CHAPTER II: REVIEW OF LITERATURE

2.1 Microbial Diversity:

All organisms in the biosphere depend on microbial activity (Pace, 1997). Soil microorganisms are vital for the continuing nutrients cycling and for driving above-ground ecosystems (Øvreås, 2000). While many anthropogenic activities, such as city development, agriculture, use of pesticides and pollution can potentially affect soil microbial diversity, it is unknown how changes in microbial diversity can influence below-ground and above-ground ecosystems (Torsvik and Øvreås, 2002).

Our knowledge of soil microbial diversity is limited in part by our inability to study soil microorganisms (Tiedje et al., 1999). Pace et al based on DNA–DNA association kinetics estimated that 1 g of soil contains approx. 4000 different bacterial ‘‘genomic units’’. It has also been estimated that about 5000 bacterial species have been described (Pace, 1996; Pace, 1997) and approximately 1% of the soil bacterial population can be cultured by standard laboratory practices.

The immense phenotypic and genetic diversity found in soil bacterial and fungal communities makes it one of the most difficult communities to study. It has been suggested that at least 99% of bacteria observed under a microscope cannot be cultured by common laboratory techniques (Amann et al., 1995). It is possible that this 1% of cultivable bacteria is representative of the entire population and that the other 99% are simply in a physiological state that eludes our ability to culture them (Rondon et al., 1999). However, it is also likely that the 99% are phenotypically and genetically different from the 1% and only the minority of the population is represented (Rondon et al., 2000; Rondon et al., 1999).

Many fungal species also elude culturing in the laboratory (van Elsas et al., 2000). To overcome problems associated with non-cultivable bacteria and fungi, various methods have been developed to identify and study these microorganisms including fatty acid analysis and numerous DNA- and RNA-based methods.

The method of DNA or RNA extraction used can also bias diversity studies. Harsh extraction methods, such as bead beating, can shear the nucleic acids, leading to problems in subsequent PCR detection (V Wintzingerode et al., 1997).

Different methods of nucleic acid extractions will result in different yields of product (V Wintzingerode et al., 1997). With environmental samples, it is necessary to remove inhibitory substances such as humic acids, which can be coextracted and interfere with PCR analysis. Subsequent purification steps can also lead to loss of DNA or RNA, again potentially biasing molecular diversity analysis.

2.1.1. 16S rDNA sequence based understanding microbial diversity:

Microbial ecology has undergone a profound change in the last two decades with regard to methods employed for the analysis of natural communities (Nocker et al., 2007). Emphasis has shifted from culturing to the analysis of signature molecules including molecular DNA-based approaches that rely either on direct cloning and sequencing of DNA fragments (shotgun cloning) or on prior amplification of target sequences by use of the polymerase chain reaction (PCR) (Kirk et al., 2004). The pool of PCR products can again be either cloned and sequenced or can be subjected to an increasing variety of genetic profiling methods, including amplified ribosomal DNA restriction analysis, automated ribosomal intergenic spacer analysis, terminal restriction fragment length polymorphism, denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, single strand conformation polymorphism (Nocker et al., 2007) (Godon et al., 1997) (Kirk et al., 2004).

PCR targeting the 16S rDNA has been used extensively to study prokaryotic diversity and allows identification of prokaryotes as well as the prediction of phylogenetic relationship. 18SrDNA and internal transcribed spacer (ITS) regions are

increasingly used to study fungal communities. However, the available databases are not as extensive as for prokaryotes (Prosser, 2002).

Initially, molecular-based methods for ecological studies relied on cloning of target genes isolated from environmental samples (McDonald et al., 1999). Although sequencing has become routine, sequencing thousands of clones is cumbersome. Therefore, many other techniques have been developed to assess microbial community diversity. In these methods, DNA is extracted from the environmental sample and purified. Target DNA (16S, 18S or ITS) is amplified using universal or specific primers, the resulting products are separated in different ways (Kirk et al., 2004).

The 16S rDNA is the most conserved (least variable) gene present in all type of cells. Portions of the 16S rDNA sequence from distantly-related organisms are remarkably similar. This means that sequences from distantly related organisms can be precisely aligned, making the true differences easy to measure. For this reason, genes that encode the rRNA (rDNA) have been used extensively to determine taxonomy, phylogeny (evolutionary relationships), and to estimate rates of species divergence among bacteria and archaea. Thus the comparison of 16S rDNA sequence can show evolutionary relatedness among microorganisms.

This work was pioneered by Carl Woese (Woese, 1987; Woese et al., 1990) who proposed the three domain system of classification - Archaea, Bacteria, and Eukarya - based on such sequence information.

Due to conservative nature of ribosomal RNA (rRNA) sequences the three domain of life i.e. Bacteria, Eukarya and Archaea can be linked together in universal tree of life (Olsen and Woese, 1993).

Later, Amann proposed that ribosomal RNA (rRNA) can serve as a gold standard for description of communities of uncultivable microbes without need of cultivation. Nucleic acid extraction and PCR amplification with domain specific primers allows the detection and characterization of microbes without need of cultivation (Amann et al., 1995).

In 1998, Großkopf et al studied the methanogenic diversity and structure in anoxic rice paddy fields using both 16SrRNA and cultivation based approach. They found four methanogens i.e. *Methanobacterium bryantii* and *Methanobacterium formicicum*, *Methanosaeta concilii*, genus *Methanosarcina* to be present in anoxic rice paddy field microcosm.

Singleton et al., (2001) proposed that sequencing of clone libraries from environmental samples can provide highest phylogenetic resolution and wealth of information about prokaryotic diversity.

Høj et al. studied archaeal methanogenic communities present in two peat lands situated at Norway. They used nested PCR for detection of methanogens from methanogenic culture as well as genomic DNA extracted directly from the peat lands (Høj et al., 2008; Torsvik and Øvreås, 2002).

Advances in sequencing technology will increasingly encourage massive efforts to capture the genomes of the total microbiota of a specific environment, termed metagenome (Riesenfeld et al., 2004; Rodríguez-Valera, 2004).

Venter et al. (2004) using next generation sequencing (NGS) reported more than one million kilobase pairs of nonredundant sequence from the entire metagenome of the Sargasso Sea. Such large scale sequencing of environmental genomes provides deeper insight into microbial diversity and might allow us to better understand the metabolic and biogeochemical potential of the examined community thereby narrowing the gap between diversity and function (Riesenfeld et al., 2004).

The Ribosomal Database Project (RDP) on DNA currently has more than 253,000 entries (as of July 2006) and is frequently updated (http://rdp.cme.msu.edu/index.jsp). Sequencing is the basis for construction of phylogenetic trees and for other comparative studies.

Advances in sequencing technology and the cost-effectiveness of high-throughput systems in large genomic service facilities have increased the popularity of

direct sequence analysis of clones as an alternative to laborious screening of clones by restriction analysis (Nocker et al., 2007).

Cadillo-Quiroz et al (2006) studied vertical profiles of methanogenesis and methanogens in McLean bog (MB) and Chicago bog (CB) peat lands in central New York State, USA both have acidic (pH 3.5–4.5). For molecular survey they used 16S rDNA based cloning and sequencing analysis.

In 2007 Høj et al. studied effect of temperature on diversity and community structure of methanogens in high Arctic peat. They used molecular techniques like fluorescent in situ hybridization (FISH), 16S rRNA gene clone libraries and denaturing gradient gel electrophoresis (DGGE) for community analysis.

In 2007, Chen et al. used molecular approach for the detection of methanotrophic diversity present in landfill cover soil. Phylogenetic analysis of mRNA revealed the presence of methanotrophs like Methylosarcina, Methylomonas, Methylocystis, Methylocella and were actively involved in methane and methanol oxidation. Transcripts of *pmoA* but not *mmoX* were readily detected by reverse transcription polymerase chain reaction (RT-PCR), indicating that particulate methane monooxygenase may be largely responsible for methane oxidation *in situ*.

Juottonen et al. (2008) studied the effect of season on the methane producing microbes by comparing archaeal communities and rate of methane production in boreal mire (Juottonen et al., 2008). They used 16S rDNA based TRFLP, cloning and reverse transcribed RNA for archaeal community analysis and found that community shifts slightly with season. The archaeal community that remained dominant throughout year was Methanosarcinaceae, Rice cluster II and Methanomicrobiales associated Fen cluster. Methane production potential, measured as formation of CH_4 in anoxic laboratory incubations, showed prominent seasonality. The potential was strikingly highest in winter, possibly due to accumulation of methanogenic substrates, and maximal CH_4 production was observed at 30°C. Archaeal community size, determined by quantitative PCR, remained similar from winter to summer.

In 2009, Riviere et al. studied the diversity of microbial consortium involved in anaerobic digestion of sludge using 16S rDNA based clone library. In this study anaerobic digester were selected from France, Germany and Chile based on technology, type of sludge, process and water quality. The sequence analysis result indicated that the archaeal community in anaerobic digester belongs mainly to Methanosarcinales, Methanomicrobiales and Arc I phylogenetic groups and bacterial community belongs to Chloroflexi, Betaproteobacteria, Bacteroidetes and Synergistetes phylogenetic groups.

Rastogi et al. (2011) investigated microbial diversity present in extremely metal contaminated Coeur d'Alene River (CdAR) sediments. To assess the indigenous microbial communities associated with metal-enriched sediments of the CdAR, high-density 16S microarray and clone libraries specific to bacteria (16SrRNA), ammonia oxidizers (amoA), and methanogens (mcrA) were analyzed. Microarray analysis indicated presence of bacterial community belonging to phylotypes Proteobacteria followed by Firmicutes and Actinobacteria. Clone libraries analysis of amoA and mcrA gene demonstrated very limited diversity of ammonia oxidizers and methanogens and only *Nitrosospira* and *Methanosarcina* related phylotypes were retrieved in amoA and mcrA clone libraries, respectively.

Brauer (2012) demonstrated the effect of metabolic substrate on methanogenesis and community composition of methanogenic archaea in three contrasting peatlands situated in New-York. The methanogenic community structure was characterized using T-RFLP analyses of SSU rRNA genes. Their result indicated that Methanogens belonging to group Methanoregulaceae were dominant in the two acidic bog peatlands with relatively greater abundance in winter. In the fen peatland the members of Methanoregulaceae and Methanosaetaceae were codominant.

Zhang et al. (2012) used polyphasic approach combining both culture and molecular methods for characterization of archaeal and bacterial diversity present in hydrocarbon contaminated soil. They found bacterial 16S rDNA gene clone library sequences belonging to 68 bacterial phylotypes related to the Proteobacteria, Bacteroidetes, Actinobacteria and Spirochaetes. The archaeal 16S rDNA gene library contained sequences belonging to 15 phylotypes belonging to the phylum

Euryarchaeota; sequences were closely related to those of methanogenic archaea of the orders Methanomicrobiales, Methanosarcinales, Methanobacteriales and Thermoplasmatales.

In 2012, 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. Each of these primers showed unique community structure in the sample. Primer set ML and MCR were better in amplification and the sequences obtained were belonging to genera Methanosaeta order Methanosarcinales indicating its dominance.

Methanogens are thought to be present in strictly anaerobic and highly reduced environment condition. But, in 2012, Angel et al. found that methanogens can be present in aerated soils and they can become active in wet anoxic conditions. Methanogens of genera Methanosarcina and Metanocella were ubiquitous in the upland soil. Analysis of the archaeal 16SrRNA gene community profile in the incubated samples through terminal restriction fragment length polymorphism and quantification through quantitative PCR indicated dominance of Methanosarcina, whose gene copy numbers also correlated with methane production rates.

In 2013, Hedlund and coworker used culture independent and enrichment technique for determination of composition and structure of microbial communities inhabiting microbial mats in two geothermal springs in Armenia. Methanogen enrichment medium was based on Medium 1 (Whitman et al., 2006) and contained per liter: 5g $NaHCO_3$, 1g NH_4Cl, 1g $MgCl_2$ hexahydrate,0.4 g $CaCl_2$ dihydrate, 0.4gK_2HPO4 trihydrate,1 mg resazurin, 2 g yeast extract, 2 g trypticase peptones, 0.5 g cysteine hydrochloride, 0.5 g Na_2S nonahydrate, 10 ml vitamin solution, 10 ml trace element solution. Vitamin and trace element solutions were prepared as described. Medium 1 was prepared anaerobically as described (Whitman et al., 2006) in 30-ml serum vials with 20 ml of headspace (200 kPa total pressure of 80:20 N_2:CO_2). Initial enrichments additionally contained one of three substrates: H_2 (replacingN_2 in headspace); 10mM sodium acetate; or 50 mM methanol. Approximately 0.1 g of hot spring sediment was added in an anaerobic chamber (headspace of 5 % H_2, 5 % CO_2,

90 % N_2; Coy Laboratory Products, Grass Lake, MI, USA) as inoculum. Cultures were incubated in the dark at 45, 55, and 65^0C for up to 7 days (Hedlund et al., 2013).

Feng et al. (2013) studied the elevated level effect of O_3 on methanogenesis and methanogenic archaeal community. They found that elevated ground-level O_3 inhibited methanogenic activity and influenced the composition of paddy methanogenic communities, reducing the abundance and diversity of paddy methanogens by adversely affecting dominant groups, such as aceticlastic Methanosaeta, especially at the rice tillering stage. Their results indicated that continuously elevated ground-level O_3 would negatively influence paddy methanogenic archaeal communities and its critical ecological function.

Chaudhary et al. (2013) emphasized on use of molecular technique like cloning of 16S rDNA and mcrA gene and denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and terminal restriction fragment length polymorphism (TRFLP) and quantitative real-time polymerase chain reaction (qRTPCR) techniques for identification, richness and diversity analysis of methanogens present in freshwater sediment. These techniques are highly sensitive, rapid and reliable as compared to traditional culture-dependent approaches. Molecular diversity studies revealed the dominance of the orders Methanomicrobiales and Methanosarcinales of methanogens in freshwater sediments.

In 2014, He et al performed 16S rDNA based phylogenetic analysis of bacterial and archaeal community in eight year old refuse samples from landfill. The results indicated that the bacterial 16SrRNA genes sequences from the aged refuse were largely affiliated with the genus Bacillus, and the archaeal sequences were closely related to the methanogenic archaea. In this study molecular evidence for the occurrence of ammonia-oxidizing archaea in aged refuse was reported, which opens up avenues for elucidating its role in ammonia transformation in landfill systems.

In 2015, Brablcová et al. studied methanogenic archaea diversity in hyporheic sediments of Sitka lake by using molecular techniques like 16S rDNA sequencing, FISH and DGGE. They found 73 bands in DGGE gel by using Gel 2k software.

Sequencing of DGGE bands revealed methanogens belonging to order Methanosarcinales, Methanomicrobiales and Methanocellales.

2.1.2. Methyl coenzyme reductase A (mcrA) gene

Genes encoding for a function specific to a functional microbial guild can overcome the problem of phylogenetic dispersal. Methyl-coenzyme M reductase (MCR E.C. 2.8.4.1) is an essential enzyme in methane production. It catalyses the final step of methanogenesis in which methyl group linked to coenzyme M is reduced with formation of methane (Cram et al., 1987).

In 1997, Reeve et al. (1997) found that mcr gene is composed of three subunits α, β and γ, encoded by an operon mcr BDCGA. For detection of methanogens specifically the primer were designed against gene sequence of α subunit of the methyl coenzyme M reductase A (*mcrA*) which is unique only in methanogens (Bapteste et al., 2005; Lueders et al., 2001; Luton et al., 2002). The highly conserved nature of *mcrA* gene makes it suitable for phylogenetic analysis.

In 2001, Lueder et al. used mcrA gene for molecular analysis of methanogens present in rice field soil and enrichment cultures. They found some novel methanogenic sequences based on TRFLP pattern. Cloning and sequencing result indicated presence of methanogens belonging to genera Methanosarcinaceae, Methanosaetaceae and Methanobacteriaceae.

Luton (Luton et al., 2002) designed a degenerate primer for *mcrA* gene having 32mer Fwd and 23mer rev primer. He compared the phylogeny obtained by using 16S rDNA primer and MCR primer against 23 known species of methanogens representing all five methanogens order using *PHYLIP* and found that tree topologies are similar. Since then these primer sets were widely used for study of methanogenic communities in various habitat.

In 2006, Juottonen et al used mcrA gene for detection of methanogenic Archaea in Peat. They also compared three sets of mcrA primer i.e. MCR, ME and ML

for checking their efficiency and coverage of methanogenic diversity. It was found after cloning and sequence analysis that ML primer set designed by Luton has better coverage and amplification range in comparison to two other sets of primer and can be used for monitoring methanogens communities in various type of habitat.

Milferstedt et al. (2010) performed molecular study to analyze spatial structure and persistence of methanogens population in humic bog lake, USA. The mcrA gene was used as a molecular marker. Patterns of diversity within methanogenic archaea in humic bog lakes are quantified over time and space using Real-time PCR.

In 2011, Supaphol et al. studied microbial community dynamics in mesophilic anaerobic digestion of mixed waste. They used 16S rDNA library, DGGE and Real-time PCR for molecular survey. They found that Methanosaeta was the predominant methanogen throughout the process suggesting that acetoclastic methanogenesis was the main pathway for methane production in anaerobic digestion of mixed waste.

Antony et al. (2012) studied molecular diversity of methanogens and methylotrophs in Lonar crater, lake sediments using 16S rDNA based cloning, DGGE and Real-time PCR. They found that 16S rDNA sequences were related to phylotypes Halobacteriales, Methanosarcinales and Methanomicrobiales. To identify the active methylotrophic archaea involved in methanogenesis, mRNA transcripts of mcrA gene were retrieved from methanol consuming and methane emitting sediment microcosms at two different time points. Reverse-transcription PCR, qPCR, DGGE fingerprint, and clone library analysis showed that the active archaea were closely related to *Methanolobus oregonensis.*

Morris et al. (2014) used mcrA gene for monitoring methanogenic community inside anaerobic digester and found that *mcrA* gene copy number is correlated with CH_4 production rates using H_2/CO_2 significantly.

2.2 Techniques used in Molecular diversity analysis

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are two similar methods for studying microbial diversity. These techniques were originally developed to detect point mutations in DNA sequences. Muyzer expanded the use of DGGE to study microbial genetic diversity (Muyzer, 1999; Muyzer et al., 1993). DNA is extracted from soil samples and amplified using PCR with primers targeting part of the 16S or 18S rDNA sequences. The 5V-end of the forward primer contains a 35–40 base pair GC clamp to ensure that at least part of the DNA remains double stranded. This is necessary so that separation on a polyacrylamide gel with a gradient of increasing concentration of denaturants (Formamide and urea) will occur based on melting behavior of the double-stranded DNA. If the GC-clamp is absent, the DNA would denature into single strands.

In denaturing gel DNA melts in domains, which are sequence specific and will migrate differentially through the polyacrylamide gel. Theoretically, DGGE can separate DNA with single base-pair difference (SNP) (Kwok and Chen, 2003).

It is estimated that DGGE can only detect 1–2% of the microbial population representing dominant species present in an environmental sample (MacNaughton et al., 1999). In addition, DNA fragments of different sequences may have similar mobility characteristics in the polyacrylamide gel. Therefore, one band may not necessarily represent one species and one bacterial species may also give rise to multiple bands because of multiple 16S rDNA genes with slightly different sequences.

TGGE uses the same principle as DGGE except the gradient is temperature rather than chemical denaturants. DGGE/TGGE has the advantages of being reliable, reproducible, rapid and somewhat inexpensive. Multiple samples can also be analyzed concurrently, making it possible to follow changes in microbial populations (Muyzer, 1999). Limitations of DGGE/TGGE include PCR biases (V Wintzingerode et al., 1997), laborious sample handling, as this could potentially influence the microbial community and variable DNA extraction efficiency (Theron and Cloete, 2000).

In 2001, Maarit-Niemi et al. used different combinations of DNA extraction and clean-up procedures and reported that the choice of method used does influence the

banding pattern on DGGE gels and thus diversity analysis. They reported that the Soil DNA Isolation Kit (MO Bio Laboratories Inc., Solana Beach, CA, USA) gave consistent, clear bands with the most extensive banding patterns.

In 2003, Knief et al. targeted catabolic genes in upland soils, such as methane monooxygenase genes using DGGE for diversity analysis of methanotrophs. This would provide information on the diversity of specific groups of microorganisms competent in a defined function such as pollutant degradation and methane oxidation.

In 2004, Koizumi et al. used DGGE for investigating changes in methanogenic archaeal community with increasing depth in freshwater lake sediment targeting 16S rDNA and internal transcribed regions. He found variation among the banding pattern in DGGE gel with depth.

In 2004, Ikenaga et al. used PCR-DGGE and sequence analysis targeting 16S rDNA for estimation of methanogenic archaeal communities present in DNA extracts from nodal roots of rice plants grown in flooded soil pots. DGGE patterns of the methanogenic archaeal communities associated with nodal roots were similar among nodal roots irrespective of the growth stage and node and were similar to the pattern observed for paddy field soils. These DGGE bands indicated that specific methanogenic archaeal members inhabited rice roots constantly and that they were mainly derived from paddy field soils.

In 2009, Han et al. used DGGE targeting 16S rDNA gene and functional gene clone libraries and microarray analyses using pmoA to investigate the methanotroph community structure in alkaline oil from a Chinese coal mine. They found Methylocella related sequences from both DGGE analysis and 16S rDNA gene clone libraries. Analyses of alkaline soil using functional gene markers for methanotrophs demonstrated presence of diverse range of bacteria capable of utilizing methane, including the type II methanotroph Methylosinus/Methylocystis, the type I methanotrophs Methylobacter/Methylosomas, Methylococcus.

In 2009, Cheng et al. evaluated of efficiency of three set of GC Clamp primers for molecular diversity analysis of rumen methanogenic archaea using DGGE. The DGGE analysis of rumen fluids conducted with three primer sets (344fGC ⁄ 915r, 1106fGC ⁄ 1378r and 519f⁄915rGC). It was found that primer pair 519f ⁄ 915rGC had better amplification ranges than the other two primer pairs. Hence, procedure of DGGE analysis with primer pair 519f⁄915rGC was more suitable for investigating methanogenic community in the rumen.

In 2013, Piterina and Pembroke used PCR-DGGE technique to monitor bacterial biodiversity within autothermal thermophilic aerobic digestion (ATAD) system treating domestic sludge by examining microbial dynamics in response to elevated temperature. For analysis of bacterial diversity in DGGE primers targeting V3-V5and V6-V8 region were used. To allow comparative analysis of DGGE patterns, a marker containing products amplified with primers from known bacterial species was used in each analysis.

Terminal restriction fragment length polymorphism (T-RFLP) is a technique that addresses some of the limitations of RFLP (Tiedje et al., 1999). It follows the same principle as RFLP except that one primer is labeled with a fluorescent dye, such as TET (4, 7, 2V, 7V-tetrachloro-6-carboxyfluorescein) or 6-FAM (phosphoramidite fluorochrome 5-carboxyfluorescein). This allows detection of only the labeled terminal restriction fragment). This simplifies the banding pattern, thus allowing the analysis of complex communities as well as providing information on diversity as each visible band representing a single operational taxonomic unit or ribotype.

The banding pattern can be used to measure species richness and evenness as well as similarities between samples (Liu et al., 1997). This procedure can be automated to allow sampling and analysis of a large number of soil samples (Osborn et al., 2000). Osborn tested the reproducibility of the method and found that banding patterns within and between samples was highly reproducible. They found that the use of different *Taq* polymerase cause variability in the same DNA sample. TRFLP is limited not only by DNA extraction and PCR biases, but also by the choice of universal

primers. None of the presently available universal primers can amplify all sequences from eukaryote, bacterial and archaeal domains.

Additionally, these primers are based on existing 16S rDNA, 18S rRNA or ITS databases, which contain mainly sequences from cultivable microorganisms, and therefore may not be representative of the true microbial diversity in a sample. In addition, different enzymes will produce different community fingerprints. It is therefore, important to use at least two to four restriction enzymes (Tiedje et al., 1999).

T-RFLP, like any PCR-based method, may underestimate true diversity because only numerically dominant species are detected because of the large quantity of available template DNA. In addition, different species will have different gene copy numbers and this could bias results (Liu et al., 1997). Incomplete digestion by restriction enzymes could also lead to an overestimation of diversity (Osborn et al., 2000). Despite these limitations, some researchers have the opinion that once standardized, T-RFLP can be a useful tool to study microbial diversity in the environment ((Liu et al., 1997);(Tiedje et al., 1999); (Osborn et al., 2000)), while others feel that it is inadequate (Dunbar et al., 2000).

Energy production from renewable raw material is of utmost importance due to increasing population and rate of production of waste which in turn leads to expansion in size of landfills. Landfill gas (biogas) can be used as an alternative source of energy which is emitted from landfill sites. Biogas is one of the major renewable energy sources ensuring an adequate energy supply for the next generations. Beside technical optimization and upgrading of biogas reactors and plants, detailed information about the diversity and composition of the participating microbial community structure is indispensable for optimizing the biogas-forming process. Furthermore, precise knowledge about the metabolic activity of the microorganisms and their optimal growth conditions, quantity are of utmost importance to ensure the maximal degradation of the substrates to biogas (Song et al., 2010; Yu et al., 2005).

In 2005, Yu Lee et al. designed group specific Taqman based probes and primers for detection and quantification of methanogens using Real-time PCR. They

designed six sets of probes for detection of four orders of methanogens. Specificity of primer and probe sets was checked by using 28 archaeal cultures.

In 2006, Schippers and Neretin used quantitative PCR for quantification of microbial communities present on the surface and in deep sediment of Peru margin. They found that the 16S rDNA copy number of all prokaryotes decrease with depth gradient. They also analyzed Eukarya diversity using 18SrDNA and found that copy numbers are lesser than bacteria and archaea in deep sediment.

In 2006, Sawayama et al., used real time PCR for studying immobilized methanogenic community in fixed bed anaerobic digester supplied with acetate. They found that the major immobilized methanogens are Methanosarcina spp. and major free living methanogens belongs to Methanosarcina and Methanobacterium spp. The immobilized methanogens are 1000 times more concentrated and cell densities were high relative to original anaerobic digested sludge.

In 2008, Shin et al., compared order specific primers and universal archaeal primers for evaluation of methanogenic diversity in acetate enrichment system. They used 16S rDNA based DGGE and real-time PCR for detection and quantification of methanogens. Real-time quantitative PCR demonstrated the existence of three methanogenic orders: Methanobacteriales, Methanomicrobiales, and Methanosarcinales throughout the reaction period. DGGE analyses with three pairs of order specific primers yielded eight operational taxonomic units (OTUs), whereas DGGE analysis with two independent Archaea-specific primers identified only five. This finding indicates that use of order specific primer can give better detail of methanogenic population than conventional archaeal analysis.

Methanogens are of great importance in carbon cycling and alternate energy production. The quantification of methanogens with traditional cultured based technique can be biased. For this region methanogens are studied using molecular techniques and can be quantified using Real-time PCR.

Lisa and Regan developed a SYBR green I quantitative PCR (qPCR) assay to quantify total numbers of methyl coenzyme M reductase alpha subunit (*mcrA*) genes and Taqman probes for nine different phylogenetic groups of methanogens present in anaerobic digester and acidic peat. They found that members of the Methanosaetaceae, Methanosarcina, Methanobacteriaceae, and Methanocorpusculaceae and Fen cluster were dominant in the environmental samples and the acidic peat samples were dominated by both Methanosarcina spp. and members of the Fen cluster (Steinberg and Regan, 2008).

In 2013, Vigneron et al. studied archaeal and methane oxidizer communities in Sonora Margin Cold Seeps, Guaymas basin using molecular techniques like Automated method of Ribosomal Intergenic Spacer Analysis (ARISA), 16S rRNA libraries, fluorescence in situ hybridization (FISH) and quantitative polymerase chain reaction (QPCR). They found that metabolically active archaeal communities of anaerobic methane oxidizers (archaeal anaerobic methanotroph (ANME)-1, -2 and -3), including a novel 'ANME-2c Sonora' lineage were dominant, metabolically active and physically associated with syntrophic bacteria in sulfate rich shallow sediment layers.

The detection and quantification via quantitative PCR (qPCR) is a promising tool to reveal conditions and alterations within microbial communities. Therefore, in 2014, Reitschuler et al. did primer evaluation and used SYBR green based qPCR for quantification of methanogens.

Molecular phylogenetic or molecular systematic is the use of molecular structure to gain information on an organism's evolutionary relationships which is expressed as a phylogenetic tree. The impact of molecular systematic on bacterial classification has been profound. In the year 1977, Carl Woese, a chemist relatiively isolated and compared 16S rRNA sequences to study the classification of microorganisms (Lynch and Neufeld, 2015; Woese and Fox, 1977). This molecular approach revealed three (Archea, Bacteria, Eukarya), rather than five (Animalia, Plantae, Fungi, Monera, Protists) primary divisions of life to describe extraordinary levels of microbial diversity. Through cells different apparatus provides numerous information related to an organism but SSU rDNAs (genes coding for small subunit

ribosomal RNA) offer a quality and quantity of information which make them one of the most useful macromolecular descriptors of microorganisms (Das and Dash, 2013; Langille et al., 2013).

Evolutionary relationships can be represented using phylogenetic trees. 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.

Most of the sequences deposited till now in public database like NCBI, Genbank corresponds to uncultured organisms. The SSU of ribosome as the tool for cultivation-independent analysis of the diversity of complex microbial communities has resulted in enormous increase in number of sequences of which only the minority of sequences corresponds to the cultured prokaryotes (Yarza et al., 2014).

"All-Species Living Tree Project" is joint initiative between the journal Systematic and Applied Microbiology and the group of scientists authoring this work. The objective was to (1) provide curated SSU database of all type strains for which sequences are available;(2) maintain an optimized and universally usable alignment; and (3) reconstruct a tree harboring reliable genealogies. ARB, SILVA, and LPSN projects have prepared a 16S rRNA database containing over 6700 sequences, each of which represents a single type strain of a classified species up to 31 December 2007. The sequences were selected manually due to a high error rate in the names and information in the publicly deposited entries. In addition, there were multiple entries for a single type strain; therefore the best-quality sequence was selected for the project. The tree reconstruction has been performed by using the maximum likelihood algorithm RAxML (Yarza et al., 2008).