozyme in 20ml of 10mM Tris (pH 8.0). Aliquot the stock and store in -20^0C.

6. 3M Sodium acetate -100ml

Add 40.83 gram of Sodium acetate in 80 ml of Mili-Q water and adjust pH 5.2 using glacial acetic acid. Then make volume upto 100ml using Mili-Q. Autoclave and store at room temperature.

7. Ethidium Bromide: 10 mg/ml

Ethidium Bromide:	100mg
Nuclease free water:	10ml

Store it in a dark bottle at 4° C.

8. Ammonium Persulphate Solution10%

Add 0.1 g Ammonium Persulphate in 1 ml of sterile water. (Make fresh when required).

9. Ampicillin stock solution (50 mg/ml)

Dissolve 2.5 g of Ampicillin sodium salt in 50 ml of deionised water. Filter-sterilize and store in aliquots at -20°C.

10. X-Gal stock solution (20 mg/ml)

Dissolve 200 mg X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (#R0401) in 10 ml N,N-dimethylformamide. Store in the dark bottle at -20°C. Use 40µl per plate.

11. IPTG stock solution (100 mM)

Dissolve 1.2 g IPTG (isopropyl- β -D-thiogalactopyranoside) (#R0391) in 50 ml deionized water. Filter-sterilize, aliquote and store at 4°C. Use 40µl per plate.

12. LB-Ampicillin+ X-Gal/IPTG Plates

• Prepare LB-agar medium (1 liter), weigh out: 25 gram of Luria-Britani. Dissolve in 800ml of water and adjust the volume with water to 1000 ml. Add 15 g of agar and autoclave.

• Before pouring the plates, allow the medium to cool to 55°C. Then, add 1 ml of Ampicillin stock solution (50 mg/ml) to a final concentration of 50µg/ml. Mix gently and pour the plates. Allow the LB-Ampicillin agar medium to solidify. Dry plates opened at room temperature under UV light for 30 min.

• Add 40µl of X-Gal stock solution (20 mg/ml) and 40µl of IPTG 100 mM or IPTG Solution, spread evenly with a sterile spatula.

3.1.4. Composition of SAB medium for cultivation of methanogens:

Sl.no.	Chemical	Quantity
1.	$NiCl_2.6H_2O$	1.5mg/l
2.	$FeSO_4.\ H_2O$	0.5 mg/l
3.	$MgSO_4.7H_2O$	0.8 g/l
4.	KH_2PO_4	0.5g/l
5.	K_2HPO_4	0.5g/l
6.	KCL	0.05g/l
7.	$CaCL_2.7H_2O$	0.05g/l
8.	NaCl	1.5g/l
9.	NH_4Cl	1g/l
10.	$MnSO_4.7H_2O$	0.6mg/l
11.	$ZnSO_4.7H_2O$	0.1mg/l
12.	$CuSO_4.5H_2O$	0.02mg/l
13.	$KAl(SO_4)_2.12H_2O$	0.02µg/l
14.	H_3BO_3	7 mg/l
15.	$CoSO_4\ .7H_2O$	4 mg/l
16.	$Na_2MoO_4.\ 2H_2O$	0.5 mg/l
17.	$Na_2SeO_3\ .5\ H_2O$	3µ g/l
18.	$Na_2WO_4.6H_2O$	4µ g/l
19.	Nitrilotriacetic acid	0.15 mg/l
20.	Sodium acetate	1g/l
21.	Trypticase	2g/l
22.	Yeast Extract	2g/l
23.	L-cysteine hydrochloride monohydrate	0.5 g/l
24.	valeric acid	5mM
25.	isovaleric acid	5mM
26.	2-methylbutyric acid	5mM
27.	isobutyric acid	6mM
28.	2-methyl valeric acid	5mM
29.	Resazurin	1 mg/l
30.	Mili-Q	1000ml
31.	$NaHCO_3$	5.0ml
32.	$Na_2S.9H_2O$	0.5ml
33.	Methanol	20ml
34.	Sodium Format	2.5ml
35.	Trace Vitamin solution	10ml
36.	Final volume	1000ml

Table 3.1. Composition of SAB media for cultivation of methanogens.

Composition of trace vitamins Solution:

1.	Biotin	2.0 mg
2.	Folic acid	2.0 mg
3.	Pyridoxine·HCl	10.0 mg
4.	Thiamine·HCl	5.0 mg
5.	Riboflavin	5.0 mg
6.	Nicotinic acid	5.0 mg
7.	Calcium pantothenate	5.0 mg
8.	Vitamin B_{12}	0.1 mg
9.	*p*-Aminobenzoic acid	5.0 mg
10.	Lipoic acid	5.0 mg
11.	Distilled water	1.0L

Table 3.2. Composition of trace vitamin solution for cultivation of methanogens.

All solutions were filter sterilized using 0.2µm filter membrane obtained from Millipore.

3.1.5. List of PCR Primers used in the study

Primer	**Specificity**	**Sequence**
MET86 F	Meth16S rDNA	GCT CAG TAA CAC GTG
MET1340 R	Meth16S rDNA	CGG TGT GTG CAA GGA
ML FWD	mcrA	GGT GGT GTM GGA TTC ACA CAR TAY GCW ACA GC
MLREV	mcrA	TTC ATT GCR TAG TTW GGR TAG TT
ML GC	mcrA.REV.DGGE	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GTT CAT TGC RTA GTT WGG RTA GTT
519 FWD	Meth16S rDNA	CAG CCG CCG CGG TAA
915 REV	Meth16S rDNA	GTG CTC CCC CGC CAA TTC CT
915GC	Meth.REV.DGGE	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GTT GTG CTC CCC CGC CAA TTC CT
Bact. 27F	Bacteria16S rDNA	AGA GTT TGA TCC TGG CTC AG
Bact.1492R	Bacteria16S rDNA	GGT TAC CTT TGT TAC GAC TT
GC968	BactREV.DGGE	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA
S-D- Bact-0011-	Lact.16S rDNA	AGA GTT TGA TCC TGG CTC AG

a-S -20		
S-G—Lab-0677-a-A-17	Lact.16S rDNA	CAC CGC TAC ACA TGG AG
S-G—Lab-0677-a-A-17GC	Lact.REV.DGGE	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GTT CAC CGC TAC ACA TGG AG

Table 3.3. List of primers for amplification of bacterial, archaeal 16S rDNA and mcrA gene.

NOTE

A. Primer set MET86F and MET1340R was used for amplification of methanogenic 16S rDNA and cloning and sequencing. It's amplicon length was about 1.2 KB.

B. Primer set ME FWD and ME REV was used for amplification and insilico analysis targeting mcrA gene which is a unique functional gene in methanogens. It gave positive result while in-silico analysis but failed in experiments.

C. Primer set ML FWD and ML REV was used for amplification, cloning and sequence analysis targeting mcrA gene. It gave positive result.

   It was also used in DGGE for specifically monitoring methanogens community profile present in MSW leachate of Delhi landfill site.

D. Primer set 519FWD and 915 REV was used for estimation of methanogens diversity using 16S rDNA based DGGE analysis and quantification of methanogens from leachate samples of Delhi landfill site and Silchar marshland using (*RTPCR*) Real-time quantitative PCR.

E. Primer set 27F and 1492R was used for estimation of bacterial diversity present in MSW leachate samples of Delhi landfill site using DGGE, cloning and sequencing.

3.1.6. Reagents for estimation of lactic acid production:

A. Tris buffer- 24.2 g of Tris (hydroxyl) amino methane and 0.19g of $Na_2EDTA.2H_2O$ were dissolved in 60 ml of water, pH was adjusted to 7.2 using 1N HCl and volume was made up to 100 ml by adding double distilled (dd) water.

B. β NAD^+ solution (0.025mol/l) – 179 mg of β-NAD^+ solution was added to 10 ml of water and stored inside refrigerator at 4^0C.

C. Lactate dehydrogenase (LDH) solution - Lactate dehydrogenase was dissolved in Tris buffer with final concentration at 150 U/ml and stored at 4^0C.

D. Lactate standard solution, 20mmol/l (stock) – 0.1920g of L-lithium lactate was dissolved in 100ml of water. Working solutions of 1, 4 and 10 milli-moles/per liter were prepared by dilution of stock solution.

3.2 METHODS:

3.2.1 Sampling:

A. Collection MSW leachate samples from Delhi landfill site and their proper preservation in the laboratory

M.S.W. leachate and sediment samples were collected from the three landfill sites situated in Delhi namely Bhalsawa, Ghazipur and Okhla with varying season and depth. Samples were collected inside airtight plastic container and immediately brought to lab. Then sample was preserved at -20°C prior to DNA extraction and further analysis. DNA isolated from the sample was preserved at -80°C.

B. Sampling from marshland and rice paddy field of Silchar Assam

Samples were collected from more than 40 different sites of Silchar marshlands, Southern Assam, India with depth varying between 50-150 cm and in different season. Samples were collected in air tight plastic container and immediately brought to lab and they are preserved at -20°C prior to DNA extraction and further analysis. DNA isolated from the sample was preserved at -80°C.

Feature	Ghazipur	Bhalsawa	Okhla	Silcoorie Lake (Silchar)	Badarpur	Karimganj
Location	28°37'22.4"N 77°19"25.7"E	28°44'27.16"N 77.9°9'27.92"E	28°30'42"N 77°16'59"E	24°45'178"9 2°46' 58.3"E	24°54' 00"N 92°36' 00"E	24° 52'00N 92° 21'00"E
Type	Leachate	Soil and leachate	Leachate	Lake Sediment	Marshy pond	Rice paddy
Depth	150cm	200cm	150cm	40cm	100cm	Surface
pH	8.1	8.4	8.3	6.27	6.89	6.69
TDS	31,469	29,700	33,657	53,282	68,293	65,312
COD	29,930	31,600	29,020	NA	NA	NA
Fe	10.32	9.81	6.51	2.81	6.17	3.89
Cl	227	1174.2	264	9.11	12.60	16.31

Table 3.4. Showing sampling location and physiochemical parameters of Delhi landfill site (Ghazipur, Bhalsawa and Okhla) and Southern Assam marshland (Silcoorie Lake (Silchar), Badarpur and Karimganj) areas. All parameters are in mg L^{-1} adapted from Ghosh et al. 2015 and Gupta et al 2012.

Place	Season	Type of soil	pH	Depth
Ramnagar	June-Aug 2011	Rice paddy	5.5-6.5	40cm-50cm
Shadinbazar	June-Aug 2011	Marshy	5.5-6.5	40cm-50cm
Tarapur	October 2011	Rice paddy,Marshy	5.5-6.5	40cm-50cm
Silicoorie lake	November 2011	Rice paddy,Marshy	5.5-6.5	40cm-50cm
Dakbanglow	December 2011	Rice paddy,Marshy	5.5-6.5	40cm-50cm
Duarband	December 2012	Rice paddy,Marshy	5.5-6.5	40cm-50cm
Barjalenga	Dec.-Jan. 2012	Rice paddy,Marshy	5.5-6.5	40cm-50cm
Chutojalenga	Jan.-March 2012	Marshy	5.5-6.5	40cm-50cm
Highway	March 2013	Marshy	5.5-6.5	50cm-100cm
Hailkandi	December 2013	Marshy	5.5-6.5	50cm-100cm
Lala	March 2013	Marshy	5.5-6.5	50cm-100cm
Karimganj	March 2013	Waste water	5.5-6.5	40cm-50cm
Irongmara	March 2013	Marshy	5.5-6.5	40cm-50cm
Neelam Bazar	March 2013	Marshy	5.5-6.5	40cm-50cm
Sonai	March 2013	rice paddy field	5.5-6.5	40cm-50cm
Panchgram	March 2013	rice paddy field	5.5-6.5	40cm-50cm
Bhanga	March 2013	Marshy	5.5-6.5	40cm-50cm
Bodarpur	March 2013	Rice paddy field	5.5-6.5	50cm-100cm
Dholi	March 2013	Marshland	5.5-6.5	40cm-50cm
Katakhal	April 2013	Wastewater	5.5-6.5	40cm-50cm
Palonghat	April 2013	Marshy land	5.5-6.5	40cm-50cm
Kabuganj	April 2013	Waste water	5.5-6.5	40cm-50cm
Chargola	April 2013	Marshy land	5.5-6.5	100cm
Bhaga	April 2013	Wastewater	5.5-6.5	50cm-100cm
Darbi	July 2012	Marshland	5.5-6.5	100cm
Sidulbi	Dec 2013	Wetland	6.5-7.1	200cm

Table 3.5. Showing sampling locations of marshland in Southern Assam, India.

A

Okhla landfill site **Bhalsawa landfill site**

Ghazipur landfill site

B

Figure 3.1. A. Showing Okhla, Bhalsawa and Ghazipur landfill sites of Delhi from where sampling was done. **B.** Showing Highway, Silicoorie Lake, Badarpur Beetle nut pond, Darbi, Sonai road and Karimganj marshland Southern Assam from where sampling was done.

3.2.2. Method for estimation of lactic acid production:

The determination of lactic acid was based on its transformation to pyruvic acid by the enzyme Lactate dehydrogenase (LDH). The following reaction was reversible reaction and we needed to run it to forward direction to obtain complete oxidation of all lactate molecules. To ensure this, NAD^+ was provided in excess. The concentration of lactate in the sample is proportional to the increase in absorbance, as NAD+ is reduced to NADH (Allain et al., 1973).

$$Lactate + NAD^+ \xrightarrow{LDH} Pyruvate + NADH$$

For lactic acid assay, the collected samples were centrifuged at 12000 rpm for 10 minutes, clear supernatant were taken for analysis. A Perkin Elmer lambda 25, UV/VIS spectrophotometer was used for measuring O.D. Following reagents were made and used.

Procedure: 2.00 ml of buffer solution was taken in a test tube and 10µl of standard or sample was added to it. After this 100 µl of LDH solution was added and the O.D. was taken at 340 nm using UV/VIS spectrophotometer after 2 minute. Standard curve was plotted and concentrations of lactic acid in unknown samples were measured using that standard curve.

3.2.3. Protocol for Genomic DNA extraction from landfill leachate sample:

DNA was extracted from the leachate samples taken out at definite interval using enzymatic and chemical lysis method. It consists of following steps-

1. Cells were pelleted in 2ml tube by centrifugation at 13000 rpm for 10 minutes and resulting pellet were dissolved in 100 µl TE buffer.
2. For chemical and enzymatic lysis 20µl of 10% SDS, 10 µl of proteinase K (20mg/l) and 20 µl of lysozyme (20 mg/ml) were added and tube was incubated for 1hour at 56°C inside water bath.

3. In next step, equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and mixed by inverting and gentle tapping with hand and centrifuged at 13000 rpm for 10 minutes.
4. Top aqueous layer was then transferred to a new tube and equal volume of Chloroform:Isoamyl alcohol (24:1) was added and again centrifuged at 13000 rpm for 10 minutes.
5. Upper aqueous layer was transferred to a new tube without disturbing interface layer. Twice volume of 90% ethanol and 1/7th vol. of Isopropanol and 1/10th 3M sodium acetate is added to the tube and allowed to stand in ice for 15 minutes.
6. Then sample was centrifuged at 13000 rpm for 15 minutes at 4°C. The supernatant was decanted carefully and pellet was washed with 70 % ethanol by centrifuging it at 12000 rpm for 10 minutes.
7. Then tube was kept for drying in laminar flow for 1 hour.
8. Pellet was dissolved in 20-30 µl of nuclease free water or TE buffer.
9. The purity of DNA extract was assessed spectrophotometrically by calculating the A260/A280 ratio on a nanodrop spectrophotometer (Applied Biosystem).

3.2.4. Protocol for Genomic DNA extraction from Landfill sediment and marshland soil sample using Fast DNA Spin Kit for Soil (MP Biomedical):

Genomic DNA from landfill sediment and marshland soil sample was extracted using Fast DNA Spin Kit for Soil MP Biomedical using protocol as recommended by manufacturer with some modification. Modification was done in first step. It was recommended to take 500mg of soil, but in case of sediment samples from marshland 1ml of sample and 1ml MiliQ was taken in 2ml eppendorf tube and homogenized using vortexer. Then tubes were centrifuged at 3000 rpm for 1 minute. After centrifugation supernatant was transferred to new eppendorf tube using micropipette and then centrifuged at 13000 rpm for 10 minutes. The resulting pellet was used for DNA extraction and supernatant was discarded. The pellet was dissolved in eppendorf tube using 500 µl of sodium phosphate buffer and mixed using micropipette. Again 478 µl of sodium phosphate buffer was added to sample. Then the sample was transferred to lysing Matrix E tube. Rest of steps was followed as advised in manufacturer protocol.

1. 122µl of MT Buffer was added to the Lysing Matrix E tube.
2. Sample was homogenized for 40 seconds vigorously using vortexer.
3. After vortexing, tube was centrifuged at 14,000 x g for 5-10 minutes to pellet debris.
4. Then supernatant was transferred to a clean 2.0 ml microcentrifuge tube.
5. 250µl of Protein Precipitation Solution (PPS) was added and mixed by shaking tube with hand 10 times.
6. After mixing sample was centrifuged at 14,000 x g for 5 minutes to precipitate protein. Then supernatant was transferred to a new 2.0 ml microcentrifuge tube.
7. Binding Matrix was resuspended and 1.0 ml of binding matrix was added to supernatant in 2 ml tube. Mixed gently by inverting tube with hands for 2 minutes to allow binding of DNA with matrix.
8. After mixing, tube was placed in a rack for 3 minutes to allow settling of silica matrix. 500 µl of supernatant was removed using micropipette carefully avoiding settled binding matrix.
9. Remaining amount of supernatant was resuspended using micropipette and approx. 600 µl of the mixture was transferred to a SPIN™ Filter and centrifuged at 14,000 x g for 1 minute.
10. Filtrate in the catch tube was discarded and the remaining mixture was added to SPIN™ filter and centrifuged as before. Filtrate in the catch tube was discarded again.
11. 500µl of SEWS-M buffer was added to the SPIN™ filter and the pellet was gently resuspended using the force of the liquid from the pipet tip.
12. Without any addition of liquid, tube was centrifuged second time at 14,000 x g for 2 minutes to "dry" the matrix of residual wash solution. Discard the catch tube and replace with a new, clean catch tube.
13. Air dry the SPIN™ Filter for 5 minutes at room temperature.
14. After drying Binding Matrix was gently resuspended (above the SPIN filter) in 50-100 µl of DES (DNase/Pyrogen-Free Water)..
15. After suspension, tube was centrifuged at 14,000 x g for 1 minute to elute DNA into the clean catch tube.
16. Discard the SPIN filter. Store at -20°C for extended periods or 4°C until use.

17. For visualizing DNA extracts 5μl of each extract was electrophoresed on 1% Agarose in 1x TAE buffer and visualized under UV light using Geldoc (Biorad).

3.2.5. Agarose Gel Electrophoresis:

Gel solution was prepared as mentioned under materials section.

1. Agarose gel was allowed to cool and then casted on tray. Then the proper comb was inserted for the particular gel rig. The gel should be allowed to cool until it has set. The amount of agarose depends on the size of the gel rig. Gels should be fairly thin, approximately 1/4 to 1/2 inch.
2. Carefully the comb was removed and the gel was placed inside the gel rig with the wells closest to the cathode (black) end. The gel was covered with 1X TAE running buffer.
3. A piece of parafilm was being cut and a 1μl drop of glycerol loading dye was placed onto the waxy side for each sample to be loaded.
4. The samples were kept on ice and pipetted upto 5μl of a sample, and are mixed with one of the drops of loading dye. Then the mixture were loaded inside well.
5. 5 μl of 1 Kb ladder was placed in first well of gel mostly left to samples.
6. The gel rig was covered and power supply was switched on for 45 minutes or according to the need.

After the appropriate migration of gel the power was switched of and gel was viewed using U.V. transilluminator and photographed using gel documentation instrument (Biorad).

3.2.6. Purity check and yield of extracted DNA:

For downstream application extracted genomic DNA should be pure. Genomic DNA isolated from environmental samples may contain PCR inhibitors like humic acid, fulvic acid etc. Therefore, before PCR step, the purity and yield of extracted environmental needs to be checked. The purity and yield of DNA can be checked by spectrophotometric analysis.

Steps for Spectrophotometric analysis:

1. Spectrophotometer was switched on for 0.30hours for prior calibration.
2. The extracted genomic DNA was diluted serially viz. 10X, 50X and 100X.
3. Optical Density was set zero using nuclease free water which was used for dilution of stock DNA.
4. Optical Density was measured at wavelength 260nm and 280nm (OD260/280) for checking the concentration and purity of DNA.
5. The yield and purity of extracted DNA was measured using:
6. Concentration of stock solution:
7. $O.D._{260nm}$ X 100(dilution factor) X 50µg/ml/1000.
8. **8.** Purity of DNA stock solution: OD260/280 (for pure DNA the ratio range between 1.6-1.8).

3.2.7. Protocol for Polymerase Chain Reaction (PCR):

A. PCR amplification of Bacterial 16S rDNA:

Universal primer pair 27Fwd and 1492Rev was used for amplification of Bacterial 16S rDNA for evaluation and community profiling of Bacterial diversity present in the landfill site.

A.1. Master mix. Composition:

10X PCR Buffer	:	5µl
dNTPs mix.(10mM)	:	5 µl
Fwd Primer (10Pmole/ µl)	:	2 µl
Rev Primer (10Pmole/ µl)	:	2 µl
DNA polymerase (5U/ µl)	:	1 µl
Template DNA (50ng/ µl)	:	1 µl
$MgCl_2$ (25mM)	:	1 µl
Nuclease Free Water	:	upto 50 µl.

A.2. Bacterial 16S rDNA amplification Profile:

The amplification profile for Bacterial 16S rDNA was 94°C for 5 min, 94°C for 30 s for 30 cycles, 55°C for 1 minute, 72°C for 2 minute and final extension at 72°C for 10 minutes followed by a cooling step down to 4°C. PCR was carried out in Veriti thermal cycler (Applied Biosystem, Inc USA). 5 µl of PCR products were checked by loading on 1.2%-1.5% agarose gel containing Ethidium Bromide and were electrophoresed in 1X TAE Buffer for 45 minutes. Then gel was visualized under UV light using Gel-Doc. (Biorad).

A.3. DGGE PCR for Bacterial community fingerprinting:

For DGGE the initial amplicons of 1.2KB retrieved from primer pair 27Fwd and 1492Rev. were reamplified using same Fwd primer i.e. 27Fwd and GC968 primer pair containing GC clamp. The amplification profile and master mix. composition for GC clamp primer pair was same as explained above.

B.1. Master mix. composition and amplification profile for *Lactobacillus* 16S rDNA:

Primer pair S-G-Lab-0677-a-A-17 REV and S-D- Bact-0011-a-S -20 FWD primers were used for detection of *Lactobacillus* bacteria present in MSW leachate, Delhi. PCR was performed in a thermo-cycler (Applied Biosystem –Gene amp 9700). The master mix. composition and amplification for *Lactobacillus*16S rDNA was same for as for Bacterial16S rDNA, except the annealing temperature was at 60°C for 1minute.

B.2. DGGE PCR for identification of Lactobacillus species present in landfill leachate using Lactobacillus Identification Ladder:

For DGGE the initial amplicons of 670BP approx. obtained from primer pair S-G-Lab-0677-a-A-17 REV and S-D- Bact-0011-a-S -20 FWD were reamplified using same forward primer but reverse primer with GC clamp.

The amplification profile and master mix. composition for GC clamp primer pair was same as explained above.

C.1. PCR amplification of methanogenic 16S rDNA:

Primer pair 86Fwd and 1340Rev was used for specifically for amplification of methanogenic 16S rDNA from both landfill leachate sample of Delhi and marshlands of Silchar, Southern Assam.

Master mixture composition:

10X PCR Buffer	:	5µl
dNTPs mix.(10mM)	:	5 µl
Fwd Primer (10Pmole/ µl)	:	2 µl
Rev Primer (10Pmole/ µl)	:	2 µl
DNA Polymerase (5U/ µl)	:	1 µl
Template DNA (50ng/ µl)	:	1 µl
$MgCl_2$ (25mM)	:	2 µl
Nuclease Free Water	:	upto 50 µl.

C.2. Methanogenic 16S rDNA amplification profile:

The amplification profile for methanogenic 16S rDNA was 94°C for 5 min, 94°C for 30 s for 30 cycles, 58°C for 1 minute, 72°C for 2 minute and final extension at 72°C for 10 minutes followed by a cooling step down to 4°C. PCR was carried out in Veriti thermal cycler (Applied Biosystem, Inc USA). 5 µl of PCR products were checked by loading on 1.2%-1.5% agarose gel containing Ethidium Bromide and were electrophoresed in 1X TAE Buffer for 45 minutes. Then gel was visualized under UV light using Gel-Doc. (Biorad).

C.3. DGGE PCR for identification of methanogens from leachate of landfill site Delhi and marshland of Silchar, Southern Assam using DGGE based Methanogen Identification Ladder (MIL):

For DGGE the initial amplicons of 1.2 KB length obtained using primer pair 86FWD and 1340Rev. were reamplified with primer pair 519Fwd and GC 915 Rev primer containing GC clamp. The master mix composition and amplification profile for the methanogenic 16S rDNA was same as explained above.

D.1. PCR amplification of methanogenic mcrA gene:

For detection of methanogens specifically, from the leachate samples of landfill site Delhi and marshland of Silchar, Southern Assam, genomic DNA was amplified using primer set ML Fwd and ML Rev designed by Luton (Luton et al., 2002).The master mix. composition and the amplification profile of the mcrA gene was same as for the methanogenic 16S rDNA except the annealing temperature was at $52^{o}C$ for 1minute and the final extension was at $72^{o}C$ for 7minutes.

D.2. DGGE PCR for community profiling of methanogens from landfill leachate of landfill site Delhi and marshland of Silchar, Southern Assam targeting mcrA gene:

For DGGE, the initial mcrA gene amplicons of length 500BP obtained from primer pair ML Fwd and ML Rev was reamplified using ML Fwd primer and GCML Rev primer having GC clamp. The master mix composition and amplification profile for GC clamp PCR of mcrA gene was same as explained above.

3.2.8. PCR purification by Gel extraction Kit (Fermentas):

1. 50 µl PCR products were loaded on 2% agarose gel and run for 2.30 hours at 40volt.
2. After electrophoresis, gel was visualized under low wavelength UV transilluminator and desired band was cut using sterilized scalpel.

3. Gel was transferred to eppendorf tube and weighed.
4. Equal volume of extraction buffer was added to the eppendorf and incubated at 56^0C for 30-45 minutes inside waterbath for gel melting.
5. After melting of gel 500 µl of ethanol (96-100%) were added and mixed using pipette. After mixing entire mixture was transferred to spin filter column.
6. Then tube was centrifuged at 13000rpm for 1minute.
7. Flow through was discarded and 500 µl of prewash buffer was added to the spin column. Then tube was centrifuged at 13000rpm for 1minute.
8. Flow through was discarded and 700 µl of wash buffer was added to the spin column. Then tube was centrifuged at 13000rpm for 1minute.
9. Above step was repeated again.
10. After washing tube was emptied and centrifuged again at 13000rpm for 1 minute to completely elute the buffer.
11. Tube was placed inside a new 1.5ml eppendorf tube and placed inside laminar to air dry and evaporate remaining ethanol.
12. 10 µl of elution buffer was added to spin column and tube was centrifuged at 13000rpm for 1 minute.
13. After elution, the purified product was checked using agarose gel electrophoresis and concentration was measured using nanodrop.

3.2.9. Protocol for preparation of competent cells:

Note: Sterilize everything (graduated cylinder, glassware, Oakridge tube, Pipettes etc.

1. Grow *E. coli* DH5α cells in a 10ml LB broth overnight.
2. Place 500 µl of O/N culture into 100ml of LB broth.
3. Monitor growth until density reaches 0.45-0.47 at OD_{600}.
4. Quickly immerse flask in ice, and swirl for 30 minutes.
5. Spin out cells at 4000 rpm for 5 minutes.
6. Resuspend cells in 24ml of ice cold $MgCl_2$ (0.1M).
7. Spin out by running centrifuge up to 4000rpm.
8. Pour off supernatant.
9. Suspend cells in 24ml of ice cold, sterile $CaCl_2$, 0.1M by gentle pipetting

10. Spin again just up to 4000 rpm.
11. Pour off supernatant.
12. Suspend in 4.3ml of 0.1M sterile $CaCl_2$, ice cold.
13. Add 700uL of sterile glycerol, mix well.
14. Dispense 200uL to sterile Eppendorf vials. Store in -80^0C.
15. Set one eppendorf tube aside on ice to test for competency and contamination, transforming a portion of cells with a known concentration of DNA, and a negative control using no DNA.

3.2.10. Protocol of Cloning:

For cloning 16S rDNA and mcrA gene TA cloning Kit K1214 was used. The InsTA clone PCR Cloning Kit takes advantage of the terminal transferase activity of *Taq* DNA polymerase and other non-proofreading thermostable DNA polymerases. Such enzymes add a single 3'-A overhang to both ends of the PCR product. The structure of these PCR products favours direct cloning into a linearized cloning vector with single 3'-ddT overhangs. Such overhangs at the vector cloning site not only facilitate cloning, but also prevent the recircularization of the vector. As a result, more than 90% of recombinant clones contain the vector with an insert. Recombinant clones are selected based on blue/white screening.

3.2.10.1. Setting ligation mixture:

A. Set waterbath at 16^0C.
B. Prepare Ligation mixture:

Composition of ligation reaction mixture:

Component	Volume
Vector pTZ57R/T	1 µl
5X Ligation Buffer	2 µl
PCR product	3 µl
Water, nuclease-free	3.5 µl

T4 DNA Ligase	0.5 µl
Total volume	10 µl

A. Incubate ligation mixture overnight at 16^0C inside waterbath.
B. Store at -20^0C prior to ligation.

3.2.10.2. Transformation protocol

1. Set water bath at 42^0C.
2. Add 40µl of X-Gal and IPTG on LB-AMP plate and spread it using spreader and incubate it at 37^0C in an incubator for 4 hour.
3. Thaw glycerol stock of competent cells for 20 minutes.
4. Add entire ligation mixture in tube containing 200µl of competent cells.
5. Give heat-shock by placing tube in waterbath at 42°C for 1minute.
6. Place the tube inside ice for 2-3 minutes.
7. Add 800µl of LB to the tube and place tube for shaking in orbital shacker for 1hour at 37°C and 200 rpm.
8. After shaking tubes were centrifuged at 12000rpm for 1 minute.
9. Supernatant was taken out using micropipette and cells pellet were mixed in remaining LB media.
10. Blue-White screening - Cells were spread on LB-AMP + X-Gal-IPTG plate using spreader and incubated overnight at 37°C in incubator.

3.2.11. Plasmid DNA isolation

Plasmid or recombinant clones were grown overnight in 10 ml LB-Amp broth overnight at 37^0C and 200 rpm in incubator shaker. 3ml of this liquid culture was used for Plasmid isolation. Plasmid was isolated using Wizard Plus SV Minipreps DNA Purification System according to manufacturer protocol.

Protocol for Plasmid DNA isolation:

1. 3ml of overnight culture was pelleted by centrifugation at 13000rpm for 5 minutes.
2. Pellet was suspended thoroughly with 250µl of Cell Resuspension Solution.
3. 250µl of Cell Lysis Solution was added to each sample; inverted 4 times to mix.
4. 10µl of Alkaline Protease Solution was added to the suspension; inverted 4 times to mix and incubated for 5 minutes at room temperature.
5. 350µl of Neutralization Solution was added to the suspension and inverted 4 times mix.
6. Suspension was centrifuged at top speed for 10 minutes at room temperature.
7. Spin Column was inserted into Collection Tube.
8. Cleared lysate was transferred into Spin Column.
9. Spin Column was centrifuged at top speed for 1 minute at room temperature.
10. Flowthrough was discarded, and Column was reinserted into collection tube.
11. Added 750µl of Wash Solution (ethanol added). Spin Column was centrifuged at top speed for 1 minute at room temperature. Flowthrough was discarded, and column was reinserted into collection tube.
12. Step 10 was repeated with 250µl of Wash Solution.
13. Spin Column was centrifuged at top speed for 2 minutes at room temperature.
14. Spin Column was transferred to a sterile 1.5ml microcentrifuge tube.
15. 100µl of Nuclease-Free Water was added to the Spin Column. Centrifuged at top speed for 1 minute at room temperature.
16. Column was discarded, and DNA was stored at –20°C or below.

3.2.12. Protocol of Denaturing Gradient Gel Electrophoresis (DGGE):

DGGE gels is formed by mixing two gel solution one with low denaturation concentration and another with higher concentration (generally 40 and 60%). Equal volume of these solutions are mixed while pouring and results in formation of a gradient of denaturation from top to bottom which allows separation of PCR products even of same size but having Single Nucleotide Polymorphisms(SNP).

Note: 6 % and 8 % gel percentages are recommended for the separation of 300 - 1000bp and 200 – 400 bp PCR products, respectively.

A. Preparation of the Polyacrylamide gel with denaturant gradient:

1. For bacterial community fingerprinting targeting 16S rDNA, 6% polyacrylamide gel with denaturing gradient 40-60% of urea and Formamide was used.
2. For methanogenic community fingerprinting targeting 16S rDNA, 8% polyacrylamide gel with denaturing gradient 30-75% of urea and Formamide was used.
3. 20 ml of both "low-denaturant" concentration solution (30-40%) and a "high-denaturant" (60-70%) concentration solution depending on the gene size was used for making gel. Two 50 ml falcon tube were labeled as "low-density' and another as "high density."Added 20 ml of each stock (low and high density) into the correct 50 ml falcon tube. The gradient wheel combines the high and low-density stocks during the pouring of the gel to create the gradient within the gel matrix.

4. Prior to pouring 100µl of 10% APS (0.1g/ 1.0 ml, made fresh) and 15µl of TEMED was added to each 50 ml falcon tubes and swirled gently to mix. These reagents initiate the polymerization of the polyacrylamide gel.

5. After addition of APS and TEMED the gel solution are taken into syringe and poured using syringe and gradient wheel. Immediately, without disturbing the gel solution, the comb is placed between the plates. The gel is allowed to polymerize for 2-3 hours.

B. Running the gel

1. Prepare approximately 7 litres of 1X TAE buffer and fill the buffer chamber; put about 0.5 L aside for later use.
2. Gently remove the comb. Using a small syringe and needle, flush each well at least twice with 1X TAE buffer to remove any unpolymerized acrylamide. Failure to do this might result in uneven well floors and unresolved bands.

3. Attach the gel plates to the core assembly. Wet the gaskets of the core assembly before attaching the gel plates. This will help to prevent leaks. Two sets of plates must be attached. If only one gel is to be run, the other set of plates can be assembled with no spacers or gel.
4. Place the core assembly into the chamber (red is on the right side of the chamber) and add enough 1X TAE buffer to the upper reservoir to cover the gel. Pre-run gel at 55ºC at 130 volts for 1hour.
5. Remove the core assembly from the chamber (pour the buffer in the upper reservoir into a beaker) and remove the buffer from each well.
6. Add an equal volume of 2X Loading Dye to each sample. Load the entire PCR product into each well. Gently add enough 1X TAE buffer to each well to bring the volume to the top of each well. Gently add the buffer back to the upper reservoir and place the core assembly back into the chamber. Reset the temperature to 60°C and pre-heat the gel for 10 minutes without any voltage.
7. Run the gel at 140 volts and 60ºC for 10 minutes before turning on the recirculation pump to minimize washing the sample from the wells. Run the gel at 140 volts and 60ºC for approximately 5 hours.

C. Protocol for Silver Staining

1. After electrophoresis, gel was removed from glass plate gently as gel may break and transferred in glass dish containing staining solution.
2. The gel was stained in staining solution by gentle shaking with hand for five minute.
3. After staining, staining solution was decanted using syringe and rinsed with autoclaved nuclease free water for 10 seconds.
4. After washing, nuclease free water was removed from the container and developing solution was added. Gel was shaked gently for 10 minutes or until black precipitate appears.
5. Then immediately developing solution was decanted and stop solution was added to the container and shaked for 1 minute.
6. Developing solution was decanted and gel was transferred to gel preserving solution. In this gel can be preserved overnight at 4°C.

7. Image was taken under white light using Gel Doc Biorad.

3.2.13. Culturing of Methanogens:

Since methanogens are anaerobic they require anoxic conditions for culturing. They are cultured using technique basically devised by Hungate (Hungate, 1969) who perfected the preparation of pre-reduced media. In this study SAB medium was used for cultivation of methanogens.

All solutions were prepared in anaerobic water, with N_2/CO_2 flushing to replace the oxygen. Appropriate concentrations of reducing agents and trace Vitamin solution was added to the autoclaved medium just prior to use and allowed the medium to sit until it becomes colorless (incubation at 37°C may accelerate this process). pH was adjusted to 7.5 with 10 M KOH (Dridi et al., 2012; Khelaifia et al., 2013).

A. Resazurin Dye:

A redox sensitive dye is usually included in media used for culturing of anaerobes to monitor the redox potential. The most commonly used redox indicator is resazurin, because it is generally non-toxic to bacteria and is effective at very low concentrations of 0.5 to 1mg/l. This indicator dye is dark blue in its inactive form and first has to undergo an irreversible reduction step to resorufin, which is pink at pH values near neutrality (the colour may change to blue under alkaline conditions). This first reduction step normally occurs when media containing an excess of organic nutrients are boiled for a few minutes or mineral media are heated under an oxygen-free atmosphere.

B. Reducing agents:

Reducing agents are added to most anaerobic media to depress and poise the redox potential at optimum levels. The most common reducing agents are sodium thioglycolate, cysteine x HCl, Na_2S x 9 H_2O, FeS (amorphous, hydrated) etc.

Serum Bottles:

Serum bottles with capacity 50ml (Sigma) were used for cultivation of methanogens. The advantage of using serum bottle is that they are cheaper, easy to handle. Serum bottles are capped using butyl rubber and sealed with alumunium crimp to avoid complete diffusion of oxygen. However, serum bottles are less stable than Balch tubes and should be handled with special care when strains are cultured that are expected to produce significant amounts of gas during incubation (Wolfe et al., 2011).

For the aseptically injection and removal of samples from anaerobic cultures Hungate technique was followed which emphasized on the use of disposable syringes and has the advantage that it provides oxygen-free atmospheres for culturing. For inoculation 1ml disposable syringe was used. The inoculation of methanogens pure culture was done inside anaerobic chamber under complete anoxic environment.

3.2.14. Methods for Quantitative Real time PCR:

For real time PCR, four methanogens pure culture were obtained from DSMZ, Germany. Their genomic DNA was isolated and subjected to 16S rDNA PCR using primer pair 519FWD and 915REV. A product size of 421 BP was obtained from all four pure cultures which is analogous to in-silico result. The PCR products were purified using the PCR purification kit (Fermentas, UK) and then ligated into pTZR57T/A cloning vector (Fermentas, UK). The ligated products were transformed inside competent *E. coli* DH5α cells by heat shock. Plasmids were purified using Wizard Plus SV Miniprep DNA Purification System (Promega) as described in methods section.

Real time PCR was done on Agilent MxPro 3000 *RT*PCR instrument using SYBR green dissociation curve with Agilent 2x SYBR Master mix. Standard curve was prepared for absolute quantification. Standard curve was made using serial dilutions of plasmid (containing 16S rDNA gene fragment) of known concentrations on tenfold basis.

Out of ten dilutions four standard dilution were selected and run in triplicate along with samples from landfill sites, Delhi and marshland, Silchar, Southern Assam. For each real-time PCR three NTC (No Template Control) was also run. Entire reaction was prepared in dark as SYBR green dye is sensitive to light. Each component was thawed properly using ice and was then reconstituted in master mix as given below:

Composition of Real-time master mix:

2XSYBR Green M.mix	:	187.5 µl
Fwd Primer	:	18.75 µl
Rev Primer	:	18.75 µl
Template	:	1 µl (25ng)
Nuclease Free Water	:	125 µl
Total	:	375 µl

3.3. Nucleotide sequence analysis:

3.3.1. DNA sequencing

Sequencing was performed for all the clones with the ABI prism 3130 Genetic Analyzer (Applied Biosystem Inc., CA) at Department of Biochemistry, South Campus, Delhi University and Department of Biotechnology, Assam University. The sequences were edited to exclude the PCR primer-binding site and manually corrected with Sequence Scanner 1.0 (Applied Biosystem).

3.3.2. *In silico* analysis of nucleotide sequences

The sequences were checked further for vector contamination using the Vecscreen tool (http://www.ncbi.nlm.nih.gov/Vecscreen). The sequences showing similarity with vector sequences from both ends were trimmed. Sequences were then compared with the available nucleotide database from the NCBI Genbank using the BLAST program (Altschul et al., 1990).

3.3.3. Alignment of sequences using Clustal X

Clustal X software was developed by Thompson et al. and it is widely used for alignment of multiple sequences. It takes fasta formatted sequence as input and performs alignment automatically and gives an output file in Clustal X format with extension .aln. This format is compatible with phylogenetic analysis software like MEGA 6.0.

Evolutionary relationships can be represented using phylogenetic tree. A tree is a 2D graph showing evolutionary relationships among organisms. The tree is composed of nodes (a point where branches bifurcate) representing the taxa and branches representing the relationships among the taxa. The lengths of the branches are often drawn proportional to the number of sequence changes in the branch and hence can represent the divergence.

Usually phylogenetic analysis methods assume that each position in the protein or nucleic acid sequence changes independently of others. A clade is a group of organisms whose members share homologous features derived from a common ancestor. In a dendrogram, the trees are constructed by similarities of sequences which do not necessarily reflect evolutionary relationships.

Distance methods also called phonetic methods compress all of the individual differences between pairs of sequences into a single number. Character-based methods are also called cladistic methods. The trees are calculated by considering the various possible pathways of evolution and are based on parsimony or likelihood methods. The resulting tree is called a cladogram. Cladistic methods use each alignment position as evolutionary information to build a tree (Das et al.,2013).

3.3.4 Phylogenetic tree and MEGA

MEGA 6.0 is an integrated program which allows all four steps in a single environment, with a single user interface eliminating the need for interconverting file formats. Thus, MEGA 6.0 molecular evolutionary genetics analysis using maximum

likelihood, evolutionary distance, and maximum parsimony methods provides a reliable platform for construction and estimation of phylogenetic tree. For calculating genetic divergence and for selection of best evolutionary model for phylogeny construction MEGA6.0 software was used (Tamura et al., 2013).

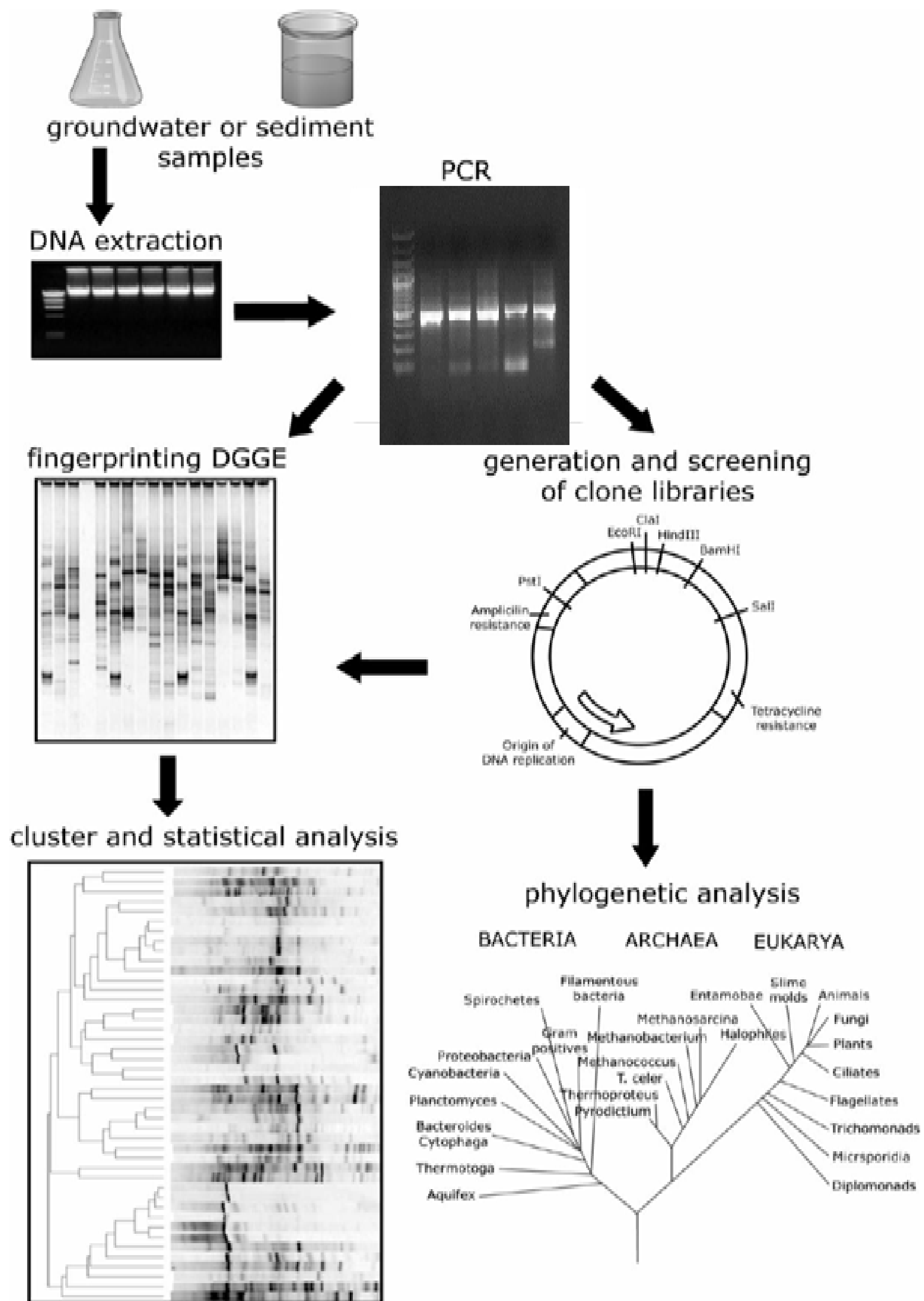

Figure 3.2. Flowchart Showing methodology adapted for identification of microbial /methanogenic diversity and universal tree having three domain of life based on 16S rDNA.