EFFECTS OF OPERATIONAL PARAMETERS ON METHANE PRODUCTION AND MICROBIAL COMMUNITY DYNAMICS IN ANAEROBIC DIGESTERS FED WITH COW MANURE AND BARLEY

by

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BS. in Biol., İstanbul University, 2012

Submitted to the Institute of Environmental Sciences in partial fulfillment of
the requirements for the degree of
Master of Science
in
Environmental Sciences

Boğaziçi University

2017

ACKNOWLEDGEMENTS

First, I would like to express my heartfelt thanks to my thesis supervisor Prof. Dr. Bahar İnce for her guidance and support through the whole study. I am very grateful to her for introducing me to a new field study and giving me the chance to part of her research group.

I would also like to kindly thank Prof. Dr. Orhan İnce for allowing me to work with the research group in Istanbul Technical University.

My sincere thanks and deep appreciation are extended to Ass. Res. Çağrı Akyol. I am eternally grateful for his continuous support and taking the time to help me. I could not finish this study without his support and encouragement.

I am also thankful to my thesis committee members Prof. Dr. Ferhan Çeçen and Prof. Dr. Emine Çokgör for sparing their time on evaluating my thesis.

I want to say thanks my colleagues, Ass. Res. Gözde Özbayram, Gülşah Günel, Elif Irmak Erdem, Mahir Bozan and Ecem Öner for their valuable help throughout laboratory works.

On a personal note, I would like to express my special thanks to my dear family; my parents Sefaret and Fevzi Güngör, my brother Halil İbrahim Güngör, my little sister Şevval Güngör and my husband Oğuz Tutal for their never ending love, patience and understanding.

This study was supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK, Project No: 115Y597) and Boğaziçi University Research Fund (Project No: 16Y00P1).

EFFECTS OF OPERATIONAL PARAMETERS ON METHANE PRODUCTION AND MICROBIAL COMMUNITY DYNAMICS IN ANAEROBIC DIGESTERS FED WITH COW MANURE AND BARLEY

In this study, effects of temperature (mesophilic vs. thermophilic), inoculum to substrate (I:S) ratios and different types of inoculums on methane production and microbial community profiles were investigated in anaerobic batch tests. The highest specific methane yield (278±12 mL CH_4/g VS) was found in the digester containing anaerobic seed sludge and cow rumen fluid as supportive inocula which was operated with an I:S ratio of 1:2 at mesophilic temperature, followed by the thermophilic digester (259±12 mL CH_4/g VS) inoculated only with anaerobic seed sludge with the same I:S ratio. The phylum *Firmicutes, Bacteroidetes* and *Proteobacteria* were major bacterial phyla for all sets. Hydrogenotrophic methanogenesis was favored in all digesters as the relative abundance of hydrogenotrophic methanogens was higher than that of acetotrophic methanogens. The most dominant methanogenic *Archaea* was *Methanobacterium sp*. in the digesters inoculated only with anaerobic seed sludge; whereas, *Methanobrevibacter spp.* dominated the digesters contained the rumen fluid. The total 16S rDNA copy number of bacteria and *Archaea* decreased in time for all digesters The results show that the methane yield can be improved with the combination of different types of inocula instead of additional energy input in thermophilic anaerobic digesters.

İNEK DIŞKISI VE ARPA İLE BESLENEN OKSİJENSİZ ÇÜRÜTÜCÜLERDE İŞLETME KOŞULLARININ METAN ÜRETİMİ VE MİKROBİYAL KOMÜNİTE DİNAMİKLERİ ÜZERİNDE ETKİLERİ

Bu çalışmada, havasız kesikli testlerde, sıcaklığın (mezofilik ve termofilik), inokulumun substrata (I/S) oranlarının ve farklı inokulumların metan üretimi ve mikrobiyal topluluk profilleri üzerine etkileri araştırılmıştır. En yüksek metan verimi (278±12 mL CH_4/g VS), destekleyici inokül olarak anaerobik aşı çamuru ve inek rumen sıvısı içeren, mezofilik sıcaklıkta ve I/S oranı 1/2 olarak işletilen çürütücüde bulunmuştur. Bir sonraki en yüksek metan verimi, aynı I/S oranına sahip, anaerobik aşı çamuruyla aşılanmış, termofilik çürütücüde (259±12 mL CH4/g VS) elde edilmiştir. *Firmicutes*, *Bacteroidetes* ve *Proteobacteria*, tüm setler için üç ana bakteri şubesidir. Hidrojenotrofik metanojenlerin göreceli oranları asetotrofik metanojenlerinkinden daha fazla olduğu için tüm çürütücülerde hidrojenotrofik metanojenez tercih edilmiştir. Sadece anaerobik aşı çamuru ile inoküle edilen çürütücü sistemlerinde en baskın metanojenik arke, *Methanobacterium sp.* iken; rumen sıvısı içeren çürütücülerde *Methanobrevibacter spp.*'di. Bakteri ve arkenin toplam 16S rDNA kopya sayısı, tüm reaktörlerde zamanla azalmıştır. Sonuçlar, termofilik anaerobik çürütücülerde ekstra enerji girişi yerine farklı inokulumların kombinasyonu ile metan veriminin geliştirilebileceğini göstermiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Units used
AD	Anaerobic Digestion	
ATP	Adenosine Triphosphate	
BMP	Bio-methane Potential	
BOD	Biological Oxygen Demand	mg L^{-1}
C/N	Carbon-to-nitrogen	
COD	Chemical Oxygen Demand	mg L^{-1}
DGGE	Denaturing Gradient Gel Electrophoresis	
DNA	Deoxyribonucleic Acid	
FISH	Fluorescent in situ Hybridization	
GC	Gas Chromatography	
GHGs	Greenhouse Gases	
I/S	Inoculum-to-substrate	
LCFAs	Long-chain fatty acids	
NGS	Next Generation Sequencing	
PCR	Polymerase Chain Reaction	
Q-PCR	Quantitative Real Time PCR	
rDNA	Ribosomal DNA	
RNA	Ribonucleic Acid	
rRNA	Ribosomal RNA	
SCFAs	Short-chain Fatty Acids	
sCOD	Soluble Chemical Oxygen Demand	mg L^{-1}
SRB	Sulphate Reducing Bacteria	
TKN	Total Kjeldahl Nitrogen	mg kg^{-1}
TS	Total Solids	%
VFA	Volatile Fatty Acids	mg L^{-1}
VS	Volatile Solids	%

1. INTRODUCTION

Biogas production from agricultural wastes, manure, crop materials, and municipal wastes is getting more important and provides several environmental benefits such as waste recycle and sustainable energy. Energy demand increases in the direction of increasing population hence biogas production offers a clean and sustainable solution to this problem (Chynoweth, 2004). Accordingly, the increase of the number of dairy farms in the proportion of the increasing human population is observed all over the world. As a result of it, a rising of animal manure can be a problem for the environment and public health. If animal wastes are failure to dispose or do not dispose properly, ecological damage will arise today and in the near future.

Anaerobic digestion is an effective method of animal waste disposal originating from dairy industries, which are hazardous in terms of the ecological balance (Li et al., 2009; Wan et al., 2011). Biogas obtained from anaerobic digestion of animal manure provides both animal wastes disposal and energy demand fulfillment sustainably. Also, it reduces the effects of harmful phenomena like acid rain and global warming caused by the burning fossil fuels (Chynoweth, 2001). In this regard, anaerobic digestion offers both economically and environmentally beneficial solutions: this process not only reduces the disposed material volume and protects water and ground from toxic materials as well as provides clean and sustainable energy such as biogas. Unlike the fossil fuels, CO_2 emission of biogas is very low (Esposito, 2012).

However, recent studies revealed that biogas yield of sole manure is relatively low due to its low carbon content. Thus, several methods have been developed to increase the biogas yield. Among several methods, anaerobic digestion with other organic waste called co-digestion has gained attention in recent years. Energy crops with high bio-methane potentials have been prominent and digestion of energy crops with animal wastes has been investigated to obtain high bio-methane yield. The most commonly cultivated energy crops include maize, herbage, clover grass, sudan grass and odder beet and all of them are seen in the literature frequently (Chynoweth et al., 1993; Vidnis et al., 2010; Menardo et al., 2015). Although barley both has

high methane yield (Braun, 2007) and is the most widely grown after wheat, maize and rice in Turkey (Akar et al, 2004), it is not studied as an energy crop commonly.

Anaerobic digestion is a process closely linked to environmental and operational conditions such as temperature, pH, nutrient contents, seed sludge characteristics, carbon/nitrogen (C/N) ratio, mixing ratio, and microelements availability. Manure and crop materials digested in anaerobic digesters contain a high proportion of lignocellulosic contents. Since the structure of lignocellulose is very refractory, its biological conversion is difficult. Gas production is low due to both rigid structure of lignocellulose and slow specific growth rates of microorganisms in bioreactors. Therefore, the biogas yield has been tried to be enhanced by adding rumen microorganisms to bioreactors in recent years (Barnes, 2003; Dalhoff, 2003; Baba et al., 2013; Wall et al., 2015). Rumen is the first part in the alimentary canal of ruminant animals. Digested foods are exposed to initial microbial fermentation by rumen microorganisms in there. Volatile fatty acids (VFA), carbon dioxide, methane, ammonia, and the microbial cells are obtained by hydrolyzing carbohydrates such as cellulose thanks to rumen microorganisms (Alataş and Umurcalılar, 2011). Biogas yield increases when ruminal fluid is added to bioreactors during anaerobic digestion (Cynoweth et al., 1993; Hu and Yu, 2005).

Methane yield in anaerobic digestion is directly linked to temperature factor. Anaerobic digestion occurs even at psychrophilic temperature (<-20°C) (Bouallagui et al., 2003). However, the temperature that methane yield is higher is generally mesophilic temperature (30-40°C) and thermophilic temperature (50-60°C) (Lettinga, 1995). Still, even in small changes in temperature can affect specific microbial populations which are active in bioreactors and consequently methane yield. In addition to all of them, if sole cow manure has low methane yield, inoculum can be added to reactors to enhance the biogas yield. Different inoculum/substrate (I/S) ratio affects the amount of methane produced (Raposa et al., 2006). From this point of view, we have the idea that the presence of microorganisms is closely linked to biotic and abiotic factors. Consequently, identification and quantification of microorganisms by the means of molecular methods are needed to better understand changes in bioreactors. Today, one of the most promising methods is next-generation sequencing technology. It is significantly faster, cheaper and more reliable than many methods. Millions of DNA fragments

in the entire genome can be sequenced in a study thank to next generation sequencing methods. Quantitative PCR (also called real-time PCR (Q-PCR)) can be used also to detect even low-level populations in samples (Hofman-Bang et al., 2003).

It can be concluded that there are many factors which affect methane production positively or negatively in bioreactors. While some energy crops as co-substrates such as maize, wheat, and grass are commonly studied, there is not enough information about barley as a co-substrate. Effects of operational conditions including temperature, I/S ratio and using rumen fluid as a supportive media for seed sludge were evaluated in anaerobic digesters fed with cow manure and barley. The data obtained in this thesis is expected to contribute to the literature.

2. THEORETICAL BACKGROUND

2.1. Anaerobic Digestion Process

Anaerobic digestion is a multi-step and natural process occurring by the help of microorganisms in the absence of oxygen. Organic matter is biodegraded roughly according to equation 2.1 (Kelleher et al., 2002; Salminen and Rintala, 2002; Chen et al., 2008).

$$\text{Organic matter} + H_2O \rightarrow CH_4 + CO_2 + \text{New biomass} + NH_3 + H_2S + \text{heat} \quad (2.1)$$

The steps of anaerobic digestion are as follows: hydrolysis, acidogenesis, acetogenesis, and methanogenesis; each step is mediated by different groups of microorganisms (Pesta, 2007). These microbes have symbiotic relationships with each other, thus the stability of anaerobic digestion is closely associated with their own nutrient and reactor conditions (Angelidaki et al., 1993; Poliafico, 2007). The degradation pathways are shown with a four stage model (Figure 2.1) and will be explained in detail in the following section.

2.1.1. Anaerobic digestion stages and microorganisms

Anaerobic digestion occurs in four steps. Polymers are converted into monomers by the means of hydrolytic bacteria in hydrolysis step. Then, acidogenic or fermentative bacteria digest hydrolyzed monomers resulting in short chain fatty acids (SCFAs) and hydrogen (acidogenesis step). Alcohols and short-chain fatty acids oxidize acetate, carbon dioxide and hydrogen by acetogenic bacteria (acetogenesis step). Finally, acetate is converted into methane and carbon dioxide by the means of methanogenic archaea in the methanogenesis step.

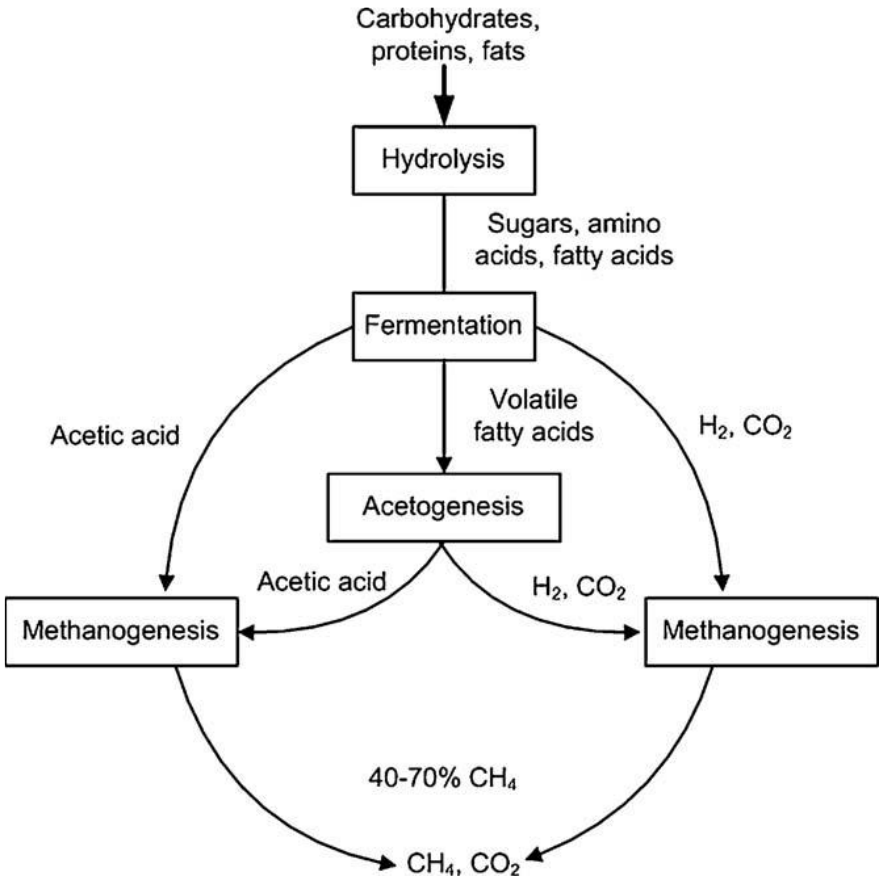

Figure 2.1. Anaerobic degradation process (Lie et al., 2011).

2.1.1.1. Hydrolysis. Hydrolysis is the first step of the anaerobic digestion process. Complex molecules must be converted to soluble monomers which can pass through the cell membrane in order to utilize by bacteria and *Archaea* (Gerardi, 2003; Madigan et al., 2008). Hydrolytic bacteria break down complex molecules such as carbohydrates, lipids and proteins into soluble monomers by the help of extracellular enzymes such as amylase, lipase as well as protease. In this step, polysaccharides are hydrolyzed to simple sugars, lipids are converted into long-chain fatty acids (LCFAs) and proteins are broken down into amino acids (Henze, 2008). The whole process is named as hydrolysis. The products produced in this step are ready for use by the acidogenic bacteria. Hydrolysis step is a process affected by temperature changes. Hydrolysis is usually considered as the slowest and rate-limiting step in the anaerobic digestion process. (Pavlostathis and Giraldo-Gomez, 1991; Henze, 2008). Also, the production of methane directly depends on the efficiency of the hydrolysis reaction (Palmisona and Barlaz, 1996).

Cellulolytic and xylanolytic microorganisms break down cellulose and xylose into glucose and xylan with the help of specific extracellular enzymes such as cellulases and xylanases; proteolytic microorganisms transform proteins into amino acids by proteases; finally, lipolytic microorganisms break down lipids into LCFAs and glycerol via lipases (Sterling et al., 2001; Salminen and Rintala, 2002; Gerardi, 2003).

Table 2.1. Specific examples of the bacterial genera of hydrolysis step (Gerardi, 2003).

Substrate to be degraded	Exoenzyme Needed	Example	Microbe	Product
Polysaccharides	Saccharolytic	Cellulase	Cellulomonas	Simple Sugar
Proteins	Proteolytic	Protease	Bacillus	Amino acids
Lipids	Lipolytic	Lipase	Mycobacterium	Fatty acids

The microbes in the hydrolytic phase are quite heterogenic. For instance, *Clostridium* degrades starch and cellulose; however, *Bacillus* degrades proteins and fats (Noike et al., 1985; Lema et al., 1991). *Clostridium thermocellum* is reported as the cellulolytic bacteria; *Clostridium bifermentas* and *Peptococcus sp*. are reported as proteolytic microbes as well as *Clostridium butyricum* and *Bacillus subtilis* are reported as aminolytic microorganisms (Payton and Handdock, 1986). Starch can be degraded by *Bacteroides amylophilus, Bacteroides ruminocola, Streptococcus bovis, Selemonas ruminatium, Succinomonas amylolytica* and some species of *Lactobacillus*. *Anaerovibrio lipotyca, Syntrophomonas wolfei* and a number of *Clostridium* and *Micrococcus* species degrade lipids. Protein degradation is carried by proteolytic clostridia such as *Clostridium bifermentas, Clostridium perfringes, Clostridium sporogenes* as well as *Clostridium histolyticum*. Also, *Streptococcus, Bacteriodes, Butyrivibrio, Fusobacterium, Selenomonas, Peptococcus* and *Campylobacter* participate in degradation of proteins to amino acids. Furthermore, these anaerobic bacteria take place in acidogenesis reaction and convert amino acids to simple fatty acids such as acetic, butyric and propionic acid. The significant hydrolytic anaerobic bacteria consuming cellulose are *Bacterioides succinogenes, Clostridium cellobioporus, Ruminococcus albus, Ruminococcus flavefaciens, Clostridium lochhadii, Clostridium thermocellum, Clostridium stercorarrium, Butyrivibrio fibrosolvens and Micromonospora bispora*. Hemicelluloses are degraded by some *Clostridium*

species and mostly rumen bacteria such as *Bacteriodes ruminicola, Bacteriodes fibrisolvens, Ruminococcus flavenfaceins, Ruminococcus albus* as well as *Streptococcus bovis* (Palmisano and Barlaz, 1996).

2.1.1.2. Acidogenesis (Fermentation). Hydrolysis products are transformed into volatile fatty acids (VFAs), alcohols, hydrogen and carbon dioxide by facultative and obligatory anaerobic microorganisms. In this step, a soluble monomer can be converted into different products by different bacteria. For example, glucose can be transformed into acetate, ethanol, and propionate in equations 2.2, 2.3, 2.4, respectively.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2.2)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (2.3)$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH3CH_2COOH + 2H_2O \quad (2.4)$$

In this process, many organic molecules are transformed into available substrates (acetate, hydrogen and carbon dioxide) for methanogenic microorganisms, yet approximately 30% of organic molecules are converted to SCFAs or alcohols (Gerardi, 2003). Ammonia and hydrogen sulfide formed during amino acid fermentation may inhibit the anaerobic digestion (Salminen and Rintala, 2002).

Acid-forming bacteria take place in this stage and convert soluble hydrolysis products into simple organic acids, mostly volatile fatty acids (short chain fatty acids) such as propionic, butyric, formic etc., ketones, alcohols, ethanol as well as carbon dioxide and hydrogen. ATP is produced during fermentation (Madigan et al., 2006). Sugars and proteins are converted into VFAs by acidogenic microorganisms. For example, sucrose is converted into different VFAs and HCO_3, H_2 and H^+ (Equations 2.5, 2.6, and 2.7, respectively). Acidogenesis is the most rapid conversion step in the anaerobic digestion process. Compared to the methanogenesis, there are 17 times more bacterial growth rate, 5 times more bacterial yield, and 6 times more conversion rate. Also, NH_3 is produced and this can balance the pH drop when acidic compounds are released at this stage (Henze, 2008).

$C_{12}H_{22}O_{11} + 9H_2O \rightarrow 4CH_3COO^- + 4HCO_3^- + 8H^+ + 8H_2$ (2.5)

$C_{12}H_{22}O_{11} + 5H_2O \rightarrow 2CH_3CH_2CH_2COO^- + 4HCO_2^- + 6H^+ + 4H_2$ (2.6)

$C_{12}H_{22}O_{11} + 3H_2O \rightarrow 2CH_3COO^- + 2CH_3CH_2COO^- + 2HCO_3^- + 6H^+ + 2H_2$ (2.7)

Several groups of bacteria are active in this step. The main species belong to the *Clostridia* group, which are bacteria that can adapt to a variety of environments. The family *Bacteroidaceae* takes part in the decomposition of sugars and amino acids (Chernicharo, 2007). Single amino acids are degraded by *Streptococci*, *Clostridia* and *Mycoplasmas*; butyric acid, butanol, acetone and isopropanol are usually generated by the genera *Clostridium* and *Butyribacterium*. *Butyribacterium* produces butyrate, *Clostridium acetobutylicum* mainly produces acetone and butanol; *Clostridium butylicum* produces hydrogen, CO_2, isopropanol and butanol.

2.1.1.3. Acetogenesis. This step is sometimes considered a part of the acidogenesis and acetate-forming microorganisms convert alcohols, VFAs other than acetate, CO_2 and a part of hydrogen into acetate. The reactions are showed in equations 2.8 to 2.15 and acetate is produced from bicarbonate, propionate, n-butyrate, iso-butyrate, n-valerate, 2-methylbutyrate and iso-valerate, respectively (Pind et al., 2003).

$CH_3CH_2OH + 2H_2O \rightarrow CH_3COO^- + 3H_2 + H^+$ (2.8)

$2HCO_3^- + 4\,H_2 + H^+ \rightarrow CH_3COO^- + 4H_2O$ (2.9)

$CH_3CH_2OOH + 2H_2O \rightarrow CH_3COOH + 2H_2 + CO_2$ (2.10)

$CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2$ (2.11)

$CH_3\,(CHCH_3COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2$ (2.12)

$CH_3CH_2CH_2CH_2COH + 2H_2O \rightarrow CH_3COOH + CH_3CH_2OOH + 2H_2$ (2.13)

$CH_3CH_2\,(CHCH_3COOH + 2H_2O \rightarrow CH_3COOH + CH_3CH_2CH_2COOH + 2H_2$ (2.14)

$CH_3(CHCH_3)CH_2COOH + CO_2 + 2H_2O \rightarrow 3CH_3COOH + 2H_2$ (2.15)

Hydrogen-producing bacteria generate acetate, H_2 and CO_2 from VFAs and alcohols; meanwhile, homo-acetogenic bacteria produce acetate from CO_2 and H_2 (Sterling et al., 2001; Lübken et al., 2007). However, most of the acetate is produced by hydrogen-producing bacteria.

Substrates other than acetate coming from the fermentation step are converted into acetate, hydrogen and carbon dioxide by acetogenic bacteria. The most important acetogenic substrates are propionate and butyrate. Also, lactate, ethanol, methanol and even hydrogen and carbon dioxide can be converted to acetate (Henze, 2008).

The acetogenic bacteria are obligate hydrogen producers and their metabolisms can be inhibited by hydrogen (Henze, 2008). Acetogenic bacteria are the intermediate metabolic group producing substrates for methanogenic microorganisms. They transform substrates generated in acidogenesis into suitable substrates for methanogenic microorganisms such as acetic acid, CO_2 and H_2. The substantial amount of hydrogen is produced during formation of acetic and propionic acid and it leads pH drop in an aqueous medium. This produced hydrogen is consumed in two ways: (i) methanogenic microbes using hydrogen and carbon dioxide to generate methane (ii) formation of organic acids like propionic and butyric formed during hydrogen, carbon dioxide and acetic acid reaction (Chernicharo, 2007). Acetic acid producing bacteria are *Methanobacterium bryantii, Desulfovibrio, Syntrophobacter wolinii, Syntrophomonas wofei* ve *Syntrophus buswellii* (Gujer et al., 1983; Stronach et al., 1986; Malina et al., 1992).

Acetogenesis is over with carbohydrate fermentation and the end products are acetate, CO_2 and H_2 which can utilize by methanogens. The existence of hydrogen is critical for acetogenesis phase. Reactions can progress only if hydrogen concentration is too low. Thus, the presence of hydrogen-producing bacteria is crucial for the continuation of the reaction (Ostrem & Themelis 2004).

Acetogenic bacteria also called obligatory hydrogen-producing acetogens are *Thermacetogenium phaeum* which is thermophilic acetate-oxidizing syntrophic bacterium (Hattori et al, 2000), *Syntrophobacter wolinii* which is a propionate-oxidizing syntrophic microorganisms, *Syntrophus aciditrophicus* which is a syntrophic bacteria degrading fatty acids and benzoate (Jackson et al, 1999), *Smithella propionica*, *Syntrophobacter strains*, thermophilic propionate-oxidizing bacteria such as *Pelotomaculum thermopropionicum* and *Desulfotomaculum thermobenzoicum*.

2.1.1.4. Methanogenesis. Methanogenesis step is carried out by obligate anaerobic microorganisms. Soluble matters are converted to methane by the help of methanogenic microbes. 70% of methane is generated from acetate (Jeris et al., 1965; Kugelman et al., 1965; Smith et al., 1966) while 30% of methane is created with the reduction of CO_2 by hydrogen or other electron donors (Hashimato et al., 1981). The other substrates converted to methane are methanol, methylamines, methyl sulfides and some alcohols; however, these reactions are less important (Gerardi 2003).

Table 2.2. Some important methane reactions carried out by different methanogens (Gerardi, 2003).

Reaction	Methanogen	Equation
$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	Hydrogenotrophic methanogens	(2.16)
$CH_3COOH \rightarrow CH_4 + CO_2$	Acetoclastic methanogens	(2.17)
$4HCOO^- + 2H_2O \rightarrow CH_4 + 3CO_2 + 2H_2O$	Acetoclastic methanogens	(2.18)
$4CO + 2H_2O \rightarrow CH_4 + 3CO_2$	Acetoclastic methanogens	(2.19)
$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	Methylotrophic methanogens	(2.20)
$4CH_3NH_3^+ + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4^+$	Methylotrophic methanogens	(2.21)
$2(CH_3)_2NH_2^+ + 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_4^+$	Methylotrophic methanogens	(2.22)
$4(CH_3)_3NH^+ + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_4^+$	Methylotrophic methanogens	(2.23)
$2CH_3CH_3OH + CO_2 \rightarrow 2CH_3COOH + CH_4$	Methylotrophic methanogens	(2.24)
$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	Methylotrophic methanogens	(2.25)

Methane reactions can be seen in Equations from 2.16 to 2.25. In equation 2.16, methane production from CO_2 and H_2 by the help of hydrogen utilizing methanogens is seen. Equations 2.17 to 2.19 show methane generation from acetogenic compounds and carbon monoxide by acetoclastic methanogens (Poliafico, 2007). Equations 2.20 to 2.25 show methane production from methanol and methylamines by methylotrophic methanogens (Gerardi, 2003). As a result of VFA consumption and ammonia (NH_3) production, pH gets slightly alkaline and it is the optimal condition for methanogens.

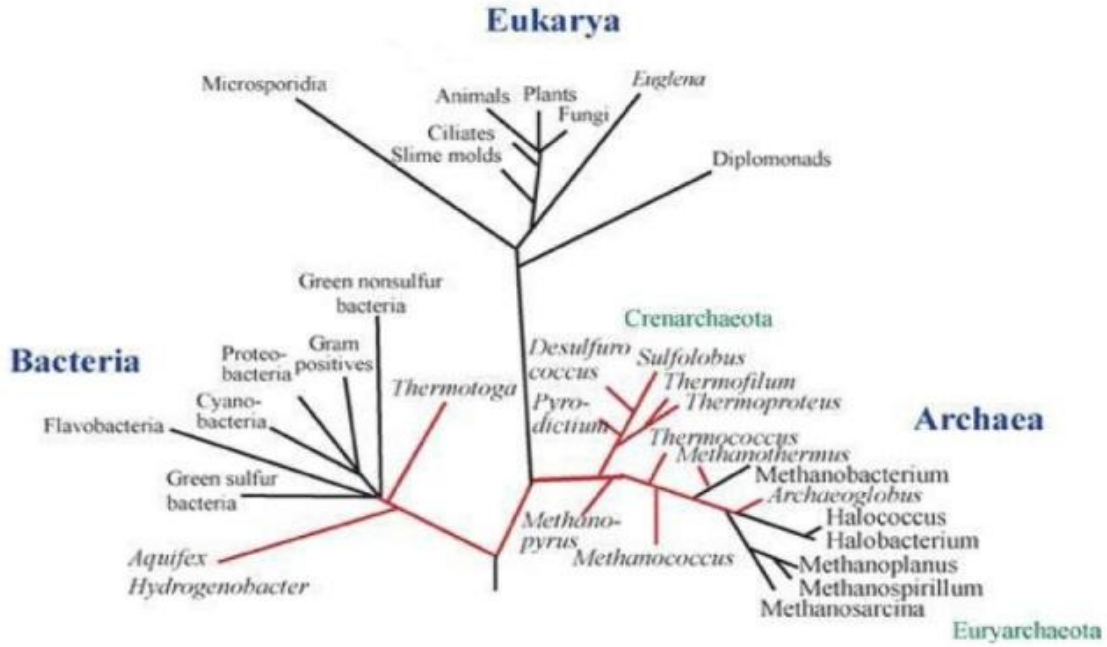

Figure 2.2. Universal phylogenetic tree (Woose et al., 1990).

Methanogenic *Archaea* completes the final stage of digestion of organic matter to CO_2 and CH_4. In this last stage, a group of methanogens both decreases carbon dioxide by using hydrogen as an electron donor (autotrophic methanogens) and decarboxylates acetate to create methane and carbon dioxide (heterotrophic methanogens).

Methanogens are obligate anaerobic *Archaea* and belong to the phylum of *Euryarchaeota* (see Figure 2.2). Methanogens are classified into five orders within kingdom *Archaebacteria*: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales* and *Methanopyrales* (Figure 2.3). Methanogens are divided into two large groups according to the substrate they use: the acetate converting or acetoclastic methanogens and the hydrogen utilizing or hydrogenotrophic methanogens.

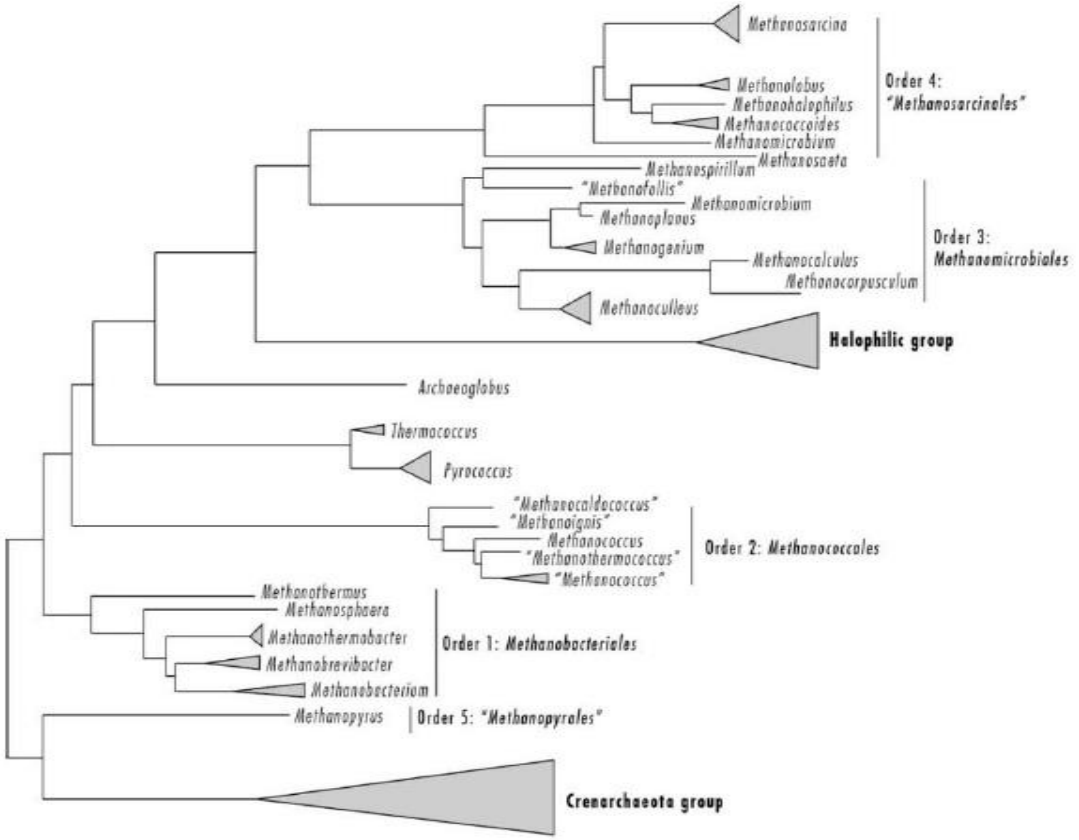

Figure 2.3. Phylogeny of methanogens, domain Archaea (Garcia et al., 2000).

Acetate-utilizing methanogens are methanogenic species that can generate methane from acetate (Eq. 2.26). There is a syntrophic relationship between acetoclastic methanogens and hydrogenotrophic methanogens. While 70% of methane is produced by acetoclastic methanogens, 30% of methane is generated by the help of other methanogens.

$$C^*H_3COOH \rightarrow C^*H_4 + CO_2 \quad \text{(acetoclastic methanogenic microorganisms)} \qquad (2.26)$$

Methanosaeta sp. and *Methanosarcina sp*. are the members of acetotrophic methanogens. *Methanobacteriales* and *Methanomicrobiales* are the hydrogenotrophic methanogens. While *Methanosaeta sp*. is favored at low acetate concentrations, *Methanosarcina* sp. is favored at high acetate concentrations (Zheng and Raskin, 2000). Besides, *Methanosaeta* sp. is affected

negatively in the presence of high SCFAs, on the other hand, *Methanosarcina* sp. dominates (Demirel and Scherer, 2008).

Table 2.3. Characteristics of methanogenic *Archaea* (Madigan et al., 2002).

Order	Morphology	Substrate for methanogenesis
Methanobacteriales		
Methanobacterium	Long rods	$H_2 + CO_2$, formate
Methanobrevibacter	Short rods	Methanol + CO_2, formate
Methanosphera	Cocci	Methanol + H_2
Methanothermus	Rods	$H_2 + CO_2$, can also reduce S^o (hyperthermophilic)
Methanococcales		
Methanococcus	Irregular cocci	$H_2 + CO_2$, formate, pyruvate + CO_2
Methanomicrobiales		
Methanomicrobium	Short rods	$H_2 + CO_2$, formate
Methanogenium	Irregular cocci	$H_2 + CO_2$, formate
Methanospirillum	Spirilla	$H_2 + CO_2$, formate
Methanoplanus	Plate-shaped cells	$H_2 + CO_2$, formate
Methanocorpusculum	Irregular cocci	$H_2 + CO_2$, formate, alcohols
Methanoculleus		$H_2 + CO_2$, formate, alcohols
Methanosarcinales		
Methanesarcina	Large irregular cocci in packets	$H_2 + CO_2$, methanol, methylamines, acetate
Methanolobus	Irregular cocci in aggregates	Methanol, methylamines
Methanohalobium	Irregular cocci	Methanol, methylamines (halophilic)
Methanococcoides	Irregular cocci	Methanol, methylamines
Methanohalophilus	Irregular cocci	Methanol, methylamines, methyl sulfides (halophilic)
Methanosaeta	Long roads to filaments	Acetate
Methanopyrales		
Methanopyrus	Rods in chains	CO_2 (hyperthermophilic)

Many studies suggest that mostly the genera of *Methanobacterium, Mathanothermobacter, Methanobrevibacter, Methanosarcina* and *Methanosaeta* are found in biogas reactor. *Mathanosarcina* and *Methanosaeta* which are acetoclastic methanogens usually dominate in large scale mesophilic and thermophilic digesters because they can widely tolerate changes in environmental factors such as nutrients and temperature (Palmisano and Barlaz, 1996).

Hydrogenetrophic methanogens generate methane from H_2 and CO_2 (Eq. 2.27). *Methanobacterium, Methanospirillum* and *Methanobrevibacter* are the most common genera in the anaerobic digesters (Chernicharo, 2007).

$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ (hydrogenotrophic methanogens) (2.27)

2.2. Environmental and Operational Parameters of Anaerobic Digestion

Anaerobic digestion (AD) is a process closely linked to the operational conditions such as nutrient contents, pH and alkalinity, temperature, mixing ratio, characteristics of seed sludge, and inoculums to substrate ratio. Digester performance can be affected positively or negatively by even small changes in conditions.

2.2.1. Nutrients

Microbes in anaerobic digesters need macro and micro nutrients to grow and survive. Macro nutrients such as carbon, oxygen, nitrogen, hydrogen and phosphorus are the main component and microbial cell includes approximately 50, 20, 12, 8 and 2 percent of those elements, respectively (Gerardi, 2003). Sulfate is also required to synthesize vital proteins (Madigan et al., 2008).

Recommended optimal C/N ratio for anaerobic digestion is in the range between 20/1 and 30/1 (Hawkes, 1980, Gerardi, 2003). Protein-rich wastes have high nitrogen content, high organic content as well as high BOD, on the other hand, have a low C/N ratio. Since these types of wastes include high nitrogen compounds, high amount of ammonia released during the

fermentation acts as an inhibitor. Anaerobic co-digestion of different organic materials is significant to achieve required the C/N ratio (Mshandete et al., 2004; El-Mashad and Zhang, 2010). Thus, when animal manure is co-digested with crops to balance C/N ratio, higher methane yield is achieved (Lehtomäki et al, 2007).

Potassium, calcium, sodium, magnesium, and iron found in trace amounts are called as micro nutrients. Also, nickel and cobalt are essential for growth of methanogenic organisms. For instance, acetoclastic microbes need nickel to synthesize cofactor F_{430} (Gerardi, 2003; Poliafico, 2007). However, if micronutrients are found in high concentrations in digesters, they inhibit the growth of fermentative and methanogenic microorganisms (Poliafico, 2007; Chen et al., 2008). On the other hand, low amount of micro nutrients can stimulate methane generation (Kugelman and Chin, 1971; Krylova et al., 1997).

Bio-methane potential (BMP) can change according to the composition of four main components such as proteins, lipids, carbohydrates and cellulose (Neves et al., 2008). For example, the highest methane yield is obtained by the degradation of lipid; on the other hand, the AD of lipid has the highest retention time. Proteins and carbohydrates are easier to degrade and also have high methane yield. However, VFA and ammonia accumulation which are inhibitory can be observed in AD of both lipids and proteins. Even though cellulose has high methane content, hydrolysis of cellulose is very slow due to its rigid structure (Neves et al., 2008).

2.2.2. Inhibitors

A substance is called as an inhibitor if it blocks the metabolism or biomass production. Inhibition of anaerobic digestion processes can easily be monitored by the methane content of the biogas or amount of volatile fatty acids accumulated in the system (Kroeker et al., 1979).

Some molecules formed during the degradation can be toxic for several microorganisms. For example, ammonia released during the degradation of protein can be inhibitory both methanogens and fermentative bacteria (Krylova et al., 1997; Chen et al., 2008). Inorganic

nitrogen is found in the forms of ammonium (NH_4^+) and free ammonia (NH_3^-) in anaerobic digesters. Methanogens are the least resistant group of microorganisms to ammonia inhibition (Kayhanian, 1994). However, there is a challenging information on the susceptibility of acetoclastic and hydrogenotrophic methanogens. In some cases, it has been reported that inhibitory effect was in general stronger for the acetoclastic methanogens than for the hydrogenotrophic methanogens (Koster and Lettinga, 1984; Sprott and Patel, 1986; Robbins et al., 1989; Angelidaki et al., 1993), while others observed the higher tolerance of acetate consuming methanogens to high ammonia levels as compared to hydrogen-consuming methanogens (Zeeman et al., 1985). Ammonia concentrations less than 1000 mg/L reported having no adverse effect on methanogens, whereas up to 3000 mg/L ammonia may have inhibitory effects at higher pH values. Ammonia inhibition in anaerobic treatment is controlled by pH, temperature, concentration acclimation and the presence of other ions (Chen et al., 2008).

Sulfate can be an inhibitor for microbes in anaerobic digesters. Sulfate is reduced to sulfide in anaerobic digestion by sulfate-reducing bacteria (SRB) (Koster et al., 1986; Hilton and Oleszkiewicz, 1988). H_2S is the toxic form of sulfide because it can penetrate into cells. Once inside the cytoplasm, H_2S may be inhibitory by denaturing native proteins through the formation of sulfide and disulfide cross-links between polypeptide chains (Conn et al., 1987). Less than 100 mg/L of soluble sulfide concentrations can be tolerated with no acclimation. Soluble sulfide concentrations between 100 and 200 mg/L do not show an inhibitory effect after an acclimation period. Sulfate concentrations higher than 200 mg/L had a direct inhibitory effect on anaerobic systems (Stronach et al., 1986).

Excess amounts of VFA produced in acidogenesis phase may inhibit the methane generation. Such an accumulation, in which VFA production is higher than the VFA consumption, can cause microbial stress if exist in elevated levels, decrease pH, and lead to failure of the digester. Therefore, the concentration of VFAs is an important consideration for the good performance of a digester (Wang, 1999). The most common VFAs found in an anaerobic digester are; acetic acid, propionic acid, butyric acid and isovaleric acid. In most cases of digester failure, acetic and propionic acid accumulate in the system and methane production decreases. Among VFAs, acetate is directly degraded by acetoclastic methanogens and

propionic and butyric acids must be first degraded by obligate hydrogen-producing acetogenic bacteria to acetate, hydrogen and carbon dioxide, which are then utilized by methanogens (Dogan et al., 2005). 35 mg/L acetic acid, higher than 3000 mg/L propionic acid and 1000 mg/L butyric acid concentrations are found as toxic for microbial growth (Ianotti and Fischer, 1983).

2.2.3. pH and alkalinity

pH is the best indicator of anaerobic digester stability (Poliafico, 2007). Especially methanogens are very sensitive to acidic conditions. Growth and reproduction of methanogens and methane generation are directly dependent on pH. Methane production is inhibited as pH decreases. Optimal pH value is different for each stage of AD. While the optimal pH of hydrolysis and acidogenesis is between pH 5.5 and 6.5, the optimum pH of methanogenesis is 7.0 (Yu and Fang, 2002; Kim et al., 2003). Thus, two-stage digester can be a good alternative to provide pH stability. Also, pH can change according to the reaction occurring at each stage. For instance, organic acids produced in acidogenesis can lower pH to less than 5, which is fatal for methanogenic microorganisms. Because methanogens diminish in low pH, they cannot consume acids in reactors and consequently acid accumulation occurs in the digester. It leads to digester failure. On the other hand, excess reproduction of methanogenic microorganisms causes accumulation of ammonia, increasing pH to higher than 8 and thus, it leads to failure of acidogenesis phase (Lusk, 1999). So as a result, constant pH is very significant in reactor installation since initially, waste will go through the hydrolysis and acidogenesis step before producing methane, which will lower pH. Bicarbonate, calcium carbonate or lime must be added to a reactor in order to balance pH. Alkalinity is very important for pH control. While fermentative bacteria need pH higher than 5 for enzymatic activity, methanogens are active at pH between 6.2 and 8. However, the most optimal pH for methanogens is between 7.0 and 7.2. (Gerardi, 2003; Poliafico, 2007).

CO_2 and VFAs produced during AD significantly affect the pH of the digester (Yadvika et al., 2004). Accumulation of ammonia may cause pH rise; on the other hand, accumulation of VFA which is generated by acidogenic microbes may cause pH drop. Nevertheless, sometimes the buffering capacity of a substrate can prevent pH drop stemming from VFA accumulation.

Alkalinity provides the buffer capacity to an anaerobic digester. Alkalinity is the equilibrium of carbon dioxide and bicarbonate ions, which prevents rapid changes in pH (Metcalf and Eddy, 2003). Thus, bicarbonate concentration is proportional to the buffering capacity (Hassan, 2003). Alkalinity values can change according to the substrate type in the digester. These values can range from 1800 to 2000 mg $CaCO_3$/L. Buffer capacity is a more reliable method of detecting digester imbalance than direct measurements of pH value (Ward et al., 2008). In AD of animal manure, alkalinity is considered necessary to neutralize the possible VFA accumulation. Under optimal conditions, VFA acidity produced by the acidogenic bacteria is utilized by the bicarbonate produced by the methanogens. Nonetheless, if VFA production is exceeded, buffering capacity can fail to lead to the collapse of the whole system.

2.2.4. Temperature

Methane yield in anaerobic digestion is directly linked to temperature factor because survival and growth of the microbial consortia directly depend on the temperature conditions (Table 2.4). Although microorganisms can survive a wide range of temperatures, mesophilic and thermophilic conditions are optimum for their growth. Actually, anaerobic digestion occurs even at a psychrophilic temperature (<-20°C) (Bouallagui et al., 2003). However, the temperature that methane yield is higher in generally mesophilic temperature (30-40°C) and thermophilic temperature (50-60°C) (Lettinga, 1995). According to Ward et al. (2008), optimum biogas is produced at temperature 35°C and 55°C for mesophilic and thermophilic microorganisms, respectively. Still, even in small changes in temperature can affect specific microbial populations which are active in bioreactors and consequently methane yield. Chae et al. (2008) observed that the biogas production decreases when the temperature is changed from 35°C to 30°C and from 30°C to 32°C in the bioreactors. Also, methane production is inhibited between 40°C and 50°C (Gerardi, 2003).

In addition to all of them, mesophilic and thermophilic conditions have both advantages and disadvantages. Thermophilic digestion provides pathogen destruction, higher substrate degradation and higher biogas production. On the other hand, thermophilic microorganisms are more sensitive to changes in environmental conditions such as pH, temperature fluctuations and

toxins (Gerardi, 2003; Poliafico, 2007; Chen et al., 2008). Thus thermophilic digestion causes process instability and poor supernatant quality. In addition, another drawback of thermophilic reactors is that these systems require additional energy input for self-heating (El-Mashad et al., 2004). Mesophilic microorganisms are more resistant and tolerate greater changes in environmental conditions. Also, mesophilic reactors do not require additional energy input for self-heating, have lower investment cost and they are easier to operate; thus these types of reactors are more favorable for commercial plants.

Table 2.4. Optimal growth temperatures for some methanogens (Gerardi, 2003).

Temperature range	**Genus**	**Optimal Temperature (ºC)**
Mesophilic	*Methanobacterium*	37-45
	Methanobrevibacter	37-40
	Methanosphaera	35-40
	Methanolobus	35-40
	Methanococcus	35-40
	Methanosarcina	30-40
	Methanocorpusculum	30-40
	Methanoculleus	35-40
	Methanogenium	20-40
	Methanoplanus	30-40
	Methanospirillum	35-40
	Methanococcoides	30-35
	Methanolobus	35-40
	Methanohalophilus	35-45
Thermophilic	*Methanohalobium*	50-55
	Methanosarcina	50-55

2.2.5. Mixing

Mixing is a significant parameter in anaerobic digesters loading with a particulate substrate. There are several benefits of mixing the digester contents:

- Enhances the anaerobic digestion process by distributing substrate and organisms uniformly, transmitting heat and equalizing temperature (Sawyer and Grumbling, 1960; Meynell, 1976; Gerardi, 2003).
- Prevents scum formation.
- Provides homogenity in the digester.
- Minimizes toxicity.
- Helps to reduce particle size as digestion progress.
- Releases biogas from the mixture.
- Prevents aggregation of mixture.

Mixing can be performed by mechanical mixers, biogas recirculation, or by slurry recirculation. A mixing system must be both productive and economically feasible. Mixing speed of the anaerobic reactors can differ from 20 to 100 rpm (Wu et al., 2010). Besides all advantages, mixing at high rpms may cause a decrease in biogas production. Low-speed mixing allows to absorb more substrate in the reactor and to produce much biogas than high-speed mixing (Stroot et al., 2001; Gomez et al., 2006).

2.2.6. Types of inoculums

Several researches have shown that biogas production and the amount of inoculum are interrelated with each other and AD reactors operating without adding inoculum are insufficient to produce methane (Luengo and Alvarez, 1988; Castillo et al., 1995, Forster-Carneiro et al., 2008). By adding inoculum, not only biogas yield increases but also retention time decreases (Kotsyurbenko et al., 1993; Kanwar and Guleri, 1995; Dangaggo et al., 1996). The source, quality and amount of inoculum are significant parameters for AD reactors because they affect

the length of operating time and steady state of reactors (Gerardi, 2003; Forster-Carneiro et al., 2007). Several types of inoculum that are rich in methanogens like sewage sludge, rumen, swine wastewater are used in the mesophilic digester (Budiyona et al., 2009; Mateescu and Constantinescu, 2011). For instance, bovine rumen fluid can be used as inoculum in AD of cattle manure and it increases the efficiency of biogas generation 2 or 3 times in compare to manure substrate without ruminal fluid (Lopes et al., 2004; Budiyono et al., 2009). Sewage sludge also can be used as inoculum in AD of swine manure (Gonzales-Fernandez and Garcia-Encina, 2009). Not only the source of inoculum but also whether inoculum is granular or suspended may affect the quality of inoculum (Neves et al., 2004). For example, Neves et al. (2004) observed that granular sewage sludge is more effective than suspended sewage sludge in methane production. Not only the inoculum characteristics but also substrate characteristics influence AD performance together because methanogens cannot degrade every substrate (Gerardi, 2003).

Manure and crop materials digested in anaerobic digesters contain a high proportion of lignocellulosic contents. Since the structure of lignocellulose is very refractory, its biological conversion is difficult. Gas production is low due to both rigid structure of lignocellulose and slow specific growth rates of microorganisms in bioreactors. Therefore, the biogas yield has been tried to be enhanced by adding rumen microorganisms to bioreactors in recent years (Barnes and Keller, 2003; Dalhoff, 2003; Baba et al., 2013, Wall et al., 2015). Rumen is the first part of the alimentary canal of ruminant animals. Digested foods are exposed to initial microbial fermentation by rumen microorganisms in there. VFA, carbon dioxide, methane, ammonia, and the microbial cells are obtained by hydrolyzing carbohydrates such as cellulose thanks to rumen microorganisms (Alataş and Umurcalılar, 2011). Hu and Yu (2005) demonstrated that biogas yield increases in the range between 55-70% when ruminal fluid is added to bioreactors during the digestion of corn stover. Except for cellulolytic bacteria, methanogens are also found in ruminal fluid and they convert acetate into methane and carbon (Hungate, 1966; Bryant, 1979).

2.2.7. Inoculum to substrate (I/S) ratio

In anaerobic digesters, there are two particles: waste particles having high biodegradability and low methanogenic activity and seed particles having low biodegradability and high

methanogenic activity (Kalyuzhnyi et al., 2000). Preventing VFA accumulation inside the seed particles in a batch digester is the very important point. Acidification can be prevented by increasing amount of inoculum during start-up (Veeken and Hamelers, 1999). Thus, inoculum to substrate ratio is a significant parameter in the assessment of anaerobic batch digesters and the I/S ratio significantly affects the biogas production (Hashimato, 1989; Neves et al., 2004; Raposo et al., 2006; Raposo et al., 2008; Liu et al., 2009b). When a large amount of seed sludge is used in batch digesters, a successful anaerobic digestion process is achieved without pH adjustment (Gunaseelan, 1995). The I/S ratio of 1 (VS basis) is usually preferred for successful digestion (Gunaseelan, 1997). In addition, for a recalcitrant substrate, I/S ratio can increase to 2 g VS/g VS to optimize the biochemical methane potential (BMP) (Chynoweth et al., 1993).

In addition to all of them, if sole cow manure has low methane yield, inoculum can be added to reactors to enhance the biogas yield. Chynoweth et al. (1993) used rumen as inoculum and observed the maximum conversion rates when the inoculum to substrate ratio is 2:1. Raposo et al. (2006) demonstrated that different I/S ratio impacts the amount of methane produced. González-Fernández and García-Encina (2009) showed that methane production rates were clearly different with the three experimental ratios and anaerobic digestion of swine slurry should be carried out at the ratio I/S of around 1, thereby avoiding reactor imbalances due to VFA accumulation. A correct concentration of substrate and inoculum is crucial in the anaerobic digestion because excess organic loading may result in VFA accumulation and biomass inhibition; on the other hand, a limitation of inoculum results in a slow methane generation (Gonzales-Fernandez and Garcia-Encina, 2009).

2.3. Various Substrates Used for Anaerobic Digestion

To meet the growing energy demand, both environmentally friendly and sustainable methods are tried to be enhanced for many years. It is agreed that fossil sources will run out in the not too distant future and greenhouse gas (GHG) emissions damage the world. AD is a better alternative method to prevent the pollution caused by fossil fuels and to maintain food safety, human and animal health. AD does not only recycle animal manure and organic waste but also meets energy demand by generating biogas (Figure 2.4).

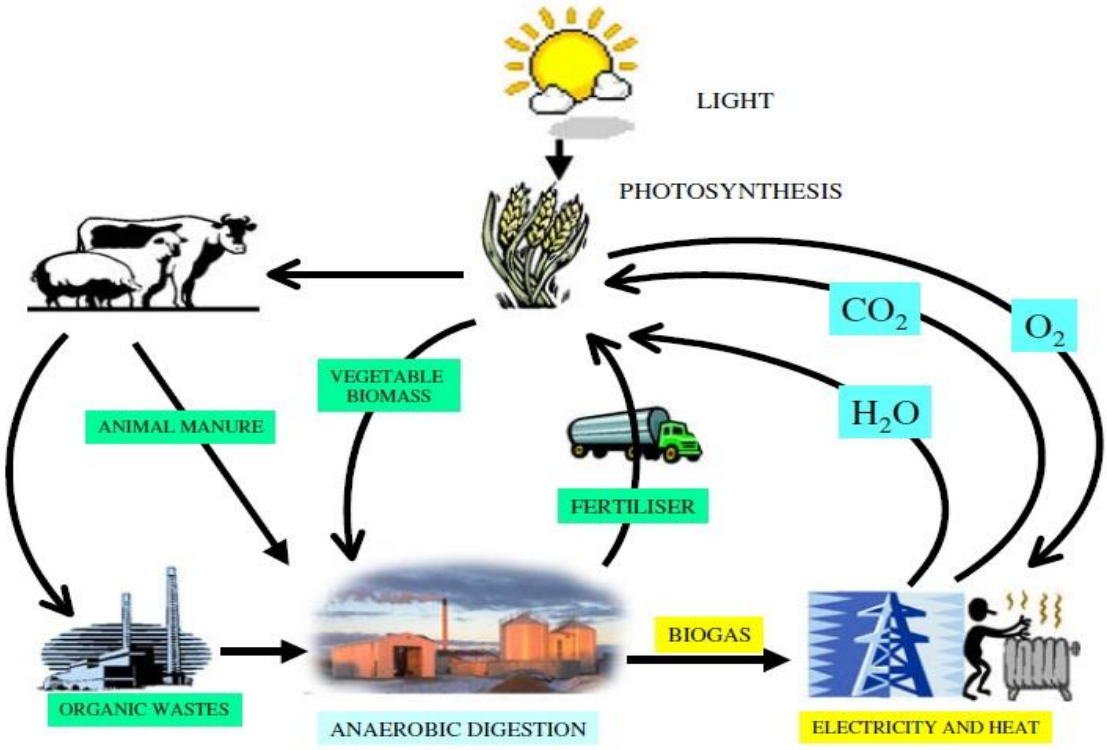

Figure 2.4. General scheme of the sustainable cycle of anaerobic co-digestion process (Al Seadi, 2002).

If animal manure is not handled properly, it may cause air and water pollution. If treated properly, animal manure is both a valuable energy source and a good source of nutrients for agricultural purpose. The slurry obtained after AD is still rich in nutrients and is used as a high quality of fertilizer for plants. So there are lots of outputs of anaerobic digestion, which are biogas, green electricity, heat, vehicle fuel and fertilizer (named as digestate). Utilization of biogas is not only cost effective but environmentally friendly and sustainable (Weiland, 2010).

Until now, several methods have been implemented for the treatment and disposal of manure like composting, land application, and anaerobic treatment. Among these options, anaerobic treatment is the most effective for pollution reduction, energy generation which contributes to the resource conservation and enhancement of the fertilizer value of the manure.

Biogas is produced from so much variety of feedstock type but the largest feedstock for AD is animal manure (cow, pig, poultry, fish, etc.). In Table 2.5, biogas yield of different animal manure is shown. After the manure, energy crops (grain crops, grass crops and maize) are used in common in AD processes (Holm-Nielsen et al., 2009). Especially maize silage is a very popular crop in biogas production systems (Braun et al., 2008).

Table 2.5. Range of biogas yield of different animal manure (Martinez and Burton, 2003).

Substrate	**Range of biogas yield (L/kg VS)**
Sheep manure	100-310
Cattle manure	150-350
Horse manure	200-350
Poultry manure	310-620
Pig manure	340-550

2.3.1. Anaerobic co-digestion of animal manure with energy crops

Low biogas production is achieved by the decomposition of the animal manure alone since carbon content of manure is low. (Ward et al., 2008; Esposito et al., 2012). Therefore, some alternatives have been tried to obtain the higher biogas generation. Among several alternatives, anaerobic co-digestion has become popular today. Various crops are used to enhance the bio-methane potentials of anaerobic digester systems. The most commonly cultivated crops include maize (*Zea mays* L.), herbage (Poaceae), clover grass (Trifolium), Sudan grass (*Sorghum sudanense*), fodder beet (*Beta vulgaris*) and all of them are seen in the literature frequently (Chynoweth et al., 1993; Weiland, 2003; Vindis et al., 2010; Menardo et al., 2015). In table 2.6, it is showed that maize, fodder beet, wheat, triticale, rye have high methane yield. Although barley both has high methane yield (Table 2.6 and Figure 2.5) and is the most widely grown after wheat, maize and rice in Turkey (Akar et al, 2004), it is not studied as an energy crop commonly.

Table 2.6. Methane yields of different crops (Braun, 2007).

Plant	**Methane yield (m^3/VS_{added})**	**Plant**	**Methane yield (m^3/VS_{added})**
Maize (whole crop)	205-450	Barley	353-658
Wheat (grain)	384-426	Triticale	337-565
Oats (grain)	250-295	Sorghum	295-372
Rye (grain)	283-492	Alfalfa	340-500
Grass	298-467	Sudan grass	213-303
Sugar beet	236-381	Straw	242-324
Fodder beet	420-500	Rhubarb	320-490

Anaerobic co-digestion of animal manure with various crops and residues has gained a wide application area in full-scale biogas plants. Anaerobic co-digestion of different organic materials is significant to achieve required C/N ratio (Mshandete et al., 2004; El-Mashad and Zhang, 2010), to improve nutrient balance, to decrease toxic effects of compounds (ammonia, sulfides, heavy metals, light metal ions, benzenes, phenols, alcohols, etc.) by dilution, to improve pH and moisture content as well as to increase buffer capacity and biodegradable parts (Mata-Alvarez et al., 2000; Kelleher et al., 2002; Lehtomäki et al., 2007; Chen et al., 2008; Li et al., 2009; Esposito et al., 2012). Several researches show that co-digestion improves gas production (Kugelman and Chin, 1971; Mshandete et al., 2004; Kaparaju and Rintala, 2005; Gelegenis et al., 2007; Alvarez and Lidén, 2008; Macias-Corral et al., 2008; Akyol et al., 2016).

Since animal manure is a protein-rich material, it includes high amount of nitrogen. Carbon to nitrogen ratio of animal manure is low. During the digestion, excessive ammonia production due to the high nitrogen content of manure may inhibit the digestion system. That's why, co-digestion process of animal manure prevents these problems and provides higher methane yield (Lehtomäki et al, 2007). Co-digestion may also reduce the inhibitory effect of ammonia and provide more stable biogas generation because it improves buffer capacity of AD (Nayono et al., 2010). A wide range of methane yields from anaerobic co-digestion of cow manure with different co-substrates is reported in the literature (Table 2.7).

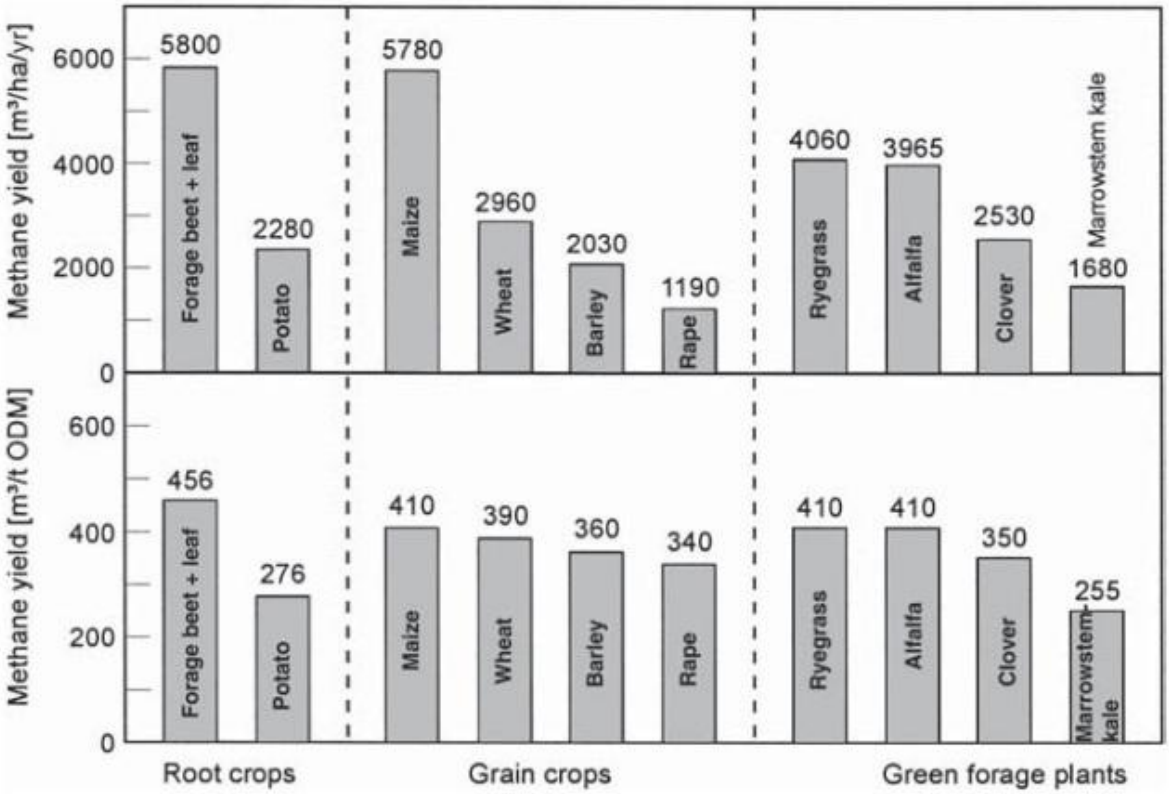

Figure 2.5. Methane yield of different crops (Weiland, 2003).

Many biogas plants use co-substrates in addition to pig, cow and poultry manure to obtain higher biogas yield. Plants producing biogas from manure as the only substrate is 7% (Weiland, 2003). Even though co-digestion has many advantages, there are several disadvantages as well (Callaghan et al., 1997; Misi and Forster, 2001; Mshandete et al., 2004). The co-digestion process requires extra equipment and investment costs. Besides, composition and energy potential of co-substrate, toxic substance content and legislative restrictions should be taken into consideration (Mata-Alvarez et al., 2000).

Table 2.7. Bio-methane potential of co-digestion of cow manure.

Co-substrates	Ratio	Operational conditions	CH_4 yield (Nm^3/ton VS)	References
Cow manure: barley straw	80:20 (on volume)	V=100 L T=35°C	160	Hills, 1980
Cow manure: wheat straw	50:50 (on VS content)	V=0.3 L T=35°C	70	Hashimato, 1983
	25:75 (on VS content)		30	
	10:90 (on VS content)		100	
Cow manure: forage beet silage	83:17 (on VS content)	V=20 L T= 35°C	400	Weiland and Hassan, 2001
Cow manure: fruit and vegetable waste	80:20 (on weight)	V=18 L T= 35±0.5°C	380	Callaghan et al., 2002
	70:30 (on weight)		340	
	60:40 (on weight)		380	
	50:50 (on weight)		450	
Cow manure: grass silage	90:10 (on VS content)	V=1.5 L T= 35±1°C	143±16	Lehtomäki et al., 2007
	80:20 (on VS content)		178±9	
	70:30 (on VS content)		268±29	
	60:40 (on VS content)		250±16	
Cow manure: straw	90:10 (on VS content)	V=1.5 L T= 35±1°C	145±9	Lehtomäki et al., 2007
	80:20 (on VS content)		159±19	
	70:30 (on VS content)		213±17	
	60:40 (on VS content)		188±19	

2.4. Molecular Methods Used in Microbial Ecology of Anaerobic Digesters

Nowadays, while much is known about the basic metabolism in anaerobic digesters, there is little information about the microorganisms responsible for the process in anaerobic reactors (Hofman-Bang et al., 2003).

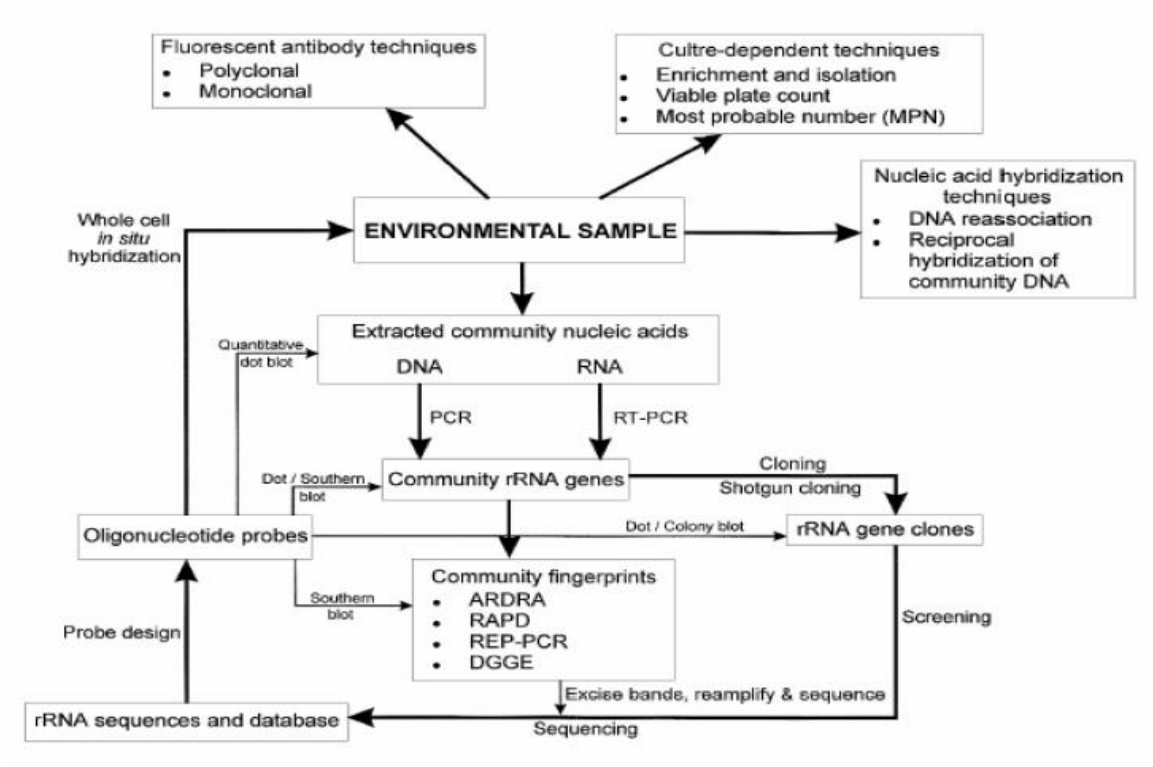

Figure 2.6. Summary of common molecular approaches used in microbial ecology (Theron and Cloete, 2000).

Identification and quantification of microorganisms by the means of molecular methods are needed to better understand of microbial changes in bioreactors. The science of microbial ecology helps us to better understand how microbes interact with each other and with their environment. Microbial activity and biodiversity can be understood with the help of microbial ecology methods. Cultivation is the most common method for identification of environmental microorganisms in the earlier of classical microbiology. The main limitations of this method are insufficient in identification of the community and time-consumption. Culture-dependent

methods can culture only a microbial community but cannot detect whole microbes in the system. On the other hand, since some groups of microorganism are difficult to grow, they cannot be identified by cultivation (Schmidt et al., 2000; Zhou et al., 2011). The cultivable microorganisms constitute 0.1-10% of all microorganisms in the world (Muyzer et al., 1993; Amann, et al., 1995; Hugenholtz et al., 1999).

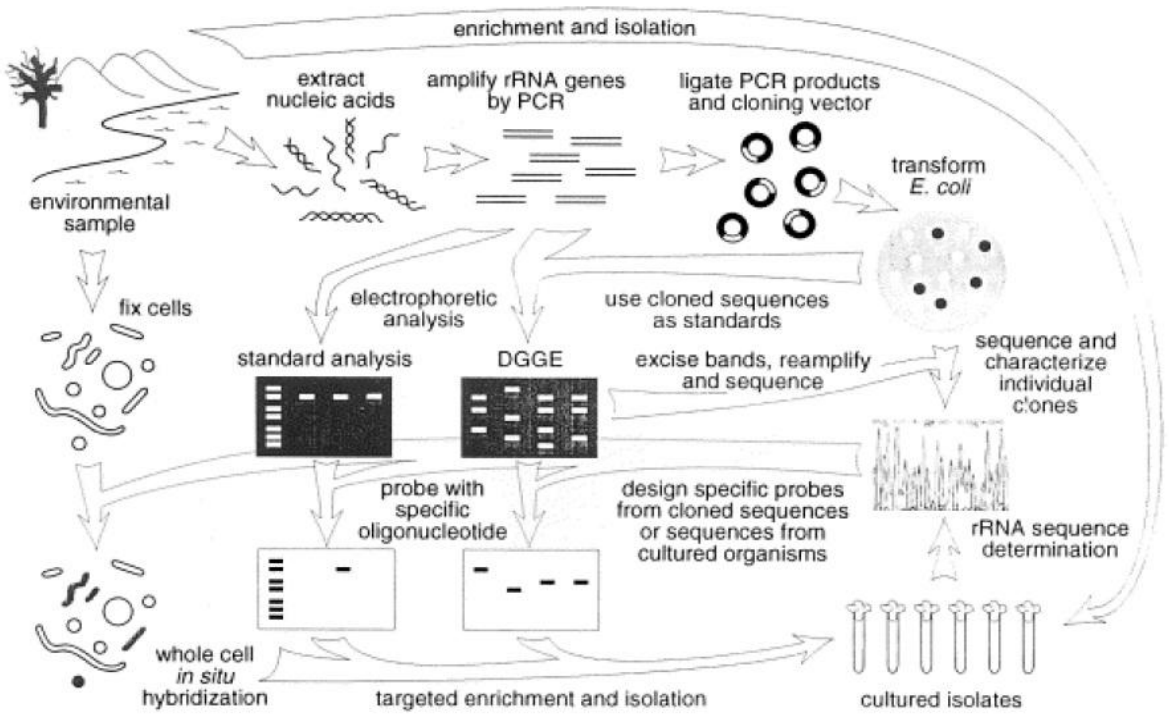

Figure 2.7. Summary of phylogenetic methodologies used in microbial ecology (Scow et al., 2004).

The non-culture based methods focus on two main points: the first one is isolation of the genetic materials and analysis of these samples to specify microorganisms and the second one is using nucleic acid based stains to visualize, count and identify microbes. Therefore, the culture-independent methods are becoming more popular in the determination of microbial communities. There are several culture-independent methods used in molecular ecology, schemes of them are shown in Figure 2.6 and 2.7, respectively.

Molecular biology methods have provided a better understanding of interactions and dynamics within microbial consortia (Zhou et al., 2011). The direct identification and quantification of microbes are possible today by the help of molecular phylogeny (Olsen and Woese, 1993). In molecular ecology, ribosomal DNA (rDNA) and ribosomal RNA (rRNA) have been the most commonly used molecular units to date.

2.4.1. Fluorescence in situ hybridization (FISH)

In Situ Hybridization (ISH) is a technique which shows specific DNA and RNA sequences morphologically in every cell, in tissue sections, in chromosome preparations or in interphase nuclei and it allows examination of nucleic acids in their natural cellular environment.

Fluorescence in situ hybridization (FISH) method is an ISH technique based on labeling the target with fluorescently labeled probes and visualizing it in the fluorescent microscope. With FISH analysis, diagnosis of the microorganisms in a short time without cultivation is achieved. There are several benefits of this method such as having high sensitivity and specificity, being inexpensive, practical and easy. It also allows identifying species in a very short time (approximately in 2-2.5 hours) (Amann, 1995; Kemph et al., 2000). It is used for the rapid identification of microorganism at the family, genus and species level and in the phylogenetic analysis of microorganisms (Amann et al., 1995; Amann et al., 1997; Zwirglmaier, 2005). FISH method allows the phylogenetic identification of microorganisms in mixed media by using probs targeting rRNA without using cultural methods. In the identification, epifluorescence microscope, confocal laser scanning microscopy or flow cytometry are used.

2.4.2. Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) method bases on the movement of the different nucleic acid sequences in the same size on the gel. That is, different rRNA sequences begin to melt in private different denaturing area on electrophoresis. The band patterns reflecting genetic diversity directly in the sample are produced. The number of bands refers to the number of the dominant species in the sample (Nicomrat et al., 2006).

DGGE consists of five basic steps:

1. DNA extraction from sample;
2. Replication of the rDNA region by Polymerase Chain Reaction (PCR);
3. The separation of PCR products by Denaturing Gradient Gel Electrophoresis (DGGE),
4. Sequence Analysis
5. Homology scanning with sequences in the gene bank of specified sequences

One of the most important advantages of DGGE is that more than one sample can be run on the same gel; thus it allows the monitoring of population changes from different environmental conditions. Furthermore, it is not an expensive, laborious and time-consuming technique like the cloning and sequence analysis (Çallı et al., 2006). DGGE is an effective method to monitor the single base changes in DNA amplified by PCR and polymorphism. It can give a good result on microbial communities in samples with the phylogenetic analysis of bands and combining sequences. Even though sequence analysis is required to identify unknown species exactly, it is a practical separation method that can directly indicate microbial diversity and shifts in population in a microbial composition.

DGGE method also allows the comparison of microbial communities with high and low activity. Unlike the cloning, DGGE is preferred when detailed information is not required. But it is still sufficient to determine the dominant species in the microbial community. This method is usually used with some methods such as in situ hybridization. The most significant application area of DGGE is monitoring the dynamic changes in the microbial community if working with more than one sample. Sometimes genomic DNA extraction and amplification can be difficult depending upon the natural structure of the sample. Also primer selection, optimization of gel conditions and in case of occurring many different bands on the gel, difficulty of comparison of these bands are important limiting factors (Hayes et al., 1999). Another limiting feature of this method is DNA fragments of different species sometimes move together on the gel. Because two different species were denatured at the same point, sensitivity of species detection may be limited (Vallaeys et al., 1997).

2.4.3. Quantitative real time polymerase chain reaction (Q-PCR)

Quantitative Real-Time PCR can be used to detect even low-level populations in samples (Hofman-Bang et al., 2003). Real-time quantitative PCR is a PCR method that could give quantitative results in a short time by measuring the fluorescent signals that increased simultaneously with the nucleic acid replication. There are three types developed commercially, which are LightCycler (Roche), TaqMan (PE Biosystem) and iCycler (BIO-RAD).

Table 2.8. Advantages and disadvantages of Q-PCR.

Advantages of Q-PCR	Disadvantages of Q-PCR
• Produces results simultaneously. • Gets faster results. • There is little risk of contamination. • Both quantitative and qualitative results can be taken. • Preventing a non-specific binding. • No need for a second technique to get results.	• The material used is expensive. • Only the recognition of known mutations. • Need to highly developed system to create the protocol.

This method makes DNA amplification and determination of DNA products in a single tube possible. Fluorescence-labeled probes and dye which can make PCR amplification visible are used in this technique. The intensity of the fluorescence signal is proportional to the amount of DNA in the sample (Bassler, 1995). This technology is also called by several names such as “kinetic PCR”, homogeneous PCR” and “Real-Time PCR”. Temperature cycles and fluorescence reading are performed within the same device and in the same tube. Thus, the target region can be determined in a short time without electrophoresis. Since both replication and identification of amplified products can be made in the same device, this method has become a very practical method. In addition, the risk of contamination is decreasing because the test is

completed without opening test tubes. In the Real-Time PCR, the determination of the product after amplification can be performed in various ways. The first is the use of double-stranded DNA dye, a non-specific method. The most commonly used dye is SYBR Green I. Since the fluorescent dye used in this method attaches only double-stranded DNA, the amount of reading fluorescence in the Real-Time PCR instrument increases proportional to the growing amount of DNA (Heid et al., 1996; Grove 1999; Kubista et al., 2006). At the beginning of the amplification, double-stranded DNA molecule, primers and "SYBR Green I" dye are found in the reaction mixture. Free DNA molecules make very little fluorescence signal. When primers begin to connect and elongate, dye molecules enter between double-stranded DNA and the fluorescence emissions begin. During the initial cycle, the signal is weak; as the amount of product increases, the amount of fluorescence increases rapidly and this increase can be viewed from the device monitor. However, there are some drawbacks of SYBR Green I method. Since the fluorescent light can be seen with the proliferation of unwanted PCR products, it does not always indicate the increase of DNA we want and thus, it is possible to get a false-positive result. Fluorescence radiation can be observed as a result of binding primers with each other (so-called as "primer dimer") and with the formation of the double-stranded DNA region in the absence of target DNA sequence in the medium. To determine if the amplified DNA is the desired target site, it is required to analyze melting curve of DNAs. If the region desired to be reproduced of DNA fragments is a specific region, fluorescently labeled probes are used for the detection of this region. TaqMan Probe Method is then can be a good alternative. In the TaqMan system, probes labeled with fluorochromes in the 5’ and 3’ ends are utilized. The TaqMan probe method contains a fluorescently labeled single-stranded probe and this probe is complementary to DNA wanted to replicate. 3’end suppressor fluorochrome (TAMRA) dye prevents the creation of dye signal of 5’end reporter fluorochrome (FAM) dye. The fluorescent signal measurement is low even in case probe binds to the target DNA. During the duplication, primers at the target nucleic acid sequences bind to “Taq-Man” probes between the binding sites. After the binding of primers, new chains form. When it is reached to the point that the probe is connected, Taq DNA polymerase enzyme separates reporter fluorochrome (FAM) from a probe by the help of 5’-3’ nuclease activity. Free FAM then signals. In each cycle, as amplicons increase, fluorescence signals keep increasing (Holland et al., 1991; Livak et al., 1995).

2.4.4. Next generation sequencing (NGS)

Unlike previous techniques, in the next generation sequencing, many parallel sequencing reactions are performed at the same time and therefore high volume and quicker results are obtained. It is significantly faster, cheaper and more reliable than other methods. More accurate analysis can be performed with less DNA. In addition to being a high-volume analysis method, it is easy to trace a DNA fragment belongs to a which sample by a barcode method, and in this process, several samples are run together in the same reaction, and the cost per sample reduces.

First, the data is formed by separating the DNA to be sequenced into pieces, then adapter arrays and barcode arrays are added to the ends of these segments. The other chain is synthesized by adding labeled bases to the single chain DNA fragments attached to the solid surface by the adapter arrays. Due to the change in light, pH or ion balance arisen from the addition of each new base, it is determined which base is added by chemical and photo sensors. When the reaction is over, complex bioinformatics analyzes are performed on the computer. At the same time, whether there is any base change is checked with the reference sequences. Thus, the diagnostic reliability and accuracy of the method is increased. Unlike other methods, it is a method that can give both qualitative and quantitative results. Thank to next generation sequencing methods, millions of DNA fragments in the entire genome, transcriptome or smaller target regions can be sequenced in a study.

Today, next generation sequencing includes several modern sequencing technologies; some of which are Illumina (Solexa) sequencing, Roche 454 sequencing, Ion torrent: Proton / PGM sequencing, and SOLiD sequencing. Ion PGM sequencing method used in this study is different from other technologies. Unlike Illumina and 454 sequencing, it does not use optical signals. This method of sequencing is based on the detection of hydrogen ions that are released during the formation of DNA. A micro-well containing a template DNA strand to be sequenced is filled with a single nucleotide type. If the inserted nucleotide can be included to extended chain, this leads to the release of a hydrogen ion, which stimulates a very sensitive ion sensor. If there are several nucleotides in the template sequence, the nucleotide sequence will be joined in one cycle. If there are few same nucleotides consecutively in the template sequence, multiple

nucleotide will be joined to the chain in a cycle. In this case, a larger number of hydrogen ions will be released and a proportionally higher electronic signal will be obtained.

3. AIM OF THE STUDY

To meet increasing energy demand, biogas production from animal manure which is environment-friendly method is an emerging application. However, since biogas yield of sole manure is relatively low due to its low carbon content, anaerobic co-digestion of animal manure with various crops is a better alternative to increase biogas yield. Moreover, several environmental and operational parameters influence biogas production. For this reason, it is important to determine the parameters that the digesters are operated most efficiently in order to meet the energy demand sufficiently. From this point of view, the aim of the thesis is to investigate effects of operational parameters including temperature (mesophilic vs. thermophilic), inoculum to substrate ratios (I/S) and inoculum types and microbial communities on methane yield in anaerobic digesters fed with cow manure and barley.

4. MATERIALS & METHODS

4.1. Sampling and Characteristics of Manure, Rumen Fluid, Barley and Seed Sludge

Fresh cow manure (CM) and cow ruminal fluid (CRF) were supplied from Veterinary Faculty of Istanbul University, Istanbul. Cow rumen fluid was taken with permission of the Animal Ethics Committee of Veterinary Faculty of Istanbul University. The cow rumen fluid was flushed with N_2 to provide anaerobic conditions after loading and sealing. It was used in the reactor set up on the same day. Barley (*Hordeum vulgare, HV*) was supplied from Faculty of Agriculture, Uludağ University and were chopped in small pieces before being added to the reactors. Seed sludge (SS) was obtained from a full-scale cow manure digester operated at mesophilic condition in Bursa. All samples were stored in containers at +4°C prior to use. Characteristics of these materials are shown in Table 4.1.

Table 4.1. Characteristics of the substrates and the inocula.

Parameter	Cow manure	Barley	Seed Sludge	Cow rumen fluid
pH	7.65 ± 0.04	5.75 ± 0.03	7.77 ± 0.02	6.81 ± 0.02
TS (g TS/g fresh sample, %)	15.1 ± 0.05	88.6 ± 0.26	4.15 ± 0.057	1.8 ± 0.016
VS (g VS/g fresh sample, %)	13.1 ± 0.08	78.7 ± 2.94	2.84 ± 0.03	1.44 ± 0.03
sCOD (mg/L)	13250 ± 315	5875 ± 72	3500 ± 331	9417 ± 289
Alkalinity (mg $CaCO_3$/L)	4750	4500	12500	5000
TKN (mg/kg)	1355 ± 103.5	1429 ± 152.1	1400 ± 98.7	51 ± 1.8

4.2. Experimental Set-ups

Digestion experiments were carried out in 1L digesters with an active volume of 750 ml. Four different sets were operated as summarized in Table 4.2. Different sets of batch digesters were performed in triplicate and continuously stirred at 100 rpm with pH=7.0±0.2 in an

incubator shaker. pH of digesters was adjusted by using 1M HCl or KOH. 3000 mg $NaCO_3$/L alkalinity were added to each reactor. Digesters were loaded with predetermined mixing ratio (Cow manure: barley, 1:1 g VS, (Akyol et al., 2016)). Tap water was used to achieve a working volume of 750 mL. After loading and sealing of the batch reactors, nitrogen gas (N_2) were flushed into the reactors for 5 minutes in order to maintain anaerobic conditions. Each reactor was connected to a milligas counters (MGC-1, Ritter Bochum, Germany) and recorded cumulative biogas production. All sets were carried out for 40 days. Samples were taken from the digesters for pH, VFA and molecular analysis.

Table 4.2. Operating conditions in batch reactors.

	Digestion components								
Experimental sets	Cow manure:barley mixing ratio (g VS)	Stirring speed (rpm)	Temperature		pH	Inoculum/substrate ratio (g VS)		Rumen fluid	Seed Sludge
			Mesophilic (37°C)	Thermophilic (55°C)		1:2	1:1		
Set 1 (CR)	1:1	100	+		7±0.2	+			+
Set 2	1:1	100	+		7±0.2		+		+
Set 3	1:1	100		+	7±0.2	+			+
Set 4	1:1	100	+		7±0.2	+		+	+

Operating conditions and loadings of digesters are summarized in Table 4.2 and 4.3, respectively. In the Set 1 (control), digesters were loaded with 27.5 g VS substrate (as 13.75 g VS cow manure and 13.75 g VS barley) and 13.75 g VS seed sludge as an I:S ratio of 1:2. After pH were adjusted to 7.0±0.2, digesters were operated at 37±1^oC and continuously stirred at 100 rpm in temperature controlled incubating shaker. Digesters were performed in triplicates. In the Set 2, digesters were loaded with 13.75 g VS substrate (as 6.875 g VS cow manure and 6.875 g VS barley) and 13.75 g VS seed sludge as an I:S ratio of 1:1. After pH were adjusted to 7.0±0.2, digesters were operated at 37±1^oC and continuously stirred at 100 rpm in temperature controlled incubating shaker. Digesters were performed in triplicates. In the Set 3, digesters were loaded with 27.5 g VS substrate (as 13.75 g VS cow manure and 13.75 g VS barley) and 13.75 g VS seed sludge as an I:S ratio of 1:2. After pH were adjusted to 7.0±0.2, digesters were operated at

55±1°C and continuously stirred at 100 rpm in temperature controlled incubating shaker. Temperature was increased by 3°C every day from 37°C to 55°C to acclimate microorganisms. Digesters were performed in triplicates. In the Set 4, digesters were loaded with 27.5 g VS substrate (as 13.75 g VS cow manure and 13.75 g VS barley) and 13.75 g VS seed sludge and cow rumen fluid as an I:S ratio of 1:2. In this set, seed sludge and cow rumen fluid were mixed with a ratio of 1:4, respectively. Thus, 11 g VS seed sludge and 2.75 g VS cow rumen fluid were added to the digesters as inocula. That is, reactors contained 25% (v/v) ruminal fluid in this set. After pH were adjusted to 7.0±0.2, digesters were operated at 37±1°C and continuously stirred at 100 rpm in temperature controlled incubating shaker. Digesters were performed in triplicates.

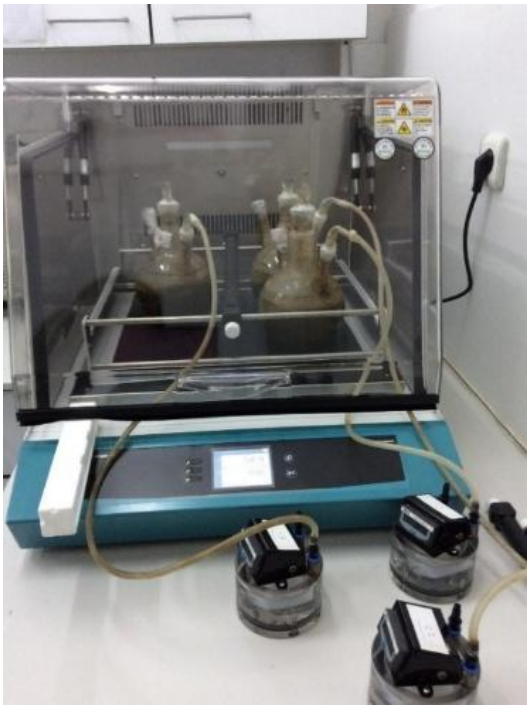

Figure 4.1. The anaerobic batch digesters in the incubator and Milli Gas Counters used in this study.

Three different blank reactors were set up, namely Blank 1 (B1), Blank 3 (B3), and Blank 4 (B4) and only inoculums were added to the blank reactors. In the B1, a digester was loaded with 13.75g VS seed sludge and active volume was finalized with tap water to 750 ml. The digester was operated at 37±1°C and continuously stirred at 100 rpm in temperature controlled incubating shaker. The amount of biogas produced in the B1 was subtracted from the amount of

biogas produced in Set 1 and Set 2. In the B3, a digester was loaded with 13.75g VS seed sludge and active volume was finalized with tap water to 750 ml. The digester was operated at 55±1^{o}C and continuously stirred at 100 rpm in temperature controlled incubating shaker. The amount of biogas produced in the B3 was subtracted from the amount of biogas produced in Set 3. In the B4, seed sludge and cow rumen fluid were mixed with a ratio of 1:4, respectively. A digester was loaded with 11 g VS seed sludge and 2.75 g VS cow rumen fluid and active volume was finalized with tap water to 750 ml. The digester was operated at 37±1^{o}C and continuously stirred at 100 rpm in temperature controlled incubating shaker. The amount of biogas produced in the B4 was subtracted from the amount of biogas produced in Set 4.

Table 4.3. Digester loadings.

Digesters	**Loadings**	**TS, %**	**VS, %**
Set 1 (CR)	13.75 g VS CM + 13.75 g VS HV + 13.75 g VS SS	7	5.5
Set 2	6.875 g VS CM + 6.875 g VS HV + 13,75 g VS SS	4.8	3.7
Set 3	13.75 g VS CM + 13.75 g VS HV + 13.75 g VS SS	7	5.5
Set 4	13.75 g VS CM + 13.75 g VS HV +11 g VS SS + 2.75 g VS CRF	7	5.5
Blank 1	13.75g VS SS	2.7	1.8
Blank 3	13.75g VS SS	2.7	1.8
Blank 4	11 g VS SS + 2.75 g VS CRF	2.7	1.8

4.3. Analytical Measurements

To determine characteristics of the substrates and the inocula, total solids (TS), volatile solids (VS), alkalinity, soluble COD (sCOD) were measured according to the Standard Methods

for the Examination of Water and Wastewaters (APHA, 2005). Total Kjeldahl Nitrogen (TKN) was measured colorimetrically using Nessler Method.

On days 0, 10, 20, 30 40, samples were taken from the digesters for analytical and molecular analyses. The gas generated from the digesters was measured by Milli Gas Counters and noted every day cumulatively. Gas compositions were measured on every 10 days using HP Agilent 6850 Gas Chromatograph (GC) with a thermal conductivity detector (HP Plot Q column 30 m x 0.53 mm). As a carrier gas, helium was used at a range of 2 ml/min. The oven temperature was 70^{o}C during the measurements. Air tight syringe (2.5 ml) was used to collect the sample accumulated in the headspace of the digesters. 0.5 ml of gas was injected to GC for the analysis. It was used to measure the percentage of the methane produced. pH was measured and adjusted using HANNA HI 221 Microprocessor pH meter. VFA concentrations were measured by Perkin Elmer Clarus 600 Gas Chromatograph equipped with a flame ionization detector. The column used was Elite FFAP (30 m, 0.32 mm). The set point of the oven and maximum temperature of inlet are 100^{o}C and 240^{o}C, respectively. Helium gas was used as a carrier gas at a rate of 0.8 ml/min.

4.4. Molecular Techniques

4.4.1. DNA extraction

DNA extraction of samples was done with Fast DNA SPIN Kit for Soil (MP Biomedicals, Germany). Firstly, samples were thawed and mixed by vortex. Approximately 500 µL sample was added up to lysing matrix tubes provided by the kit. The tubes contain mixture of ceramic and silica particles to lyse all microorganisms in sample. 978 µL sodium phosphate and 122 µL MT buffer solution were added to samples in lysing matrix tubes. All tubes were shaken vigorously and then the lysing matrix tubes were spun in Ribolyser (Fast Prep TM FP120 Bio 101 Thermo Electron Corporation) for 45 seconds at speed of 6.5 m/s. The tubes were centrifuged at 14000 x g for 5 minutes. After centrifugation, supernatants were transferred to clean 2 mL microcentrifuge tubes and added 250 µL PPS (Protein Precipitation Solution) reagent. The tubes were shaken by hand 10 times to mix composition. After mixing, the tubes

were centrifuged at 14000 x g for 5 minutes for precipitation of pellets. Supernatants were transferred to 15 mL conical tubes and 1 mL of binding matrix suspension was added to the supernatants. The tubes were inverted for 3 minutes to allow binding of DNA to matrix. To settle the silica, matrix tubes were incubated at room temperature for 3 minutes. 500 µL of supernatant was removed carefully without disturbing settled silica matrix. Then the remaining supernatants were mixed by pipetting up and transferred to spin filter tubes. All mixtures were filtered by centrifugation at 14000 x g for 2 minute. After centrifugation, all tubes were emptied and this step was repeated until samples in 15 mL conical tubes were depleted. Filters were washed by 500 µL SEWS-M by pipetting up. After washing, filters were dried by centrifugation at 14000 x g for 1 minutes. The tubes were emptied and centrifuged again for 2 minutes. Spin filters were transferred to a clean 2 mL catch tubes. The tubes were dried for 10 minutes at a room temperature. 100 µL DES (DNase/Pyrogen free water) was added to the spin filters in the catch tubes and waited for 3 minutes while the caps were closed. Then the filters with DES were centrifuged at 14000 x g for 2 minutes to elute the DNA into the catch tubes. Spin filters were removed. Finally, DNA was ready for application. Extracted DNA sample was stored at -20°C for use when needed.

4.4.2. Next-generation sequencing

In this study, 16S universal Eubacterial primers Bac515F and Bac806R were used. For the archaeal study, Arc349F and Arc806R primer sets were used. These primers sequences used in the PCR amplifications can be seen in Table 4.4. In the first step, single-step 30-cycle PCR was performed separately for each sample by using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) (94C-3dk / 28 x 94C-30sn / 53C-40s / 72C-1dk).

All amplicons obtained after PCR were diluted to be at the same concentration and purified using Agencourt Ampure beads kit (Agencourt Bioscience Corporation, MA, USA). The samples were then sequenced using the Ion PGM ™ platform and chemicals, following the manufacturer's protocols. The Q25 sequence data obtained after the sequencing was analyzed by the determined workflow. In the sequence data, short (<200bp) sequences without barcodes

were removed from the data. Similarly, the data having incoherent base readings and including high homopolymeric region (> 6 bp) were removed from the analysis process.

Table 4.4. Used primer sequences for PCR amplifications in NGS assays.

Primer Type	Target Molecular Unit	Sequences
Bacterial	Bac515F	GTGCCAGCMGCCGCGGTAA
	Bac806r	GGACTACVSGGGTATCTAAT
Archaeal	Arc349f	GYGCASCAGKCGMGAAW
	Arc806r	GGACTACVSGGGTATCTAAT

4.4.3. Quantitative real time PCR

Roche LightCycler DNA Master SYBR Green I kit (5 µl master mix, 0.5 µl Primer F and R, 3 µl H_2O, 1 µl sample) and Roche Light Cycler 2.0 (Roche Diagnostics GmbH, Mannheim, Germany) were used for the Q-PCR analyses. Q-PCR assays were performed using specific primers. The primers used in this study are in Table 4.5. Light Cycler Software 4.05 program provided by Roche was used to analyze Q-PCR results. The program was performed under the following cycle conditions; denaturation at 95°C for 10 min followed by 40 cycles at 95°C for 1 min, 53 - 60°C (see annealing temperature in Table 4.4) for 1 min and 72°C for 1 min.

Table 4.5. 16S rDNA specific primers used for PCR amplifications in Q-PCR analysis

Target microorganism	Target gene	Target Molecular Unit	Sequence of the primer (5'-3')	Annealing (°C)	Reference
Bacteria	16S rDNA	Bac519f	CAGCMGCCGCGGTAANWC	53	Lane, 1991
		Bac907r	CCGTCAATTCMTTTRAGTAT		
Archaea	16S rDNA	Arc349f	GYGCASCAGKCGMGAAW	60	Takai and Horikoshi, 2000
		Arc806r	GGACTACVSGGGTATCTAAT		

4.5. Statistical Analysis

Stata/SE 12.0 statistical program were used in order to carry out statistical and canonical analysis. First, the structure of data was examined and it was determined whether the present data meets the preconditions. It was observed that the variables were normally distributed by using the Shapiro-Wilk test. At the same time, the histogram graphs were depicted. There were no outliers in the data set and there was no complete correlation between the variables. According to the results of independent-samples t-test, the difference between the means of the different sets is statistically significant. Wilks' Lambda significance test of all canonical correlations were performed. P value was obtained at 95% significance level.

5. RESULTS AND DISCUSSION

In this study, four different anaerobic digesters operated under different operating conditions in order to understand the impacts of parameters on biogas and methane production. Thus, anaerobic digesters fed with cow manure and barley were operated under different temperature (mesophilic vs. thermophilic), different inoculum to substrate (I:S) ratios and different types of inocula. Digestion stability was controlled with measurement of pH and VFA content. Biogas and methane generation was measured to evaluate the performance of anaerobic digesters. Finally, microbial community dynamics of anaerobic digesters were analyzed by the help of Next Generation Sequencing (Ion PGM^{TM}) and Q-PCR methods.

5.1. Performance of Batch Digesters

5.1.1. Digestion stability

The VFA production and pH are significant indicators of anaerobic digestion process since changes in VFA and pH during the process indicate the changing conditions (Song and Zhang, 2015).

Initial pH of all digesters was set to 7±0.2 because the most optimal pH for methanogens is between 7.0 and 7.2. pH to less than 5 can be fatal for methanogenic microorganisms and increasing pH to higher than 8 leads to failure of acidogenesis phase (Lusk, 1999). pH level was in the range of 7.1 - 7.4 for Set 1, 7.1 - 7.5 for Set 2, 7.2 - 7.9 for Set 3 and 7.1 - 7.7 for Set 4, respectively. There was no drop below to 7.0 and rise above 7.9 in pH values of all reactors during the digestion. The initial and final pH values of all reactors can be seen in Table 5.1. At the beginning of the work, 3000 mg $NaHCO_3$/L were added to all reactors and it provided the buffer capacity to all digesters because although much VFAs were generated especially in Set 3 and Set 4, no significant decrease in pH was observed.

Table 5.1. Initial and final pH of reactors.

Reactor	Initial pH	Final pH
Set 1	7.11 ± 0.07	7.24 ± 0.06
Set 2	7.10 ± 0.02	7.25 ± 0.07
Set 3	7.15 ± 0.02	7.27 ± 0.05
Set 4	7.10 ± 0.04	7.25 ± 0.03

VFAs were measured on days 0, 10, 20, 30 and 40 of the anaerobic digestion. While major VFAs found in the digester are acetic and propionic acid; isobutyric, butyric, isovaleric, valeric, isocapric, caproic and heptonic acid are the minor VFAs found in the digester. In Figure 5.1, the total VFA concentrations as equivalent of acetic acid in all sets during 40 days are depicted. First ten days, rising in total VFA concentrations was observed. Total VFA was produced most in thermophilic reactor (Set 3) as 6720 mg acetic acid /L. Set 4 (including rumen fluid) had the second most VFA production (4496 mg acetic acid/L). The amount of total VFA was reached up to 1704 and 1351 mg acetic acid/L in Set 2 and Set 1, respectively. After 10th day, total VFA concentrations decreased substantially in all sets. It can be clearly seen that VFAs were consumed efficiently and VFAs accumulation was not detected.

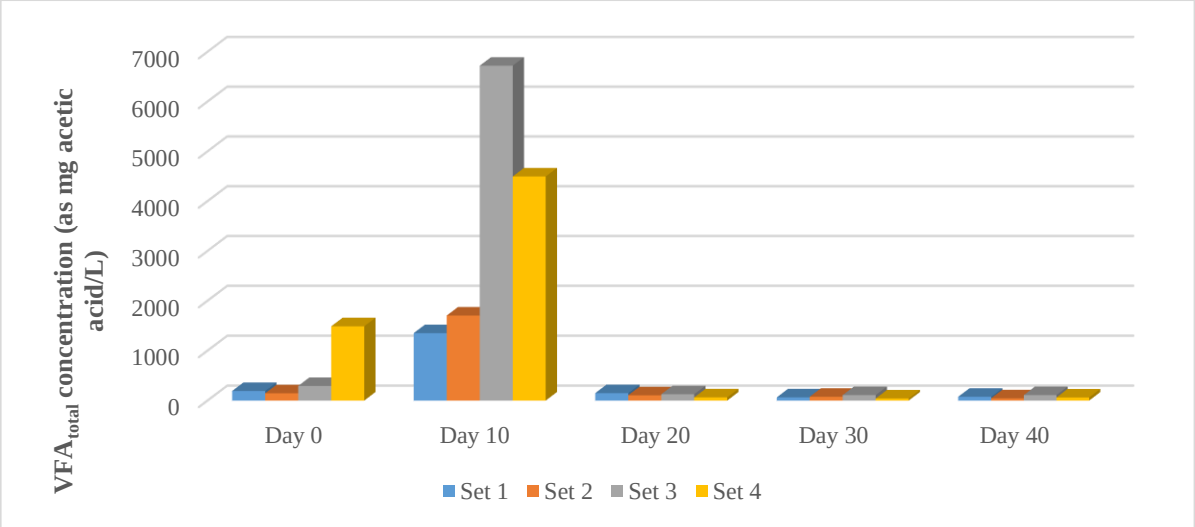

Figure 5.1. Total VFA concentrations of reactors during anaerobic batch digestion.

Also, the amount of VFA species of four sets in different sampling time can be seen in the Figure 5.2. Acetic and propionic acids were measured in major amounts in the all reactors; on the other hand, isobutyric, butyric, isovaleric and valeric acids were detected in minor amounts. In the thermophilic reactor (Set 3), the increase of the propionic acid was reached up to 4000 mg/L on 10^{th} day, but most of it was consumed on day 20. Likewise, propionic acid concentration detected up to 2500 mg/L on day 10 in Set 4, greater part of it was consumed in ten days. The amount of propionic acid was reached up to 700 and 1100 mg/L in Set 1 and Set 2, respectively on day 10 as well, and it showed a rapid decline on day 20. As can be seen, we can say that there is no VFA accumulation at the end of the process. During the start-up phase, Set 4 had the higher amount of acetic, propionic and butyric acid in a considerable amount when compared the other sets. This may be due to the presence of the ruminal fluid in the Set 4.

Wang et al (2009) observed that acetic acid concentrations up to 2400 mg/L did not cause a failure in the digester system. For this reason, we can easily say that there is no acetic acid accumulation in the system. Several studies in the literature have suggested that acetic acid concentration is a more important parameter for digester stabilization (Boe, 2006), while others argue that propionic acid is a more significant parameter (Lyberatos and Skyatas, 1999; Roy et al., 2009). It has even been shown that up to 6000 mg/L acetic acid and 3000 mg/L propionic acid did not cause system failure (Ahring et al, 1995). Nevertheless, it is said that these values may be different in each system, and that the system does not inhibit buffer capacity even though it has a high VFA formation (Franke-Whittle et al., 2014). With the exception of Set 3, no accumulation occurred because the other sets were below the propionic acid concentration indicated in the literature. However, propionic acid concentration of set 3 was up to 4000 mg/L per day on day 10. In literature, high accumulation of propionic acid in thermophilic digesters were often mentioned (Kugelman and Guida, 1989; Kim et al., 2002; Speece et al., 2006 and Wilson et al., 2008). Nonetheless, inhibition caused by VFA accumulation cannot be said due to the continuation of methanogenesis step and stability of pH and the methane percentages in biogas. Addition of alkalinity to the digesters in the set up phase may have prevented to VFA accumulation in the system.

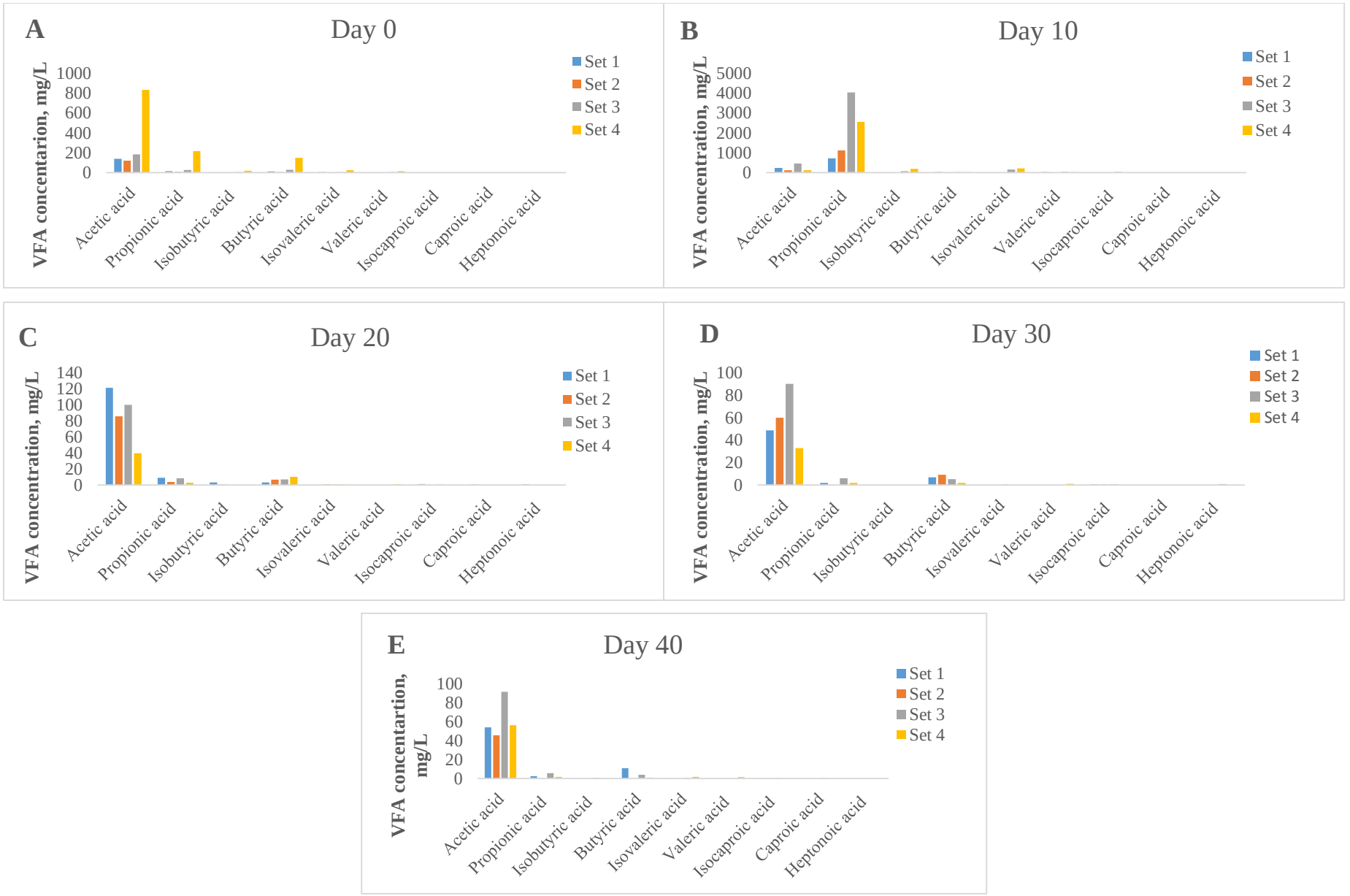

Figure 5.2. The amount of VFA species on the 0th (A), 10th (B), 20th (C), 30th (D) and 40th (E) day.

5.1.2. Biogas and methane production

The cumulative biogas and methane production of anaerobic batch reactors for 40 days are depicted in Figure 5.3 and 5.4, respectively. The highest biogas production was observed in the Set 3, digester operated at thermophilic (55°C) conditions with an I:S ratio of 1:2, as 12,82 L biogas. In Set 3, the highest biogas production was observed within days 4-15, the methane content of the biogas ranged between 54 and 61 % and cumulative methane production was obtained as 7,11 L CH_4. Relatively lower biogas production value was observed as 12,66 L biogas in Set 4, where the mesophilic digester containing anaerobic seed sludge and cow rumen fluid as a supportive inocula which was operated with an I:S ratio of 1:2. However, the highest cumulative methane production (7,64 L CH_4, see Figure 5.4) was reached in Set 4 due to higher methane content, because the methane content of Set 4 ranged between 55 and 66 %.

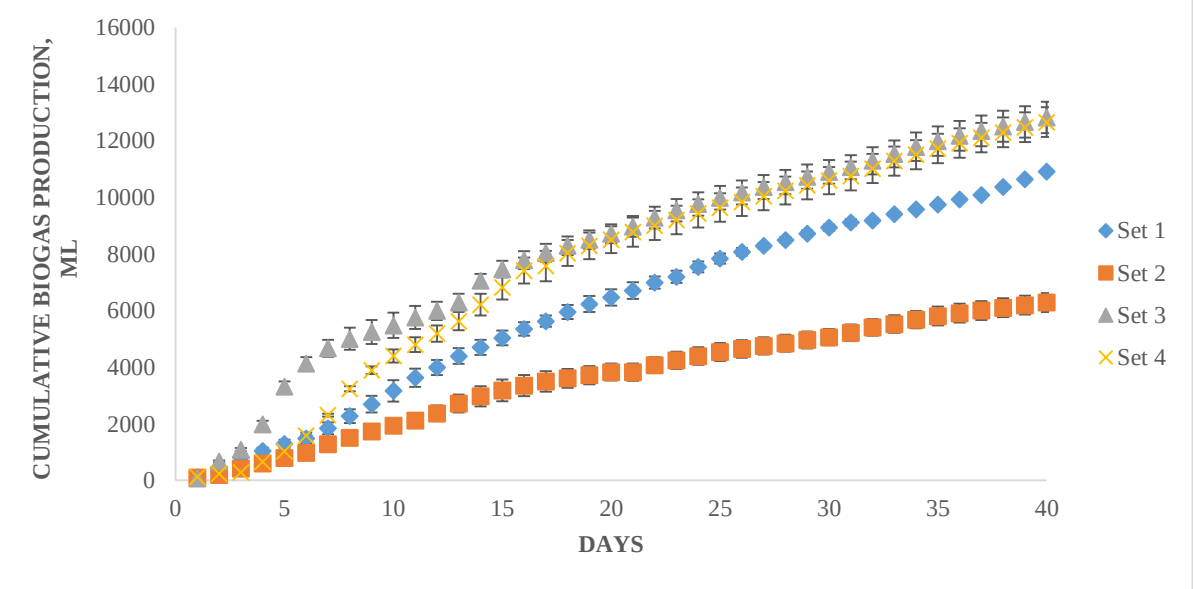

Figure 5.3. Cumulative biogas production in anaerobic batch reactors.

Besides, the first six days, while daily biogas production in Set 3 was more than Set 4, in between 7 and 13 days, highest daily biogas production belonged to Set 4. However, in Set 1 (Control), 10,92 L biogas and 6,47 L CH_4 were generated. The least biogas and methane production were observed in Set 2 operated at mesophilic condition with an I/S ratio 1/1. As Set 2 comprised 50% lower solid content than Set 1, the total biogas and methane production in Set

2 were 42% and 46% lower, respectively. On the other hand, Set 3, which had the same content with Set 1 but was operated at thermophilic condition, had a 18% higher biogas and 10% higher methane production than Set 1. The methane increase was not as much as the biogas increase; the reason for this was that methane content reached up to 61% in Set 3, whereas methane content of Set 1 reached 70%.

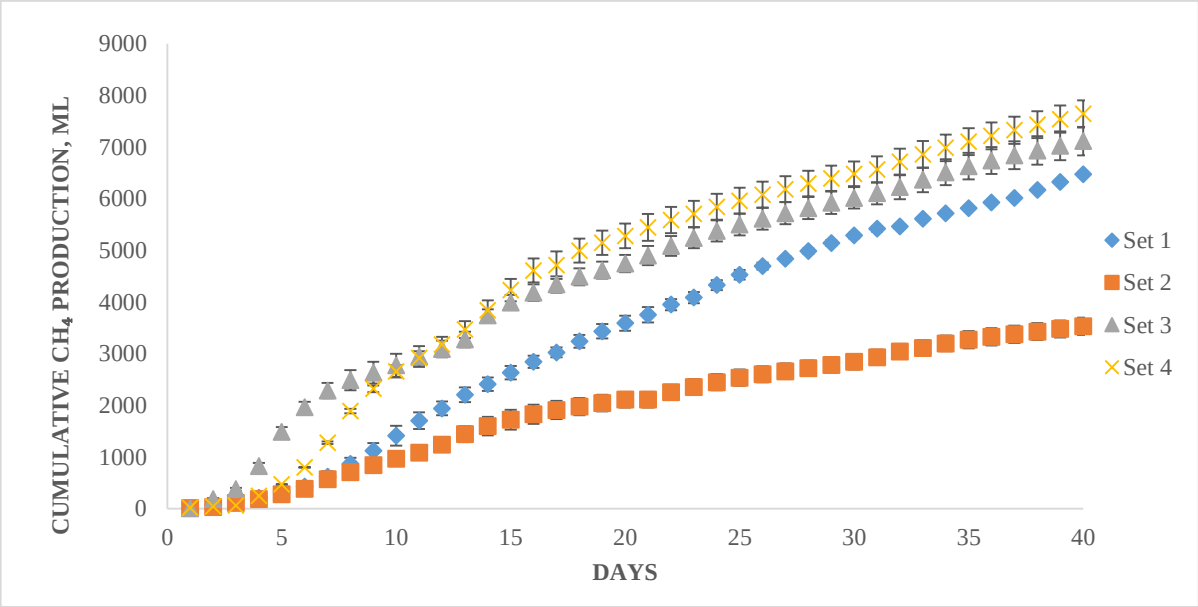

Figure 5.4. Cumulative methane production in anaerobic batch digesters.

Cavinato et al. (2010) showed 38% increment in the biogas yield with the help of thermophilic conditions in the anaerobic co-digestion of cattle manure with agro-wastes and energy crops. This increase is not the same as in our work, this may be due to the differences in substrates used and operating conditions. Set 4 produced 16% more biogas and 18% more methane than Set 1. That is, the addition of 25% cow ruminal fluid to the digesters enhanced the methane generation, resulting in a methane yield of 7,64 L CH_4/g VS, which was an increase of 18% compared with Set 1. Methane content of Set 4 was up to 66%. Despite lower biogas production compared to those of Set 3 and Set 4, the methane content of the Set 1 ranged between 54 – 70%. While highest biogas production was obtained in Set 3, highest methane production was observed in Set 4. Similarly, Mwazighe and colleagues (2014) studied with sewage wastewater as substrate and sludge/rumen as inoculum. They observed the highest

biogas production at thermophilic digesters containing sludge as inoculum when inoculum to substrate ratio of 1/3, 1/1 and 3/1, respectively. Also they showed that the higher biogas yield was achieved at mesophilic digesters containing ruminal fluid than digester containing sludge. While the best mesophilic digesters were the ones loaded with the I:S ratio of 1:3, digesters loaded with the I:S ratio of 1:1 and 3:1 had the lower biogas yield.

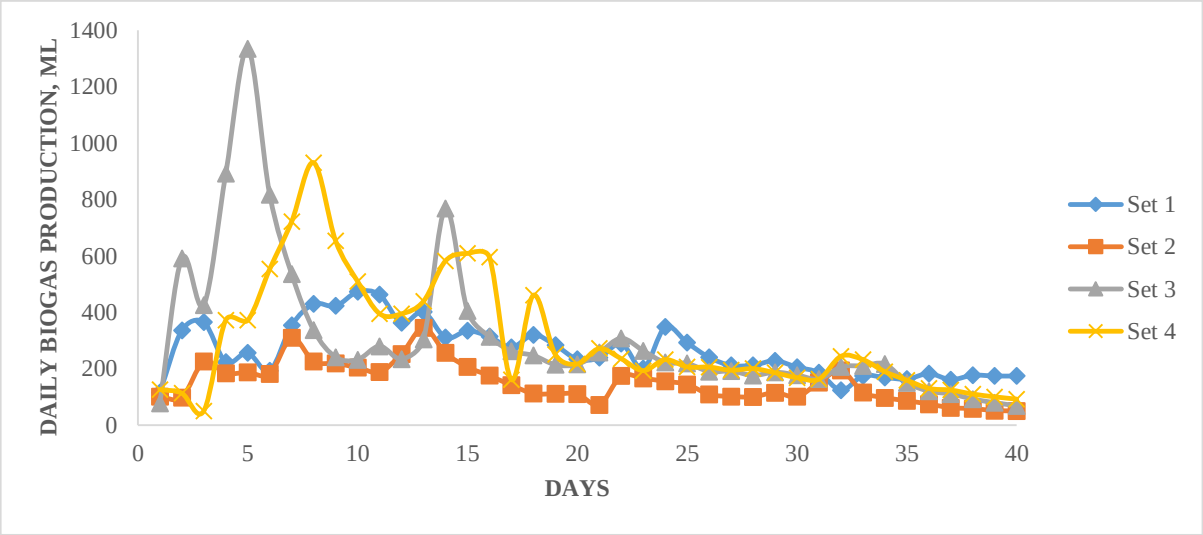

Figure 5.5. Daily biogas production in anaerobic batch reactors.

In Figure 5.5 and 5.6, daily biogas production and daily methane production of anaerobic batch reactors during 40 days are shown. As Figure 5.5 showed that even though all reactors reached first peak within the first five days, each reactor gave its first peak in different days. While the highest daily biogas was produced on the 5th day in Set 3, next higher daily biogas was generated on the 8th day in Set 4, on the 10th day in Set 1 as well as on the 13th day in Set 2, respectively. Daily methane production values showed a similar pattern with daily biogas production values (Figure 5.6). In Set 1, the daily methane production began to decrease after the first three days, nevertheless, on 7th day it rose again and until day 12th the highest daily methane was produced. In Set 2, daily methane production was less than that produced in all sets. Also, that the methane produced in Set 3 and Set 4 showed less stable trend than other sets was seen in Figure 5.6. When Set 3 operated at thermophilic condition gave the highest peak on the 5th day, Set 4 containing the anaerobic seed sludge and ruminal fluid as the inocula and operated at mesophilic condition gave the highest peak on the 8th day. It was earlier than the

Set 4 due to the increase in the temperature. Similarly, several studies indicated that thermophilic reactors give peaks earlier and are less stable than mesophilic reactors (Speece et al., 2006; Chen et al., 2008; Wilson et al., 2008; Labatut et al., 2014). It is also apparent that the decrement in the methane production in Set 4 was much lower than Set 3.

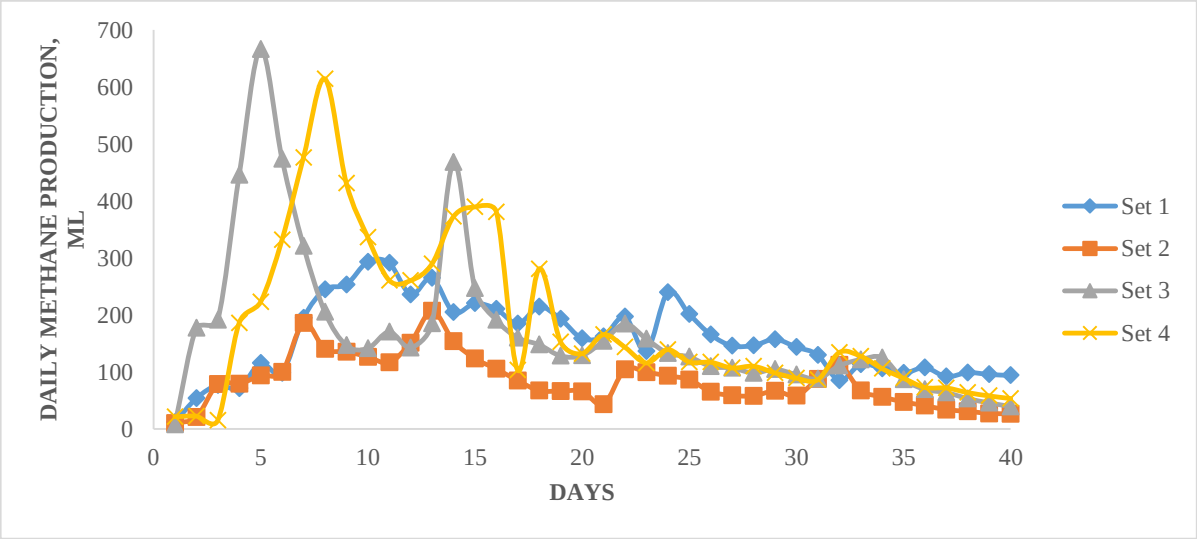

Figure 5.6. Daily methane production in anaerobic batch reactors.

The methane content, biogas and methane yields for the anaerobic batch reactors are seen in Figure 5.7 and Table 5.2. In all digesters, the percentage of methane produced in biogas increased up to the highest value during the first ten days for all sets. While the biogas and methane yields of Set 1 were 397 mL biogas/g VS and 235 mL CH_4/g VS, respectively, the biogas and methane yield of Set 2 were 457 mL biogas/g VS and 257 mL CH_4/g VS, respectively. On the other hand, the biogas and methane yields for Set 3 were calculated as 466 mL biogas/g VS and 259 mL CH_4/g VS, respectively. In Set 4, the biogas and methane yields were observed as 460 mL biogas/g VS and 278 mL CH_4/g VS, respectively as well. While the highest biogas yield was obtained in thermophilic reactor (Set 3), the highest specific methane yield (278 mL CH4/ g VS) was found in the digester containing anaerobic seed sludge and cow rumen fluid which was operated with an I:S ratio of 1:2 (Set 4). Surprisingly, although Set 1 and Set 4 were operated under mesophilic condition and I:S ratio of 1:2, addition of rumen fluid to inoculum in Set 4 increased biogas and the methane yield in a considerable amount. The

addition of 25% (v/v) cow rumen fluid to inoculum effectively improved the methane production especially in 20 days, resulting in a methane yield of 278 mL CH_4/g VS, which was 18% higher than Set 1. Similarly, Sunarso et al (2012) showed a positive effect of ruminal fluid addition to the anaerobic digesters. They observed a 111% increase in biogas production in the digester including 25% ruminal fluid when compared to digester containing only cattle manure. In terms of methane production and a being low cost method, rumen fluid addition was better for anaerobic co-digestion development. The results show that we can increase the methane yield with the combination of different types of inocula instead of energy input in thermophilic anaerobic digesters.

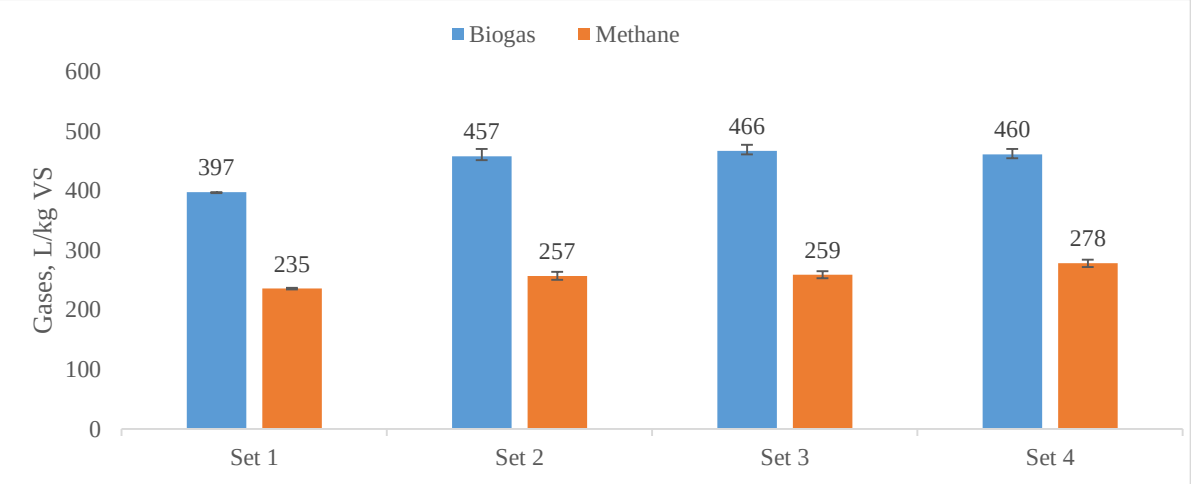

Figure 5.7. Biogas and methane yield of anaerobic batch digesters.

Table 5.2. A comparison of the biogas and bio-methane potential of the digesters.

	Specific biogas production (mL biogas/g $VS_{initial}$)	CH_4 content range (%)	Specific CH_4 production (mL methane/g $VS_{initial}$)
Set 1	397±1	54-70	235±2
Set 2	457±24	59-66	257±13
Set 3	466±20	54-61	259±12
Set 4	460±19	53-66	278±12

Wei et al. (2014) carried out a study at a different barley straw/cow manure and inoculum/substrate ratio in the psychrophilic (15°C) anaerobic condition and they found that the highest methane volumetric productivity at the barley straw/cow manure ratio of 1/1 and inoculum/substrate ratio of 1/2 (based on TS). On the other hand, when barley straw/cow manure ratio was 1/1, the highest methane yield was observed at the I/S ratio of 2/1, 1/1 and 1/2, respectively. Dinuccio et al. (2010) investigated bio-methane potential of barley straw at mesophilic condition (40°C) and they reported that barley straw had the 417 mL biogas/g VS and 229 mL CH_4/g VS in the 2L digester with the I:S ratio of 2:1 at the end of the 40 days.

5.1.3. Results of molecular analyses

5.1.3.1. Next Generation Sequencing (NGS) Results. The bacterial characterization of anaerobic seed sludge analyzed by the next-generation sequencing technology is illustrated in Figure 5.8 as the phylum distribution.

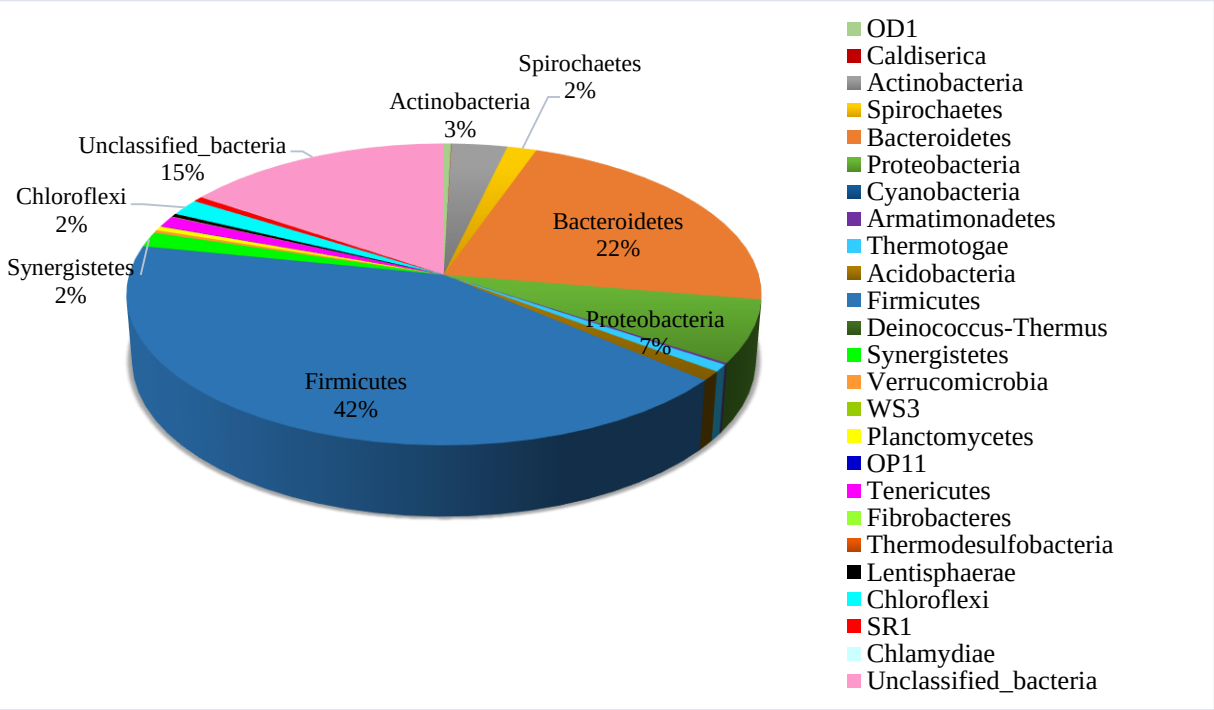

Figure 5.8. Bacterial phyla of anaerobic seed sludge.

It is identified that *Firmicutes* (42%), *Bacteroidetes* (22%) and *Proteobacteria* (7%) were dominant in anaerobic seed sludge. Bacteria that cannot be included in any phylum constitute 15% of the total sequences. The remaining portion, which constitutes less than 14% of the pie chart were found mostly *Actinobacteria* (3.2%) and *Cloroflexi* (1.8%). In addition to phylogenetic analysis, taxonomic characterization in lower levels has also been performed in order to better identification of the bacterial community. Most of the bacterial classes are composed of *Clostridia* (44%), *Bacteroidia* (12%), *Flavobacteria* (6.5%), *Deltaproteobacteria* (5.9%) and *Actinobacteria* (4.7%). The distribution of the bacterial compositions of the seed sludge taken from the full-scale mesophilic anaerobic digester is similar to the literature (Regueiro et al., 2012; Wang et al., 2010; Kröber et al., 2009).

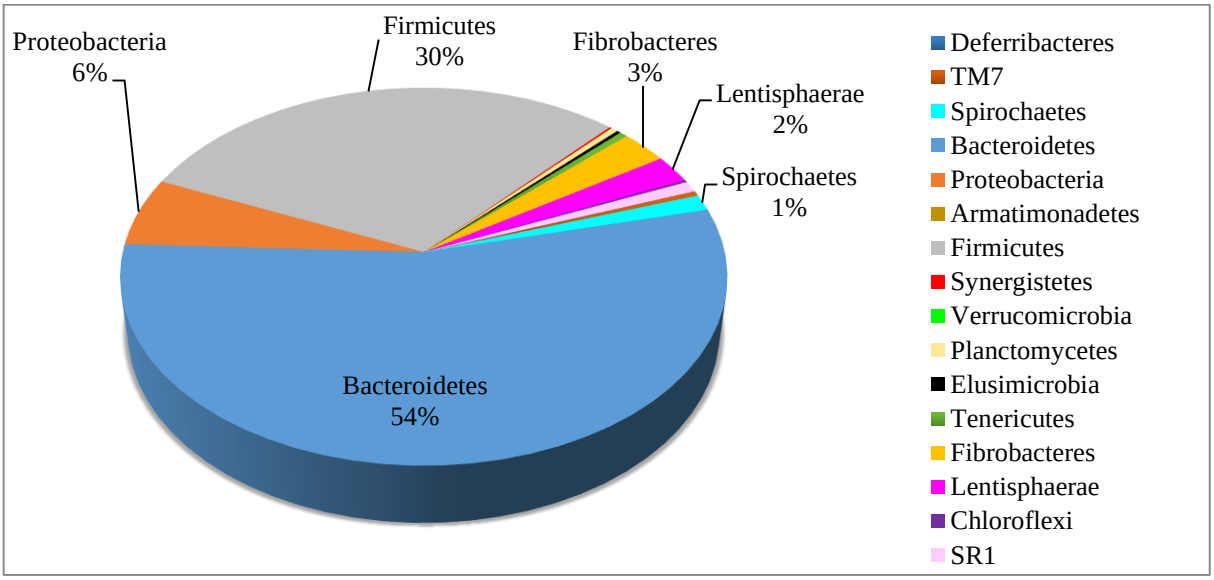

Figure 5.9. Bacterial phylum of ruminal fluid.

In the ruminal fluid used only in digester Set 4, bacterial phylum composition can be seen in Figure 5.9. Unlike the bacterial composition of anaerobic seed sludge, it was identified that *Bacteroidetes* (54%) was the most abundant phylum. *Firmicutes* (30%) was the second most dominant phylum as well. *Proteobacteria* (6%), *Fibrobacteres* (3%), *Lentisphaerae* (2%) and *Spirochaetes* (1%) were also found in a significant amount. Bacterial classes were mostly composed of *Bacteroidia* (46%), *Clostridia* (30%), *Sphingobacteria* (8%), *Alphaproteobacteria* (4%), *Fibrobacterio* (3%) and *Lentisphaeria* (3%).

Microbial community dynamics of four different sets were determined through Ion PGM^{TM}. Variations of bacterial and archaeal communities in the digesters were revealed in this context. NGS analyses were done to samples taken at 10th day.

According to the results of bacterial analyses, in the Set 1, also called as control reactor (with the I:S ratio of 1:2, operated at mesophilic condition), 18 phyla, 37 class, 78 order, 185 families, 455 genera and 782 species were identified. On the other hand, 19 phyla, 38 class, 75 order, 176 families, 404 genera and 685 species were found in the Set 2 (with the I:S ratio of 1:1). Similarly, in the Set 3, which is the thermophilic reactor, 19 phyla, 37 class, 75 order, 169 families, 402 genera and 680 species were detected; whereas, 18 phyla, 38 class, 74 order, 172 families, 417 genera and 714 species were found in the Set 4 containing ruminal fluid with seed sludge.

According to results of archaeal analyses, while in the Set 1, 2 phyla, 4 class, 6 order, 8 families, 9 genera and 12 species were identified, 1 phylum, 3 class, 5 order, 7 families, 8 genera and 11 species were detected in the Set 2. On the other hand, in the set 3, 1 phylum, 3 class, 5 order, 7 families, 8 genera and 9 species were found. In the Set 4, 2 phyla, 4 class, 6 order, 8 familiy, 9 genera and 10 species were detected.

The abundance rate of bacterial phyla, bacterial genera and archaeal species in the Set 1, Set 2, Set 3 and Set 4 were illustrated in the Figure 5.10-5.12. It can be clearly seen that the phylum *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were “major” bacterial phyla for all sets, respectively. It was noticed that next-generation sequencing results reflected bacterial phylum distribution in the seed sludge and ruminal fluid used in this study. Likewise, in literature, these three phyla were generally dominant in the anaerobic digesters (Krause et al., 2008; Schlüter et al., 2008; Kröber et al., 2009; Regueiro et al., 2012; Wirth et al., 2012). Moreover, these bacterial community compositions were frequently encountered in the inoculum and cow manure (Wang et al., 2010).

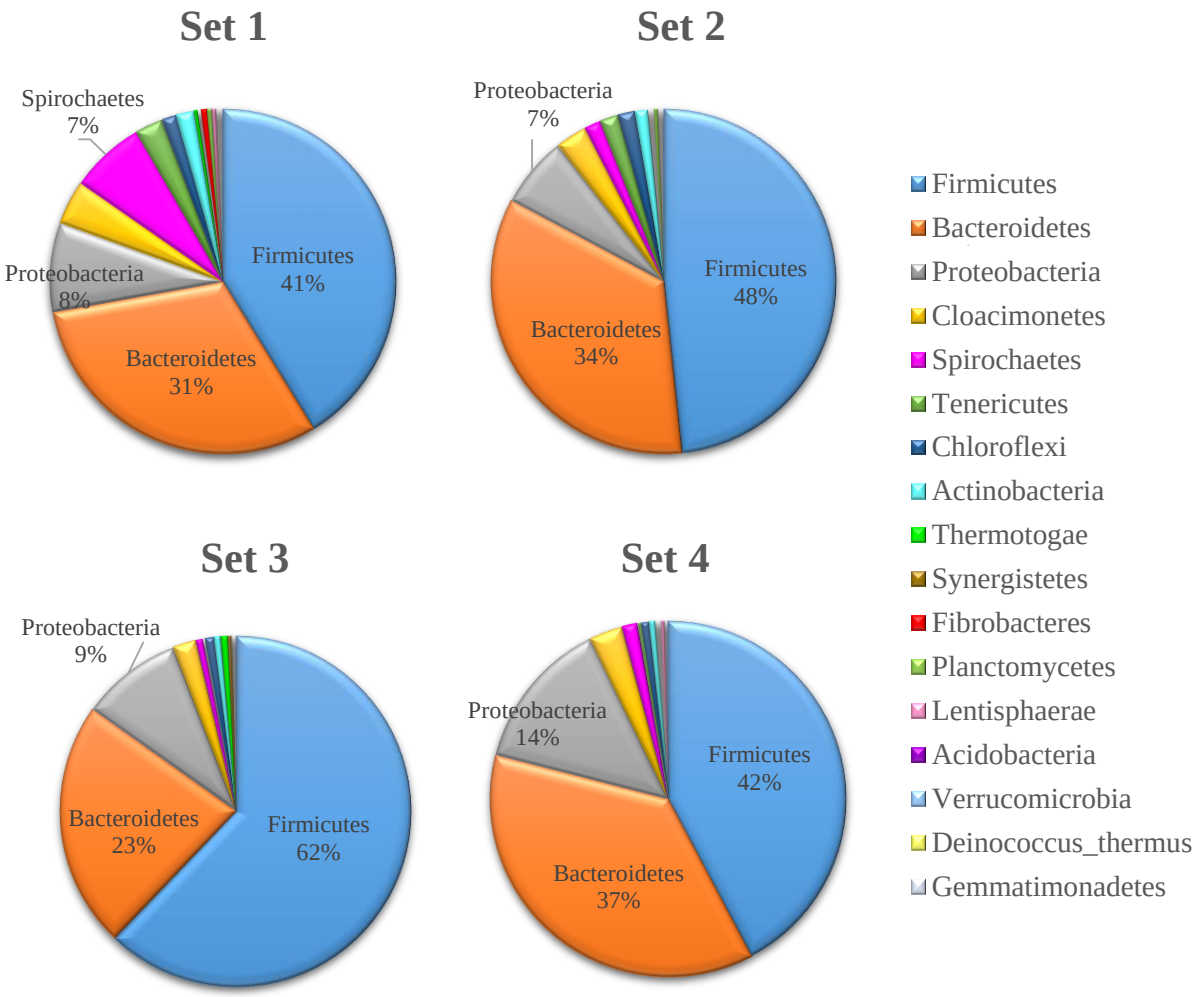

Figure 5.10. Bacterial phylum in the four different sets at the 10th day.

The relative abundance of bacterial phyla in four sets were similar. We can obviously say that there is no significant difference between the all four sets in bacterial phyla variations. However, in Set 1, the abundance ratio of *Spirochaetes* was more than other sets. According to the study of Liu et al. (2009a), 13.3% of Spirochaetes was found in an anaerobic mesophilic digester fed with pig manure. 46.5 % of *Firmicutes*, 35.2 % of *Bacteroidetes*, 13.2 % of *Spirochaetes* was detected in the system, and it is similar with bacterial phylum distribution of Set 1.

Although Set 3 is a thermophilic reactor, it was similar with microbial community of mesophilic sets on the basis of phyla. This may be due to the fact that the seed sludge added to the thermophilic reactor (Set 3) was taken from the full-scale mesophilic reactor. The phylum *Firmicutes* includes microorganisms that can live in a wide variety of environments and can degrade complex substances such as carbohydrates and protein (Wiegel et al., 2005). It was the most abundant phylum in seed sludge and all sets and the second most dominant phylum in ruminal fluid. The *Bacteroidetes* which are hydrolytic and acidogenic bacteria mostly active in anaerobic conditions are facultative anaerobic microorganisms and constitutes a very diverse group (Delbès et al. 2000). They can be found abundantly in intestine and manure of warm-blooded animals (Wang et al., 2010). The *Bacteroidetes* was the most dominant phylum in ruminal fluid and second most dominant phylum in seed sludge in the present study and four sets. This phylum is considered to be able to degrade carbohydrates and long chain fatty acids.

The abundance ratio of phylum *Firmicutes* (62%) was most found in Set 3 which the most biogas is produced and the phylum *Bacteroidetes* (37%) was most abundant in Set 4 which the second most biogas is produced. Since the most abundant phlum is *Bacteroidetes* in ruminal fluid (see Figure 5.9), Set 4 containing ruminal fluid in addition to seed sludge had the highest abundance of *Bacteroidetes*. Also, *Proteobacteria* may have ben affected by the addition of ruminal fluid because the ratio of *Proteobacteria* was the highest in Set 4. The total percentage of the major three phyla (*Firmicutes*, *Bacteroidetes* and *Proteobacteria*) was 89% in Set 3, and 93% in Set 4. If the combination of *Firmicutes*, *Bacteroidetes* and *Proteobacteria* is present in excess, biogas production may be positively affected. These three phyla may have provided more biogas production in Set 3 and Set 4 by contributing to hydrolysis and acidogenic reactions.

The most abundant classes were *Clostridia* (belonging to the phylum *Firmicutes*), *Bacteroidia* (Phylum: *Bacteroidetes*) and *Bacilli* (Phylum: *Firmicutes*) for all four sets, respectively (Figure 5.11). *Clostridia* (37%) and *Bacilli* (23%) were most found in the digester Set 3. The distribution of bacterial communities in Set 3, a thermophilic reactor, is similar to that of various studies (Tang et al. 2004; Weiss et al 2008). In the thermophilic reactor (Set 3), compared to other reactors, a relative large number of the class *Clostridia* within the phylum

Firmicutes was identified. This results is in agreement with study of Weiss et al (2008). One of the reasons for the greater presence of *Clostridia* in thermophilic conditions is thought to be the increase in spore-forming microorganisms within *Clostridia* by the help of heat activation (Mead, 1992). We can say that it provided positive effect of biogas production on Set 3. In Set 4, where the most methane production was seen, there were more *Gammatoproteobacteria* (Phylum: *Proteobacteria*), *Cytophagia* (belonging to the phylum *Bacteroidetes*) and *Epsilonproteobacteria* (Phylum: *Proteobacteria*) than the other digesters. Although these classes are found in other sets, they were the minor amounts. Since these groups of bacteria were also present in the rumen fluid, these classes increased in Set 4 with addition of the rumen fluid, and they contributed to the production of more biogas and therefore methane.

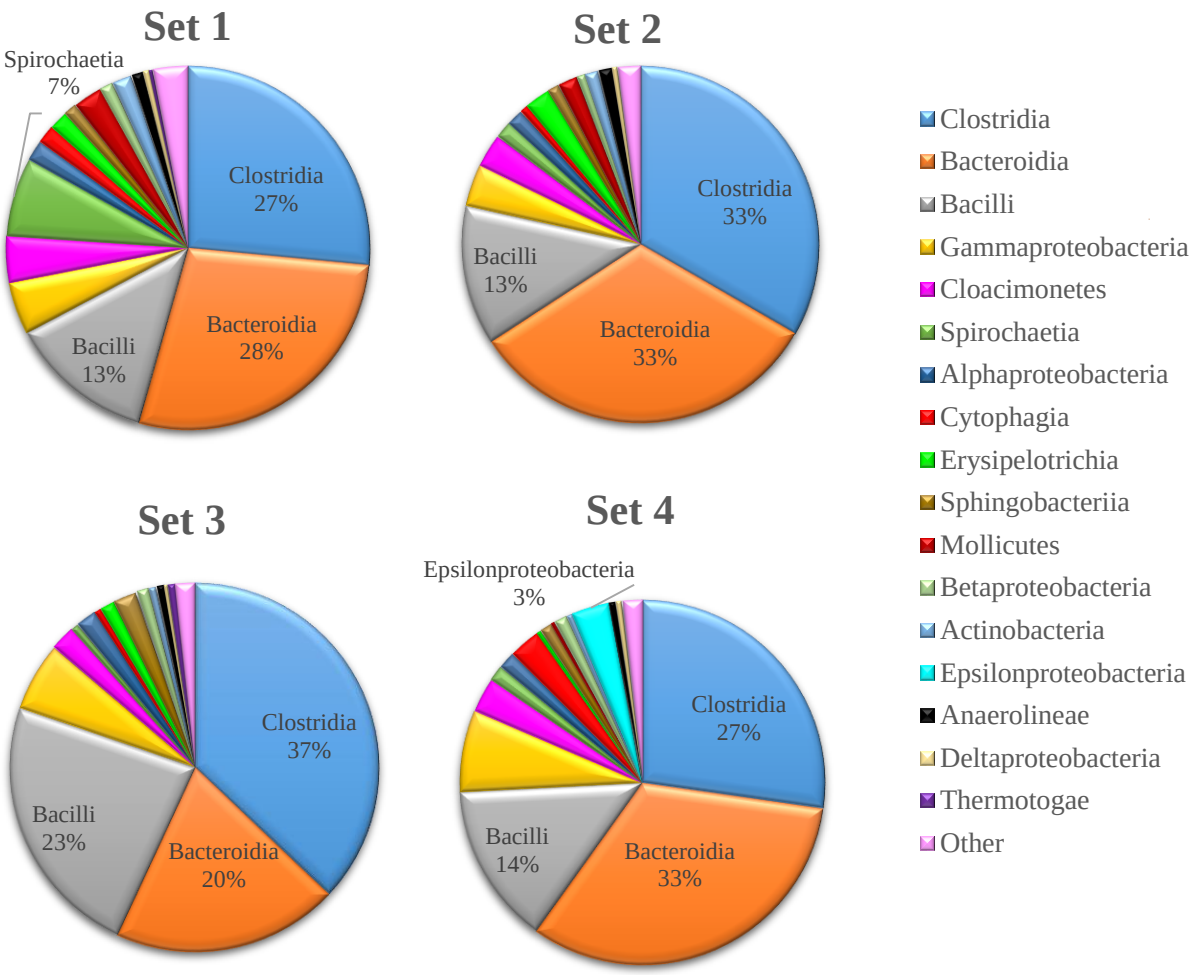

Figure 5.11. Bacterial class in the four different sets at the 10th day.

We can notice that Set 2, which produces least biogas and methane, was not significantly different from other reactors in terms of bacterial community. However, the difference in archaeal community may be the main reason for the low production of methane. This situation can be seen more clearly in Figure 5.12.

Archaeal community was less diverse than the bacterial one. Although bacterial community profiles were quite similar in the digesters, methanogenic profiles varied significantly. The most dominant methanogenic *Archaea* was *Methanobacterium sp*. (Order: *Methanobacteriales*) in the digesters inoculated only with anaerobic seed sludge (Set 1, Set 2, Set 3); whereas, *Methanobrevibacter spp.* (Order: *Methanobacteriales*) dominated the digesters contained the rumen fluid (Set 4). Likewise, Yanagita et al. (2000) found that the genus *Methanobrevibacter* was identified as a major methanogen in the rumen fluid. The second most abundant archaeal species for Set 1 was *Methanosarcina spp*. (Order: *Methanosarcinales*), for thermophilic reactor (Set 3) was *Methanoculleus bourgensis* (Order: *Methanomicrobiales*) and for Set 4 (incluiding ruminal fluid) was *Methanobacterium sp.*. Moreover, the second most common species in Set 2 (I/S:1/1) were two, which were *Methanosarcina spp.* (2.6%) and *Methanobrevibacter spp.* (2.5%). Although Set 2, which has the lowest biogas and methane production, shows similarity with other reactors in the results of bacterial analysis, we can see that the results of archaeal analysis of Set 2 were significantly different from those of other reactors. It is seen that several different types of methanogens were dominant in other sets, but in Set 2, the *Methanobacterium sp.* dominates the digester. We can say that the dominance of a single species influences negatively the performance of anaerobic digester. However, the most diverse digester was belonged to Set 4. The addition of ruminal fluid and therefore combination of seed sludge and ruminal fluid affected methane production positively in Set 4. The most productive digesters in terms of methane generation was Set 4, Set 3, Set 1 and Set 2, respectively.

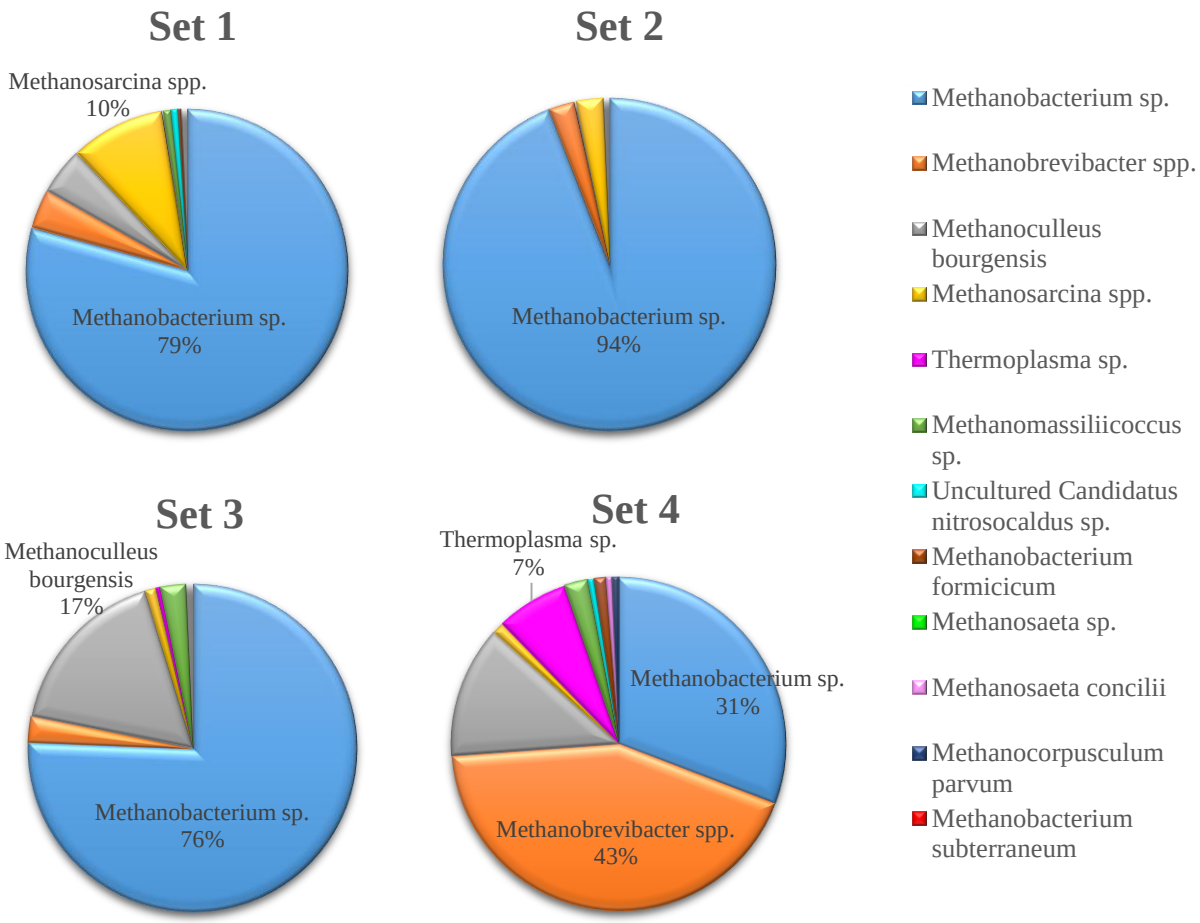

Figure 5.12. Archaeal species in the four different sets at the 10th day.

Similar with the results of the thermofilic reactor (Set 3), Tang and collegues (2004) observed that as long as the population of genus *Methanocullues* increased, the population of the genus *Methanosarcina* decreased in the thermofilic digesters. Although the content of Set 3 is exactly the same as that of the control reactor (Set 1), when we compare the two sets, we can clearly see that *Methanosarcina sp.* decreased at higher temperature. Also, the study of Weiss et al (2008) shows that *Methanoculleus bourgensis* was identified at the thermophilic reactor in the ratio between 9.6% and 18.9% of the archaeal community. This is similar to our results. While acetogenotrophic *Methanoculleus bourgensis* most of which are found in the thermophilic reactor (Set 3) uses acetate to produce methane, the genus *Methanobrevibacter* most found in Set 4 (the reactor including rumen) is the hydrogenotrophic methanogen responsible to generate methane from CO2 and H2 (Krumholz and Bryant 1986, Weiss et al.,

2008). *Methanobacterium sp.* found in all reactor in large amounts uses hydrogen and carbon dioxide to produce methane; whereas *Methanosarcina* most found in control reactor (Set 1) generate methane from H2, CO2 and acetate. Yet, *Methanosaeta* uses only acetate to produce methane. Therefore, we can talk about that all digesters had hydrogenotrophic and acetoclastic activites. However, as the relative abundance of hydrogenotrophic species was higher than that of acetotrophic species, it can be said that hydrogenotrophic activities were more common in all digesters. This result is consistent with last studies (Nettmann et al. 2010; Zhu et al. 2011).

It has been acknowledged by many studies that archaeal community diversity is less than the bacterial community diversity. Moreover, thermophilic digesters have less archaeal diversity than mesophilic ones (Karakashev et al.,2005). In accordance with this results, the number of archaeal species detected in the thermophilic digester (Set 3) was the least, compared the other reactors. On the other hand, the genus *Methanosarcina* and the genus *Methanosaeta* compete with each other. *Methanosarcina* shows a faster growth in high acetate concentration. Unlike the genus *Methanosarcina*, *Methanosaeta* is more common at low acetate concentration (McMahon et al. 2001). When we look at the Figure 5.12., it can be seen that the abundance of *Methanosarcina spp*. is more than *Methanosaeta sp*. Since the results of day 10^{th} samples which have the highest acetic acid concentration (up to 457 mg/L, see Figure 5.1.b) were measured, the relative abundance of *Methanosarcina spp.* was higher than that of *Methanosaeta sp.* for all sets.

It is acknowledged that factors such as types of substrates and inocula and operational parameters impacts microbial community dynamics in the anaerobic systems (Demirel and Scherer 2008). The NGS results of this study was also revealed that different operational parameters and inoculum types impacts microbial community distrubition in the anaerobic digesters.

5.1.3.2. Q-PCR results. To quantify the total copy number of the 16S rDNA gene of the bacteria and archaea, quantitative real-time PCR assays were applied. 16S rDNA sequence specific primers were used to quantify total bacteria and archaea present in the anaerobic digesters for the Q-PCR assays. Results of real-time PCR analyses of are shown in Figure 5.13 and 5.14.

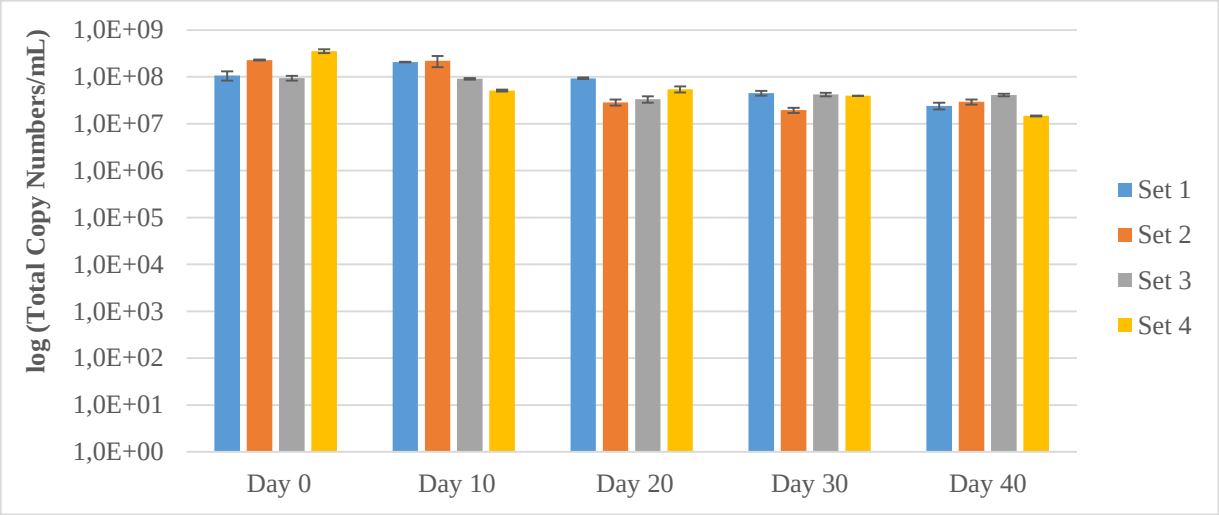

Figure 5.13. Total 16S rDNA copy number of bacteria.

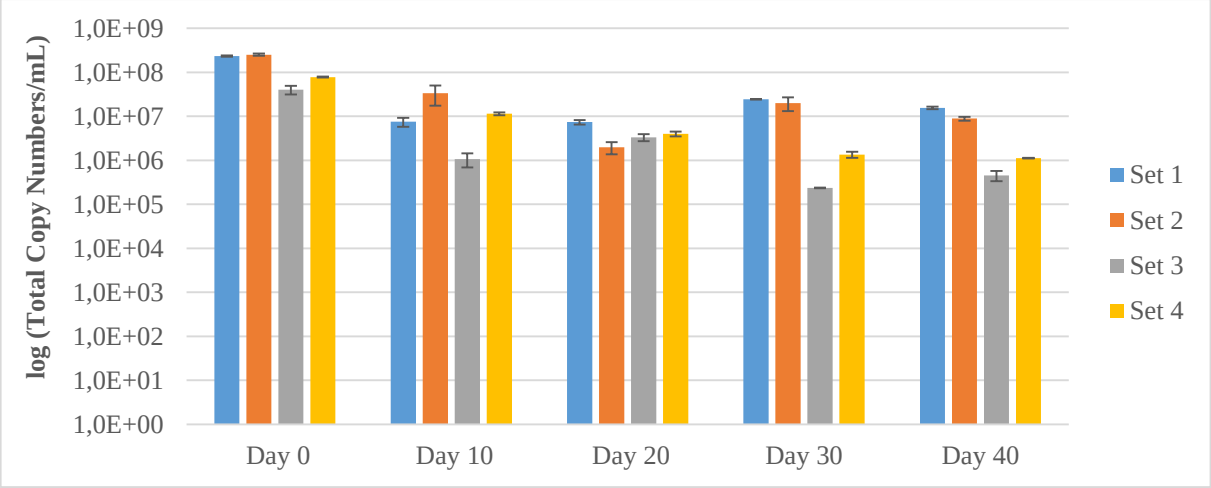

Figure 5.14. Total 16S rDNA copy number of Archaea.

According to Q-PCR assays, total numbers of bacteria was found in the highest amount in Set 4 at the beginning. This may be due to the rumen fluid addition. The digesters containing the same amount of seed sludge (Set 1, Set 2, Set 3) had almost same total numbers of bacteria at the day 0th. The total 16S rDNA copy number of bacteria declined in time in all digesters, however, this decline was not very sharp.

The total 16S rDNA copy number of *Archaea* decreased in time in all digesters. At the day 10, the lowest copy number belonged to the Set 3 (thermophilic digester), this may be reason

that increase in temperature damaged some microorganisms. However, at the day 20, the total copy number of *Archaea* increased in the Set 3, and this may be due to their adaptation to the high temperature over time and therefore their growth.

Total 16S rDNA copy numbers of bacteria and *Archaea* were not influenced significantly by different operating conditions. There is no significant change between digesters as in the NGS results, which may be due to the fact that remarkable changes mostly occur among subgroups.

6. CONCLUSIONS AND RECOMMENDATIONS

Operating conditions significantly impact the performance of anaerobic digesters. In this study, the biochemical methane potentials of cow manure and barley with respect to four different operational parameters were evaluated so as to achieve the most efficient methane yield. Effects of temperature (mesophilic vs. thermophilic), inoculum to substrate (I/S) ratios and different types of inocula were investigated in anaerobic batch tests. Within this context, digester performances in terms of biogas and methane generation and microbial community profiles were observed.

According to the findings of the study, the highest specific methane yield was found in the Set 4, digester containing anaerobic seed sludge and cow rumen fluid as a supportive inocula which was operated with an I/S ratio of 1:/2. Addition of cow rumen fluid (25%) to inoculum effectively improved the bio-methane yield especially in 20 days, resulting in a methane yield of 278 mL CH_4/g VS, which was 18% higher than Set 1 (Control). The next highest specific methane yields were obtained in the Set 3, digester operated at thermophilic temperature (55°C) and in the Set 2, digester operated with an I:S ratio of 1:1, as 259±12 CH_4/g VS and 257±13 CH_4/g VS, respectively. In the control reactor (Set 1), the methane yield was found as 235±2 CH_4/g VS. As a major indicator of stability in anaerobic digesters, no volatile fatty acids accumulation was observed in the digesters at the end of the operation period.

Changes of bacterial and methanogenic profiles among digesters were detected through NGS-based metagenomics analysis applied using Ion PGM™ platform. The microbial profile was closely related to bacterial community distribution in the seed sludge and ruminal fluid used in this study. The phylum *Firmicutes, Bacteroidetes* and *Proteobacteria* were determined as the most dominant three bacterial phyla for all sets, respectively. The most abundant classes were *Clostridia* (Phylum: *Firmicutes*), *Bacteroidia* (Phylum: *Bacteroidetes*) and *Bacilli* (Phylum: *Firmicutes*) for all four sets, respectively. Archaeal communities were less diverse than the bacterial ones. On the other hand, although bacterial community profiles were quite similar in

the digesters, methanogenic profiles varied significantly. The difference of methanogenic profiles among digesters was more obvious than that of the bacterial community profiles. Hydrogenotrophic methanogenesis was favoured in all digesters as the relative abundance of hydrogenotrophic methanogens was higher than that of acetotrophic methanogens. *Methanobacterium sp*. (belonging to the order *Methanobacteriales*) dominated the digesters inoculated only with anaerobic seed sludge (Set 1, Set 2, Set 3); whereas, *Methanobrevibacter spp.* (belonging to the order *Methanobacteriales*) was the most dominant archaeal species in the digesters including rumen fluid in addition to the anaerobic seed sludge. According to the Q-PCR results, the total 16S rDNA copy numbers of bacteria and *Archaea* were not influenced significantly by different operating conditions.

Since the methane yield was enhanced effectively by adding rumen microorganisms, the combination of different types of inocula can be applied in the anaerobic digestion process instead of additional energy input in thermophilic anaerobic digesters to improve methane yield. The findings of this study can contribute to further bio-augmentation applications in anaerobic digesters treating lignocellulosic feedstocks. For further investigations, different types of animal manure and crops can be co-digested to observe the change of the methane yield. In the Q-PCR analysis, 16S rDNA-based quantification of bacteria and Archaea were evaluated. RNA based quantification of these domains can be analyzed. Also, in this study, the anaerobic seed sludge taken from the mesophilic digester was inoculated to the thermophilic reactor. The inocula taken from a thermophilic digester can be added to digester operated at thermophilic temperature to provide better microbial community profiles and to enhance the methane yield.

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