CHAPTER V: DISCUSSION

Methane is an important greenhouse gas because it is 25 times more potent gas than CO_2 in global warming potential (i.e., the ability of the gas to trap heat in the atmosphere) and thus plays a crucial role in climate change and carbon cycling (Bridgham et al., 2013; Yvon-Durocher et al., 2014). Methane emission has contributed approximately 20% to global climate change from pre-industrial times (Houghton et al., 2001; Yvon-Durocher et al., 2014). About 500–600 Tg of methane is emitted annually to the atmosphere of which 74% is biogenic, i.e. is produced by methanogenic archaea (Solomon, 2007).

Landfill sites contribute about 7% of the total anthropogenic source of methane emission (Conrad, 2009). Landfills are most commonly used for disposal of municipal solid waste (MSW) because of its economic advantage in a developing country like India. Landfill gas generated from these sites poses potential risk to surrounding ecosystems (Singh and Mittal, 2011). Despite of economic advantages, landfills are important anthropogenic sources of the greenhouse gas methane (CH_4). At present there are three functional landfill sites in capital city, Delhi (Bhalsawa, Ghazipur and Okhla). All these sites are uncontrolled dump sites where municipal solid waste (MSW) is indiscriminately and haphazardly disposed. The total composition of organic waste in MSW of India is around 40-60%, which gives it more potential for greenhouse gas (GHG) emission (Rawat and Ramanathan, 2011). The average composition of landfill gases is 45-60% methane and 40- 60% carbon dioxide, 2-5% Nitrogen gas, < 1% hydrogen sulphide and non-methane organic compounds (NMOCs) (Ranjan et al., 2014; Singhal and Pandey, 2001).

Similarly, wetlands (marshland) are the largest source of natural methane emissions and contribute about 100-231 Tg CH_4/yr (per year) accounting 20-39% of annual global CH_4 emission (Liu et al., 2012; Solomon, 2007). Methanogens in the moist, anoxic (oxygen-free) wetland soil produce CH_4 as they decompose dead plant material.

The CH_4 emission from wetland, increased by 7% from 2003 to 2007 (Bloom et al., 2010; Bridgham et al., 2013). Methane is produced by the anaerobic digestion process which is comprised of four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Each step is catalyzed by a different set of microbial guilds and products of each step serves as substrate for the next step.

In first three steps, anaerobic degradation of polymers such as carbohydrate, lipids and proteins take place by hydrolysis and fermentation. In fourth step, i.e. methanogenesis, substrates are utilized by methanogens for methane production (Alvarado et al., 2014; Purwantini et al., 2014). The rate of production of methane depends upon methanogenic diversity and activity which in turn is influenced by the presence of other syntrophic bacteria in vicinity and climatic conditions. Therefore, objective of this study was to analyse microbial diversity with emphasis on detection of methanogens from both landfill site and marshlands.

5.1 Cultivation independent, molecular analysis of microbial diversity

Microbes dominated in the history of living organisms and they are a fundamental part of the biosphere. The study of microbial diversity has been, therefore, essential for understanding the evolution of life. Traditionally, cultivation based methods have contributed to our knowledge about their whereabouts and diversity of microbes in naturally occurring communities. However, only a small fraction of the prokaryotes has been cultivated *in vitro* by standard methods. Therefore, this knowledge may not reveal the actual composition and/or diversity associated with an ecosystem (Amann et al., 1995; Pace, 1996). In the present study, culture independent molecular techniques like 16S rDNA PCR, cloning-sequencing, DGGE and qPCR were used for estimation of the richness and diversity of the methanogenic archaea in the landfill site of Delhi and marshland areas of Southern Assam. These techniques are widely used for molecular community analysis of microbes present in various types of habitats (Chaudhary et al., 2013; Kirk et al., 2004; Piterina and Pembroke, 2013; Watanabe et al., 2006; Watanabe et al., 2007; Watanabe et al., 2010). A combination of DNA sequencing, DGGE and Quantitative PCR (qPCR) can provide valuable information about microbial consortia associated with a specific ecosystem. Denaturing gradient gel electrophoresis (DGGE)

is used to determine the genetic diversity of microbial communities. The procedure is based on electrophoresis of PCR-amplified 16S rDNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants. In DGGE, DNA fragments of the same length but with different base-pair composition can be separated. Separation is based on the electrophoretic mobility of partially melted DNA molecules in a polyacrylamide gel and resulting into a band pattern (Brablcová et al., 2014; Muyzer, 1999; Muyzer et al., 1993). DGGE can reveal 1-2% of the actual diversity present in the samples (MacNaughton et al., 1999).Bacteria and archaea plays important role in biogeochemical processes like degradation and recycling of organic and inorganic substances (Bardgett and van der Putten, 2014). Very few studies have been carried out in India on landfills and marshlands.

Studies which have been done are mainly limited to toxicity analysis, methane emission potential and other physiochemical aspects avoiding the key players (i.e. microbes) which play active role in the entire process (Gupta et al., 2007; Rawat and Ramanathan, 2011; Singh and Mittal, 2011). Therefore, in the present study, cultivation independent molecular analysis techniques were applied for microbial diversity analysis. Specially designed glass bioreactors were manufactured for enrichment and to carry out anaerobic digestion experiment.

5.2 Bacterial diversity analysis by clone library method in landfill site Delhi

Bacterial 16S rDNA sequences obtained under this study were mostly affiliated to aerobic or obligate anaerobic bacteria, firmicutes. These microbes primarily participate actively in the first three steps of anaerobic digestion i.e. hydrolysis, acidogenesis and acetogenesis reaction (Kalia, 2008).

Phylogenetic analysis of bacterial 16S rDNA clones showed four clusters in the tree belonging mainly to genus *Bacillus and Clostridium* indicating their abundance in the landfill. Members of both genus *Bacillus and Clostridium* are acidogenic/acetogenic bacteria that produce volatile fatty acids like acetic acid and lactic acid and H_2 which is subsequently utilized by methanogens for production of methane during methanogenesis (Huang et al., 2010; Hung et al., 2011).

Presence of some novel taxa like *Bacillus licheniformis and Thermoanaerobacter tengcongensis* were also detected which has potential for industrial applications. *Thermoanaerobacter tengcongensis* MB4 is an obligate anaerobic, rod-shaped, gram-negative and thermophilic eubacterium belonging to family**.** *Clostridium* and *Eubacterium* like *Thermoanaerobacter tengcongensis* MB4, anaerobic microbes play crucial role in anaerobic cellulose degradation in landfills (Desvaux et al., 2001; Liang et al., 2011). *Bacillus licheniformis* are utilized in enzyme industry for production of extracellular enzymes. Presence of *Clostridium botulinum* in landfill site has been reported previously (Van Dyke and McCarthy, 2002). To our knowledge, this is first study reporting presence of *Clostridium botulinum* in landfill leachate samples of Delhi, India. DGGE based lactobacillus identification ladder was created for identification of lactobacillus species present in the Okhla landfill site. It indicated presence of *Lactobacillus acidophilus*, *Lactobacillus casei* and *Lactobacillus fermentum* species were present in the MSW leachate. Accumulation of lactic acid was also found during anaerobic digestion of MSW leachate in lab scale bioreactor.

Though lactic acid accumulates in animal tissues during oxygen starvation, such accumulation has not been reported in anaerobic reactors such as activated sludge reactors or cow-dung digester for biogas. This may be because that some bacteria like the Sulphate Reducing Bacteria (SRB) or the denitrifying bacterium (D.N.B.) present in the complex consortia consumes/converts the lactic acid. Two more reactors having cow-dung only and another with cow-dung with leachate were set up.

The accumulation of lactic acid was found to be much lower in reactor having only cow-dung than in the third reactor having cow-dung with leachate. This clearly indicates that MSW leachate has some constituent (heavy metals like Molybdate) that may suppress the growth of SRB (Nemati et al., 2001). Further, SRB bacteria population in landfill is limited because of sulphate and it allows methanogenesis to predominate. This may have a good application in India's rural economy. We know that SRBs present in cow-dung digesters (gobar gas plants) lead to production of H_2S. This leads to bad odour and rusting of iron caps of "gobar gas plant" besides reduced productivity of methane. Beside this methanotrophic bacteria were also detected from Ghazipur landfill sites which plays crucial role in global carbon cycling and methane

emission by acting as a natural source of methane mitigation which has been explained in later section.

5.3 Methanogenic diversity in landfill and marshland

5.3.1 Evaluation of methanogenic diversity in landfill and marshland by 16S rDNA

Phylogenetic analysis of 16S rDNA clones indicates the presence of methanogens belonging to the phylum Euryarchaeota, order Methanomicrobiales, Methanobacteriales-1, and seventh order of methanogens in the landfill sites (Borrel et al., 2012; Juottonen, 2008; Watanabe et al., 2010). Both *Candidatus Methanomethylophilus alvus Mx1201* and *Methanomassiliicoccus luminyensis* represent a monophyletic lineage that is not phylogenetically associated with any of the previously known orders of methanogens or the anaerobic methanotrophic ANME1 lineage (Borrel et al., 2012; Borrel et al., 2013). They belong to the Mx order clusters with two lineages: the planktonic Marine Group II (MG-II) and the sediment dwelling Marine Benthic Group D (MBG-D) (Borrel et al., 2013; Iverson et al., 2012; Lloyd et al., 2013). The other five sequences from Ghazipur landfill sites revealed presence of methanotrophs belonging to class *Betaproteobacteria*, family Methylophilaceae. 16S rDNA clones obtained from marshland sites of Southern Assam revealed a cluster of archaea that are distantly related to two different phylum, Crenarchaeota & Thaumoarchaeota. Microorganisms belonging to the phylum Thaumarchaeota recently proposed are thermophilic and mesophilic in nature and are found to be present in a wide variety of ecosystems, including marine and fresh waters, soils and also hot environment (Anderson et al., 2011; Brochier-Armanet et al., 2012; Galand et al., 2005; Ikenaga et al., 2004; Juottonen, 2008; Spang et al., 2012)..

Methanogens pertaining to both acetotrophic and hydrogenotrophic pathways were detected in MSW landfills, which have been reported in earlier studies (Huang et al., 2003; Laloui-Carpentier et al., 2006; Watanabe et al., 2010). Acetate serves as a precursor for more than 70% of CH_4 formation in the most anaerobic digestion process

(Yu et al., 2005). Therefore, acetoclastic methanogens, which utilize acetate as substrate, plays a key role in stabilizing the pollution load of wastewater by methanogenesis. Methanogens belonging to order Methanosarcinales i.e. *Methanosarcina mazei* (aceticlastic) were detected in both Okhla and Bhalsawa landfill and Silicoorie Lake of southern Assam by qPCR. The copy number of *Methanosarcina mazei* was highest in Bhalsawa landfill than Okhla landfill which in turn was higher than Silicoorie Lake.

The copy number of hydrogenotrophic methanogens *Methanobacterium bryantii* was found to be higher in both Okhla and Bhalsawa landfill of Delhi and Silicoorie Lake of Southern Assam, India. The abundance of hydrogenotrophic methanogens has been found to be associated with landfill and wetlands in earlier studies indicating that it may be the main reaction for of methane emission from landfills, rice paddy fields and wetlands (Galand et al., 2005; Huang et al., 2003; Laloui-Carpentier et al., 2006). Quantitative PCR indicates the higher methanogenic richness in both landfill site Delhi than of Silicoorie Lake, Silchar of Southern Assam.

Microbial diversity within contaminated ecosystems like landfill should be less diverse than those in natural systems like a wetland. Because the diversity may be influenced by the complexity of toxic chemical mixtures, heavy metals present and the duration of time the populations have been exposed. But in the present study, after analyzing DGGE gel banding pattern and number of bands we found that methanogenic diversity present in both landfills (anthropogenic system) and marshland (natural) is quite similar, except the samples obtained from the Karimganj rice paddy field in which only four bands appeared. The number of total bands observed in this study was in accordance with the number of DGGE bands reported in previous studies (Brablcová et al., 2014; Ikenaga et al., 2004; Watanabe et al., 2007; Watanabe et al., 2010). It indicates that the methanogenic archaea diversity in both landfill and marshland is influenced by sampling location rather than type.

5.3.2. Methanogenic diversity in landfill by mcrA gene

McrA sequences obtained from the landfill leachate samples of both Ghazipur and Bhalsawa landfill site revealed the presence of methanogens species belonging to order Methanothermobacter, Methanobacteriales and Methanosarcinales group which is congruent with previously published studies (García-Maldonado et al., 2012; Juottonen et al., 2006; Morris et al., 2014).

Phylogenetic analysis of mcrA gene clones derived from Ghazipur and Bhalsawa landfill site indicated presence of methanogens belonging to order Methanothermobacter and Methanomicrobiales. The results obtained in this study are in accordance with the previously published research where methanogens belonging to genera Methanomicrobiales have been detected (Huang et al., 2003; Laloui-Carpentier et al., 2006). Methanogens belonging to family Methanosarcina i.e. acetoclastic methanogens produce 72% of total methane from waste and plays crucial role in stabilization of waste. Only 28% methane is produced by hydrogenotrophic methanogens via hydrogen pathway (Kalia, 2008).

It is believed that acetoclastic methanogens commonly dominate in anaerobic systems and contribute about 70% of the methane produced. But in present study, it has been found that hydrogenotrophic methanogens were more abundant in both landfill site and marshland. But mere abundance cannot be correlated with the methanogenic potential and the predominant pathway. McrA gene expression in real time can be used as a potential tool for determining active members of methanogens and methane dynamics in the sample.

Community profiling of methanogens present in both landfill site Delhi and marshland samples of Southern Assam using mcrA gene revealed higher diversity than 16S rDNA gene. When we compare the banding pattern and total no. of bands in two figures of DGGE gel i.e. the 16S rDNA and mcrA gene in the respective samples, more bands were detected in the mcrA gel than 16S rDNA.

It has been reported in earlier studies that 16S rDNA library of the bio digester showed less diversity than the library of *mcr*A gene (Steinberg and Regan.2008). Therefore *mcr*A gene is used as a molecular marker for methanogenic archaea (Alvarado et al., 2014; Lueders et al., 2001). Eillis et al. used three sets of mcrA gene (ML, MCR and ME) primers for identification of methanogenic diversity present in an anaerobic digester fed with wastewater algae using 454 sequencing. They found that each of these primers showed unique community structure in the sample. Primer set ML designed by Luton was better in amplification and the sequences obtained were belonging to genera Methanosaeta order Methanosarcinales indicating its dominance (Ellis Joshua et al., 2012). Methyl coenzyme reductase A (mcrA) sequences from various type of environmental samples like in anaerobic digester (Morris et al., 2014) humic bog lake (Milferstedt et al., 2010), landfill(Luton et al., 2002), rice paddy fields (Lueders et al., 2001), in peat (Juottonen et al., 2006) etc. Therefore, the *mcr*A gene, which encodes the α-subunit of MCRI mcrA gene, was used as a tool for identification and phylogenetic analysis of methanogenic community (Alvarado et al., 2014).

5.3.3. Methanotrophs and methane mitigation

Methanotrophs have gained attention since last decades, as they have considerable potential for the oxidation of methane and in bioremediation processes (Schimel, 2000). The slow growth rate of methanotrophs hinders their study by conventional culture dependent techniques. Methods such as the MPN technique, FISH can be biased by selective culture conditions or require too much manual effort. Therefore, PCR based molecular techniques are widely used for their identification and diversity analysis (Murrell et al., 1998). In present study we used 16S rDNA based cloning-sequencing and DGGE for identification and diversity analysis of methanotrophs present in the leachate samples of Ghazipur, Delhi. They are extensively studied in a wide variety of environments because of their critical role in the global carbon cycle and methane mitigation. Culture-independent surveys showed that methylotrophs belonging to family Methylophilaceae are ubiquitous, thriving in a variety of natural as well as man-made environments. Sequences obtained in this study were distantly related to the cultured species of Methylophilaceae (similarity below 96% for the 16S rRNA gene). This indicates that the diversity of Methylophilaceae may be much greater than the

diversity covered by the cultured species (Lapidus et al., 2011). Novel Type 1 obligate methanotrophs belonging to class Betaproteobacteria has been also found to be associated with leachate samples of Ghazipur landfill site which can utilize methane and formaldehyde as energy source (Chistoserdova et al., 2007; Gogleva et al., 2011). Methanotrophic community diversity present in all five samples on the basis of band pattern were found to be homogenous in the Ghazipur landfill site.

Methane as a resource occurs in two alternative forms in nature: natural gas (non-renewable fossil fuel) and renewable biogas (by-product of human activity). Since methane can be used as an alternative source of energy, interest in new technologies for effective conversion of waste sources of methane into chemical compounds or next-generation fuels has been increased (Kalyuzhnaya et al., 2013). The composition of methane in landfill gases produced in Ghazipur landfill is around 40-50% (v/v). Presence of methanotrophs in the Ghazipur landfill can be utilized for biological mitigation of emitted methane in the landfill gas.

The biological oxidation of methane at landfill sites has advantages over combustion that a wide range of methane concentrations and toxic pollutants can be oxidized in eco-friendly manner. By providing facility like biocovers or bioreactors, optimum conditions for microbial processes and by efficiently collecting landfill gases to sites where they are produced a biological system can be developed for mitigation of methane from the landfill. Methanotrophic communities enriched from the environment can also be used for bioremediation of the toxic chemicals and compounds present in contaminated environments more economically and in eco-friendly manner (Jiang et al., 2010; Singh, 2011).