IMPROVEMENT OF BIOMETHANE PRODUCTION USING RUMEN BACTERIA IN ANAEROBIC CATTLE MANURE DIGESTERS

by

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In the memory of Ali İsmail Korkmaz…

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IMPROVEMENT OF BIOMETHANE PRODUCTION USING RUMEN BACTERIA IN ANAEROBIC CATTLE MANURE DIGESTERS

One of the most important difficulty of energy production from animal manure in anaerobic conditions is the high cellulose and lignin concentration that limits the speed in hydrolysis stage. Rumen bacteria which reside in the digestive system of herbivores are indicated as an alternative solution to this case. In this study, effect of rumen fluid addition on biogas production and microbial dynamics in batch-wise operated serum bottles with cow manure was investigated. Three experiment sets were conducted, namely Set 1, Set 2 and Set 3 and operated at 36 ^{o}C, 41 ^{o}C and 43 ^{o}C, respectively for 40 days. The highest specific methane yield was found in the Set 2, digester containing 40% as a supportive inocula. Addition of rumen fluid to inoculum effectively improved the bio-methane yield especially in 20 days, resulting in a methane yield of 262 mL CH_4/g VS, in which methane content of the biogas was around 52%. Bacterial and methanogenic profiles were detected through NGS-based metagenomics analysis. The phylum *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were determined as the most dominant bacterial phyla. The results from Q-PCR showed that set3, with a high heat of 43 ^{o}C, could not set favorable conditions for the rumen bacteria.

ANAEROBİK İNEK DIŞKISI ÇÜRÜTÜCÜLERİNDE RUMEN BAKTERİLERİ KULLANILARAK BİYOMETAN ÜRETİMİNİN İYİLEŞTİRİLMESİ

Anaerobik koşullarda hayvan gübresinden enerji üretiminin en önemli zorluklarından biri, hidroliz aşamasındaki hızı sınırlayan yüksek selüloz ve lignin konsantrasyonudur. Otoburların sindirim sisteminde bulunan rumen bakterileri, bu davaya alternatif bir çözüm olarak gösterilir. Bu çalışmada, inek gübresi ile kesikli olarak işletilen serum şişelerinde, rumen sıvısı ilavesinin biyogaz üretimi ve mikrobiyal dinamikler üzerine etkisi araştırılmıştır. Set 1, Set 2 ve Set 3 olmak üzere üç deney seti kurulmuş ve 40 gün boyunca sırasıyla 36 ^{o}C, 41 ^{o}C ve 43 ^{o}C'de çalıştırılmıştır. En yüksek spesifik metan verimi, % 40 aşı içeren Set 2 reaktöründe bulunmuştur. Rumen sıvısının aşı olarak eklenmesi, biyometan verimi özellikle 20 gün içinde etkili bir şekilde artmıştır, biyogazın metan içeriği % 52 civarında ve metan verimi 262 mL CH_4 / g VS olarak elde edilmiştir. NGS tabanlı metagenomik analiz ile bakteri ve metanojenik profiller tespit edilmiştir. *Firmicutes, Bacteroidetes* ve *Proteobacteria* en baskın bakteri filumları olarak belirlenmiştir. Q-PCR sonuçlarına göre, 43 ^{o}C gibi yüksek sıcaklıkta kurulan set 3'ün, rumen bakterileri için uygun koşulları sağlamadığı tespit edilmiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Units used
AD	Anaerobic Digestion	
ATP	Adenosine Triphosphate	
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	
LCFAs	Long-chain Fatty Acids	
NGS	Next Generation Sequencing	
PCR	Polymerase Chain Reaction	
Q-PCR	Real Time PCR (Quantative 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 Kjehldahl Nitrogen	mg kg^{-1}
TS	Total Solid	%
VFA	Volatile Fatty Acids	mg L^{-1}
VS	Volatile solid	%
HRT	Hydraulic Retention Time	
OLR	Organic Loading Rate	g TVS/L-day
SRT	Solid Retention Time	day

1. INTRODUCTION

Emission of greenhouse gases worldwide has shown a significant increase and become one of the major global concerns in the twenty-first century. The hardest challenge in restricting the emissions of greenhouse gases and pollution is considered to be the world population, which demands more and more energy consumption with higher living standards. Furthermore, the limited availability of fossil fuels also brings along increasing prices. Thus, humankind is compelled to replace new alternative sources of energy with fossil fuels (Teghammar, 2013).

Biogas produced from animal waste is widely used as a renewable bio-fuel source. This source of energy is regarded as cheap and clean and is also known to produce a residue with a high fertilizer value for crop production (Albihn and Vinneas, 2007). In developed countries, the biogas technology is used on a large scale for power and heat production. It is also one of the technologies supported by governments and the international organizations UN and EU because it reduces GHG emissions from manure and produces renewable energy (Moller et al., 2004; Sommer et al., 2004).

Recovery of biomass from animal manure is the most important application of anaerobic digesters. Biomass stands out as an effective biological material source of energy owing to its potential ability to provide energy as fuel and power as sustainable energy. Decomposition of the biological materials in the conditions of anaerobic environments lead to the production of biogas (Chum and Overend, 2001). Sources of biomass have a great variety as they include residues from animals and industries, sewage, agricultural crop wastes, municipal wastes. However, lignocellulosic compounds, as sources of biomass, are more favorable for producing biogas than the other sources. Lignocellulosic biomass resources are, in definition, those that contain plant dry materials, and they are considered to be the most widespread bio-renewable biomass in the World.

Anaerobic digestion (AD) is a technology widely used for treatment of organic waste for biogas production. AD that utilizes manure for biogas production is one of the most

promising uses of biomass wastes because it provides a source of energy while simultaneously resolving ecological and agrochemical issues. The anaerobic fermentation of manure for biogas production does not reduce its value as a fertilizer supplement, as available nitrogen and other substances remain in the treated sludge (Alvarez and Lide´n, 2008).

Production of biogas from animal manure, especially cow is very potential and has an advantages, energy derived from it is very environmentally friendly since in addition to utilizing the waste from livestock, left over from the process (biogas slurry) can be used as organic fertilizer that is rich in the elements required by plants (Putri DA et all, 2012). Animal manure is usually disposed into the land. Despite the fact that disposal of animal manure has an advantage for soil fertilizer and harvesting nutrients in feed crops, recent studies showed that limited land for disposal of large amount of wastes and limited feeding processes have become a problem in time. (Bhattacharya and Taylor, 1975). In addition, public health and environment are threatened because animal manure is main source of foul odor, harmful pathogens and noxious gases which are toxic and harmful to living organisms (Sorathiya et al., 2014). Therefore, use of animal manure as a bio-fuel source became crucial in order to prevent accumulation of wastes and environmental damages.

It can be concluded that lignocellulosic compounds which lead rate limitation in hydrolysis step of anaerobic digestion process, negative effect on the performance of anaerobic digesters and reduction in yield is the major problem in energy production from animal manure. Despite the fact that there are many physical and chemical pretreatment studies and some biological pretreatment studies which usually contain anaerobic bacteria for improvement biomethane potential of anaerobic digesters, there is not enough information about using rumen microorganisms at mezophilic stage, method with NGS and QPCR of rumen microorganisims of anaerobic digesters.

2. THEROTICAL BACKGROUND

2.1. Fundamentals of Anaerobic Digestion

The production of biogas from residuals is in many ways an optimum treatment. The gas can be used for electricity and heat production, or can be upgraded for use as vehicle fuel. Furthermore, the waste product from the anaerobic digestion of "clean" substrates, such as; manure, municipal solid waste, and plant residues can be used as a fertilizer on agricultural land (Teghammar et al, 2013). Biogas is formed naturally in different natural environments, such as swamps, the rumen of ruminants, rice fields, landfills, and other anaerobic environments (Angelidaki et al 2003).

Especially methane which is one of the end products of anaerobic digestion is quite important biogas because it provides a renewable alternative for utilization of heat and power. In addition to important renewable energy source, fertilizer production, pathogen removal, pollution control, waste stabilization and odor reduction can be obtained due to anaerobic digestion (Lusk and Moner, 1996).

2.1.1. Biochemistry of Anaerobic Digestion

Anaerobic digestion is used worldwide as a unit treatment for industrial, agricultural and municipal wastes. It involves the degradation and stabilisation of an organic material under anaerobic conditions by microbial organisms and leads to the formation of methane and inorganic products including carbon dioxide:

Organic matter+H_2O $\longrightarrow$ CH_4 +CO_2 + New biomass+NH_3 +H_2S+heat (Kelleher et al., 2000)

Trace gases such as hydrogen sulfide, nitrogen, ammonia, and hydrogen are also formed in the same process (Angelidaki et al., 2003).

The process can be divided into four phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 2.1); in each individual phase, different groups of facultative or obligatory anaerobic microorganisms work together (Gerardi, 2003). The microorganisms use their substrate for a source of energy as well as a carbon source for growth (Tegammar et al., 2013). Hydrolysing and fermenting microorganisms initiate the attack on polymers and monomers found in the waste. Mainly, acetate and hydrogen is produced with varying amounts of volatile fatty acids (VFA) such as propionate and butyrates as well as some alcohols are produced at the end of this stage. The obligate hydrogen producing acetogenic bacteria convert propionate and butyrate into acetate and hydrogen and two groups of methanogenic Archaea produce methane from acetate or hydrogen (Ahring et al., 2003).

The carbon flow in an anaerobic reactor is mainly between the fermentative microorganisms and the methanogens. Only between 20% - 30% of the carbon is converted into intermediate products before being metabolized to methane and carbondioxide (Mackie and Bryant, 1981). The stages of the anaerobic digestion process are hydrolysis, acidogenesis, acetogenesis and methanogenesis. The last stage is considered the rate limiting stage of the process, in which the organic matter is reduced to methane. In the first two stages, organic polymers are hydrolyzed into their monomers and/or fermented into intermediate short chain fatty acids. In acetogenesis stage they are further converted into acetate and H_2/CO_2 and finally in the methanogenesis stage methane is produced from acetate and H_2/CO_2 (Liu et al., 2002).

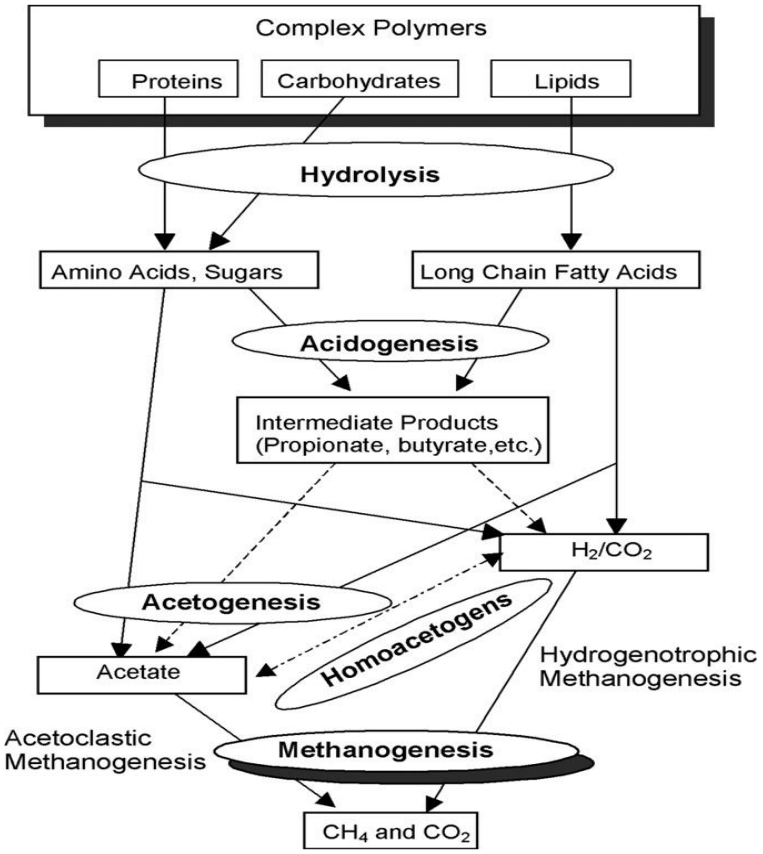

Figure 2.1. Anaerobic degradation pathway (Gujer & Zehnder, 1983).

Three-stage model (Gerardi, 2003), Six-stage model (Lester et al., 1986) and Nine-stage model (Harper and Pohland, 1986) are the models developed for a detailed explanation of biochemical steps in anaerobic digestion. Figure 2.2 is a description of the Nine-step model.

i. Hydrolysis of organic polymers to intermediate organic monomers,

ii. Fermentation of organic monomers,

iii. Oxidation of propionic and butyric acids and alcohols by obligate H2 producing acetogens,

iv. Acetogenic respiration of bicarbonate by homoacetogens,

v. Oxidation of propionic and butyric acids and alcohols by sulphate reducing bacteria (SRB) and nitrate reducing bacteria (NRB),

vi. Oxidation of acetic acid by SRB and NRB,

vii. Oxidation of hydrogen by SRB and NRB,

viii. Acetoclastic methane formation,

ix. Methanogenic respiration of bicarbonate.

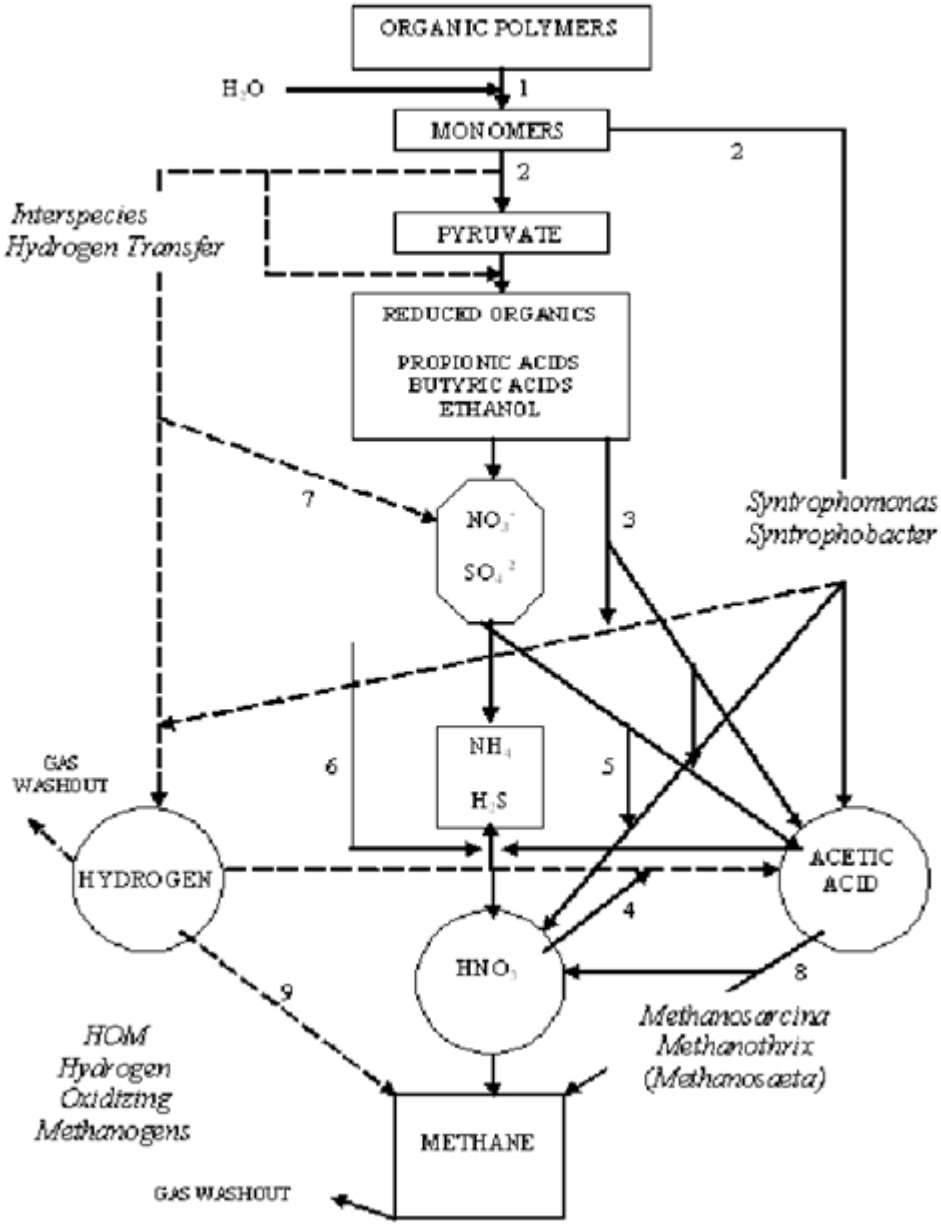

Figure 2.2. Substrate conversion patterns associated with the anaerobic digestion (Harper and Pohland, 1986).

2.1.1.1. Hydrolysis. Hydrolysis is the first step in the anaerobic digestion. During this phase, undissolved compounds, such as polysaccharides, proteins, and fats get degraded into their monomers, such as sugars, amino acids, and fatty acids. This is performed by extracellular hydrolytic enzymes, which use water to cut the covalent bonds in the polymers. The hydrolytic enzymes include cellulases, hemicellulases, amylases, lipases, and proteases (Parawira et al., 2008). Many cellulose-degrading organisms have their enzymes in exoenzyme complexes, called cellulosomes. These complexes are attached to the cellular wall and simultaneously they attach to the substrate for a more effective degradation (Bayer et al., 2008). The hydrolysis of complicated structures, like lignocelluloses, can require weeks, and the degradation is often not complete (Gerardi, 2003). As such, the hydrolysis is the rate-limiting step, while the methanogenesis is considered as the rate-limiting step for readily available substrates (Teghammar et al., 2013; Vavilin et al., 2008).

Some parameters important for the rate of hydrolysis process are: pH, size of particles, production of enzymes, diffusion and adsorption of enzymes on the particles of wastes that go through the digestion process. The group of relative anaerobes of genera has the bacteria carrying out hydrolysis: Streptococcus, Enterobacterium (Bryant, 1979; Smith, 1966). The microbial community of the hydrolysis stage features a significantly heterogenic character. The result of some studies suggested that the degradation of protein and fats is led by *Baccilus spp*., while the compounds containing cellulose are degraded by *Clostridium spp*. (Noike et al., 1985; Lema et al., 1991). The classification of the most widespread hydrolytic microorganisms is as follows: proteoytic (*Clostridium bifermentas, Peptococcus spp*.), lipolytic (genera of *Clostridia* and *Micrococci*), aminolytic (*Clostridium butyricum*, *Bacillus subtilis*), and cellulytic (*Clostridium thermocellum*) bacteria (Payton and Haddock, 1986). The soluble products from the hydrolysis phase are metbolised by the fermentative bacteria inside their cells and converted into several more basic compounds and then the cells excrete them. Carbon dioxide, hydrogen, ammonia and hydrogen sulfide, lactic acid, alcohols, volatile fatty acids, as well as new cells, are the compounds produced (Chernicharo, 2007).

2.1.1.2. Acidogenesis. During anaerobic digestion, following hydrolysis, glucose is fermented primarily to lactic, butyric, or propionic acid. These are in turn fermented to acetic acid. Co-products released from these reactions include CO_2 and H_2 gases.

The first phase, called acidogenesis, converts the products formed during hydrolysis to short chained organic acids. This process is conducted by facultative and obligate anaerobic organisms under anaerobic conditions generated by the consumption of dissolved oxygen by the facultative bacteria. During this stage, the acidifying bacteria convert water-soluble chemical substances, including hydrolysis products to short-chain organic acids (formic, acetic, propionic, butyric, pentanoic), alcohols (methanol, ethanol), aldehydes, carbon dioxide and hydrogen in equations 2.2, 2.3, 2.4, respectively.

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

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

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (2.4)$$

From decomposition of proteins, amino acids and peptides arise, which may be a source of energy for anaerobic microorganisms. Acidogenesis may be two-directional due to the effects of various populations of microorganisms. This process may be divided into two types: Hydrogenation and dehydrogenation. The basic pathway of transformations passes through acetates, CO_2 and H_2, whereas other acidogenesis products play an insignificant role. As a result of these transformations, methanogenes may directly use the new products as substrates and energy source. Accumulation of electrons by compounds such as lactate, ethanol, propionate, butyrate, higher volatile fatty acids is the bacteria's response to an increase in hydrogen concentration in the solution (Equations 2.5, 2.6, and 2.7, respectively).

$$C_{12}H_{22}O_{11} + 9H_2O \rightarrow 4CH_3COO^- + 4HCO^-_3 + 8H^+ + 8H_2 \quad (2.5)$$

$$C_{12}H_{22}O_{11} + 5H_2O \rightarrow 2CH_3CH_2CH_2COO^- + 4HCO^-_2 + 6H^+ + 4H_2 \quad (2.6)$$

$$C_{12}H_{22}O_{11} + 3H_2O \rightarrow 2CH_3COO^- + 2CH_3CH_2COO^- + 2HCO^-_3 + 6H^+ + 2H_2 \quad (2.7)$$

The new products may not be used directly by methanogenic bacteria and must be converted by obligatory bacteria producing hydrogen in the process called acetogenesis. Among the products of acidogenesis, ammonia and hydrogen sulfide which give an intense unpleasant smell to this phase of the process should also be mentioned. The acid phase bacteria belonging to facultative anaerobes use oxygen accidentally introduced into the

process, creating favourable conditions for the development of obligatory anaerobes of the following genera: *Pseudomonas, Bacillus, Clostridium, Micrococcus* or *Flavobacterium* (Zieminski and Frack, 2012).

Acetic acid producing bacteria are *Methanobacterium bryantii, Desulfovibrio Syntrophobacter wolinii, Syntrophomonas wofei* and *Syntrophus buswellii* (Gujer et al., 1983; Stronach et al., 1986; Malina et al., 1992).

2.1.1.3. Acetogenesis. This step is sometimes considered as a part of the acidogenesis and acetate-forming microorganisms that 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; Angelidaki et al., 2007).

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

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

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

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

$$CH_3(CHCH_3)COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2 \quad (2.12)$$

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

$$CH_3CH_2(CHCH_3)COOH + 2H_2O \rightarrow CH_3COOH + CH_3CH_2CH_2COOH + 2H_2 \quad (2.14)$$

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

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 (Angelidaki et al., 2007).

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 and 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 propionate-oxidizing syntroph, *Syntrophus aciditrophicus* which is a syntroph 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. This phase consists in the production of methane by methanogenic archaea. Methane in this phase of the process is produced from substrates which are the products of previous phases, that is, acetic acid, H_2, CO_2 and formate and methanol, methylamine or dimethyl sulfide. Despite the fact that only few methanogens are capable to produce methane from acetic acid, a vast majority of CH_4 arising in the methane digestion process results from acetic acid conversions by heterotrophic methanogens (Demirel and Scherer, 2008). During this process H_2 is used up, which creates good conditions for the development of acid bacteria which give rise to short-chain organic acids in acidification phase and consequently – too low production of H_2 in acetogenic phase. A consequence of such conversions may be gas rich in CO_2, because only its insignificant part will be converted into methane (Ziemisnki et al., 2012).

Methanogenesis is an energy yielding metabolism for every methanogen, which is used for the synthesis of ATP, and occur via two conversion pathways. First one is the decarboxylation of acetic acid and the second one is the reduction of carbon dioxide in the absence of other electron acceptors such as oxygen, nitrate, and sulfate and only bicarbonate and protons act as terminal electron acceptors (Garcia et al., 2000; De Bok et al., 2004; Stams et al., 2006). The substrates for methane fermentation can be divided into three groups (Deublein and Steinhauser, 2008);

- CO_2 type: CO_2, $HCOO^-$, CO
- Methyl type: CH_3OH, CH_3NH_3, $(CH_3)_2NH_2^+$, $(CH_3)_3NH^+$, CH_3SH, $(CH_3)_2S$
- Acetate type: CH_3COO^-

In methanogenesis from $CO_2 + H_2$, electrons for the reduction of CO_2 to CH_4 comes from H_2. However, formate, carbon monoxide and some organic compounds like alcohols can give electrons for CO_2 reduction in some methanogens.

$$CO_2+4H_2 \rightarrow CH_4+2H_2O \qquad \Delta G°=-131kJ \qquad (2.16)$$

In methanogenesis from methyl compounds and acetate, methyl group substances which are listed above as the second class of methanogenic substrates are reduced to methane by two mechanisms. The formation of methane by reducing methyl group substances using an external electron donor such as H_2 is the first mechanism.

$$CH_3OH+H_2 \rightarrow CH_4+H_2O \qquad \Delta G°= -113\ kJ \qquad (2.17)$$

Also, the methyl group substances can be oxidized to CO_2 in order to generate the electrons needed to reduce other molecules of CH_3OH to CH_4 in the absence of H_2.

$$4CH_3OH \rightarrow 3CH_4+CO_2+2H_2O \qquad \Delta G°= -319\ kJ \qquad (2.18)$$

Acetate is the final methanogenic substrate. The conversion mechanism of acetate to methane and carbondioxide called the acetotrophic reaction (Pavlostathis and Gomez, 1991).

$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$ $\Delta G^\circ = -31$ kJ (2.19)

Each of the above reactions is exergonic and can be used to synthesize ATP. Concerning carbon for cellular biosynthesis, CO_2 is the precursor for all cellular components when growing on CO_2+H_2. If methanogenic substrates are acetate or methylated compounds, these compounds are also used in the organic cell components with the fixation of some CO_2. Characteristics of methanogens take place in the process are expalined further down.

Methanogens are obligate anaerobes and they are very hard to culture. Most of them thrive under extreme coditions. All methanogens, belong to the Euarchaeota group of Archaea and they exist in diverse types of habitats and show different morphologies. Anoxic sediments, hydrotermal vents and the digestive tracks of animals are only some places they are present and these places are the main source of biogenic methane in nature. (Madigan, 2008). Also, methanogens are diverse in terms of cell wall chemistries. For example, *Methanobacterium* species and relatives have pseudomurein cell wall whereas *Methanosarcina* and relatives have cell walls that contain methanochondroitin. A summary of the characteristics of methanogens is shown on Table 2.1.

Woese et al. (1990) proposed a new classification for living organisms, dividing life on earth into three major domains: bacteria, Archaea and eukarya (Figure 2.1). The unique phylogenetic status and evolutinary divergence of Archaea suggest that they should exhibit wide physiological diversity. However, traditional culture-based studies have led to belief that opposite was the case. Two major lineages of Archaea are Crenarchaeota and Euryarchaeota. The first kingdom, Crenarchaeota derived from being phylogenetically close to ancestor or source of Archaea (Woese et al., 1990). It was believed to include only sulphur-dependent extreme thermophiles. Euryarchaeota is a heterogenous group comprimising a broad spectrum of organisms with varied patterns of metabolism from different habitats. It includes extreme halophiles, methanogens and some extreme thermophiles so far. Moreover, a third archaeal kingdom has been discovered which is reported isolation of several archaeal sequences evolutinary distant from all Archaea known to date by Barns et al. in 1994 and then in 1996. The new group was placed on phyologenetic tree under Crenarchaeota/Euryarchaeota and named as Korarchaeota.

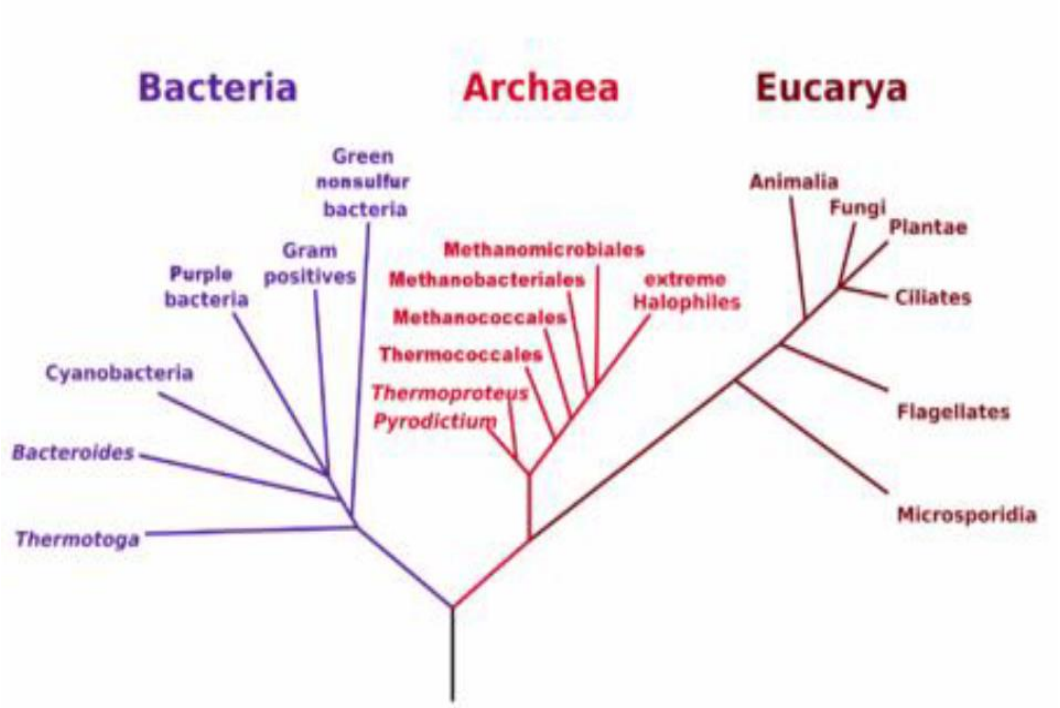

Figure 2.3. Universal phylogenetic tree (Woese et al, 1990).

Characteristics of the population of bacteria and archaeanes in anaerobic thermophilic processing of manure indicated a dominance of two species: *Methanoculleus thermophilicus* (hydrogenotrophic) and *Methanosarcina thermophila* (acetotrophic). The main hydrogentrophic microorganisms, participating in anaerobic processing of fruit and vegetable wastes comprise *Methanosphaerastadtmanii* and *Methanobrevibacterwolinii* (Bouallagui et al., 2004). Methanogens are classified into five orders within kingdom *Archaeobacteria*: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales* and *Methanopyrales*(Garcia et al., 2000).

Table 2.1. Characteristics of the methanogens (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
Metahanoplanus	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		
Methanosarcina	Large irregular cocci in packets	$H_2 + CO_2$, methanol, methylamines, acetate
Methanolobus	Irregular cocci in aggregates	Methanol, methylamines
Methanohalobium	ırregular cocci	Methanol, methylamines (halophilic)
Methanococcoides	ırregular cocci	Methanol, methylamines
Methanohalophilus	ırregular cocci	Methanol, methylamines, methyl sulfides (halophilic)
Methanosaeta	Long roads to filaments	Acetate
Methanopyrales		
Methanopyrus	Rods in chains	CO_2 (hyperthermophilic)

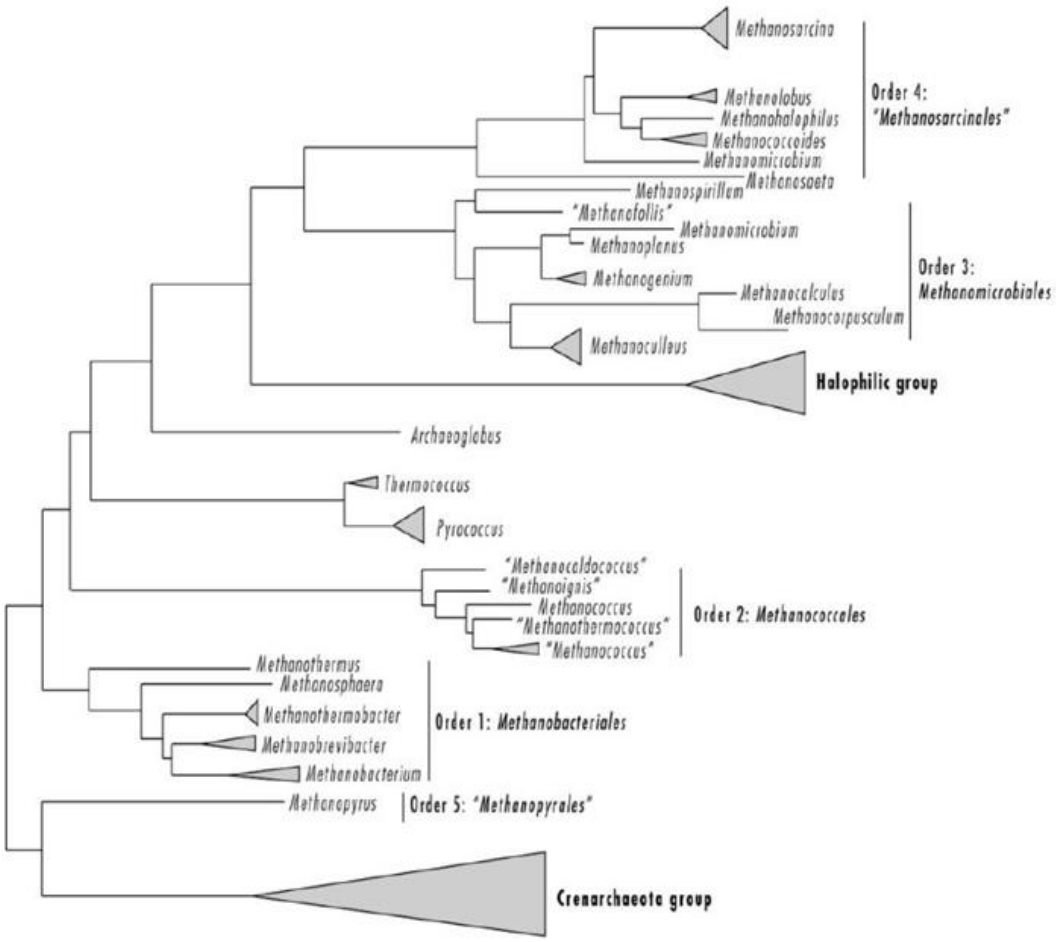

Figure 2.4. Phylogeny of methanogens, domain Archaea (Garcia et al., 2000).

Methanogens can only use a limited number of substrates, comprising acetic acid, hydrogen/carbon dioxide, formic acid, methanol, methylamines and carbon monoxide. Methanogens are divived into two main groups according to their affinity for these substrates, one that forms methane using acetic acid or methanol, and the other one that produces methane from hydrogen and carbon dioxide:

- acetate-using microorganisms (acetoclastic methanogens)
- hydrogen-using microorganisms (hydrogenotrophic methanogens)

Acetoclastic methanogens are only a few of the methanogenic species that are capable

of forming methane from acetate, which usually dominate the anaerobic digestion systems. They are responsible for about 60 to 70% of all methane production. Two genera utilize acetate to produce methane: *Methanosarcina* prevails above methane, while *Methanosaeta* prevails below this acetate level (Zinder, 1993). *Methanosaeta* usually have lower yields and is more sensitive to pH changes, comparing to *Methanosarcina* (Schmidt and Arhing, 1996). While *Methanosarcina* has a greater growth rate, *Methanosaeta* needs longer solids retention time. *Methanosaeta* genus is characterized by exclusive use of acetate and having a higher affinity with it than *Methanosarcina* genus. *Methanosarcina* genus are considered as the most versatile ones among the methanogenic microorganisms, since they can also use hydrogen and methylamines (Soubes, 1994).

$$C^*H_3COOH \rightarrow C^*H_4 + CO_2 \quad (2.1)$$

(Microbial group involved: acetoclastic methanogenic microorganisms)

Hydrogenotrophic methanogens are capable of producing methane from hydrogen and carbon dioxide. *Methanobacterium*, *Methanospirillum* and *Methanobrevibacter* are the genera more frequently isolated in anaerobic reactors (Chernicharo, 2007).

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (2.2)$$

(Microbial group involved: hydrogenotrophic methanogenic microorganisms)

2.1.2. Environmental and operational factors affecting anaerobic digestion process

There are various parameters affecting the performance of anaerobic digestion such as temperature, hydraulic retention time (HRT), solid retention time (SRT), organic loading rate (OLR), pH, alkalinity, micro and macronutrients.

Temperature is one of the most important parameters which affect the anaerobic digestion process in many ways such as; ionization equilibrium, solubility of substrates, substrate removal rate and other constants such as specific growth rate, decay biomass yield, and half saturation constant. Although it is known that anaerobic digestion process can take place within a large temperature range, it is optimal at mesophilic (35 ºC - 42 ºC) and thermophilic (45 ºC - 60 ºC) conditions. It is important to maintain a constant temperature

during the anaerobic digestion process, for biogas production it is negatively affected by temperature fluctuations. In most cases thermophilic reactors exhibit less methanogenic diversity and therefore it can be said that thermophilic reactors are more vulnerable to temperature fluctuations (Karakashev et al., 2005; Leven et al., 2007). Mesophilic microflora are able to tolerate temperature fluctuations within ±3ºC without considerable reductions in methane production. Under thermophilic conditions, growth rate of anaerobic microorganisms are higher, therefore process is faster and more efficient. Under optimal operating conditions, a thermophilic reactor can be fed with higher organic loading rates at lower HRT than mesophilic reactors, however higher temperatures makes the system imbalanced and susceptible to failure (Weiland, 2010).

In addition to all of them, mesophilic and thermophilic conditions have both advantages and disadvantages, as well. Thermophilic digestion provides pathogen destruction, higher substrate degradation and higher biogas production. On the other hand, thermophilic microorganisms are more sensitive to changes is environmental conditions such as pH, temperature fluctuations and toxins (Gerardi, 2003; Angelidaki et al., 2007; 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.

The hydraulic retention time (HRT), described as a criterion on biogas production and waste stabilization, is another factor affecting anaerobic digestion (Thakur, 2006). Because optimum HRT depends on substrate characterization and temperature, it can be different from various substrates and temperature conditions. However, it should be sufficiently long in order to provide microbial growth for processes of anaerobic digestion. If the HRT is too short, the organic material cannot be completely degraded and it leads to low biogas production, washout of the microorganisms and inhibition of the process. It was proven that the retention times range between 25 - 35 days at mesophilic and thermophilic conditions (Kim et al., 2006).

The solids retention time (SRT) relating to growth rate of microorganisms and to anaerobic digester volume is also one of the significant parameters for anaerobic digestion. It is the same with HRT if there is no recycling or supernatant withdrawal (Bolzonella et al., 2005). It is required to properly choose SRT and volume of digester because the digestion process is a function of time required by microorganisms to digest the organic material. It was found that the optimum SRT in digesters is about 30 days for mesophilic digestion and longer for low-temperature digestion (Metcalf and Eddy, 2003).

One of the other important parameters for anaerobic digestion performance is the organic loading rate (OLR). It is a certain amount of organic matter which is fed daily per m^3 of digester working volume and generally described as volatile solids (VS). If nutrients in digester can be easily degraded, anaerobic digestion process can be affected because of acidification phase which has more end-products. Thus, OLR is considerably important for methanogenic activity and biogas production (Rincón, 2008).

pH is also another significant factor that affects anaerobic digestion systems. 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). It is considerably pivotal parameter because the solubility of matters and reaction potential of microorganism and so all digestion performance are directly influenced by pH. It was investigated that the optimal range of pH is 6.5–7.5 in anaerobic digestion to provide maximum biogas efficiency, however, the range of pH can be relatively wide due to different types of substrate and digestion techniques (Liu et al., 2008). Microorganisms in anaerobic digesters are also important factor to determine the optimum pH. While most methanogens function in a pH between 6.5 and 7.5, anaerobic bacteria can generally grow in pH between 6-8. However, fluctuations from optimum range of pH can cause excessive production and aggregation of acidic or basic conversion products like organic fatty acids or ammonia. On the other hand, the accumulation of VFA cannot usually result in a pH drop owing to the buffering capacity of the substrate (Zoetemeyer et al., 1982). In addition, if alkalinty is not sufuciently high, organic acids producing by acidogenic bacteria lead yo decrase ph. However, the

biocarbonate which is produced by methanogens can be buffered the reduction of pH under normal conditions. If buffering capacity cannot be enough, especially unfavorable environmental conditions, acidity can lead inhibitory effect on methanogens. Yet, acidogenetic bacteria and anaerobic fungi are more resistant to acidity (Malina and Pohland, 1992).

Mixing is a very important parameter in anaerobic digesters, especially operating with particulate substrate like manure. Thorough mixing of the substrate in the digester distributes organisms uniformly and also transfers heat, and thus is regarded as essential in high-rate anaerobic digesters (Sawyer and Grumbling, 1960; Meynell, 1976). Furthermore, agitation helps to reduce particle size as digestion progresses and to release biogas from the mixture. The importance of mixing in achieving efficient substrate conversion has been noted by many researchers (Casey, 1986; Smith et al., 1996), although the optimal mixing pattern is a subject of much debate. An intermediate degree of mixing appears to be optimal for substrate conversion (Smith et al., 1996).

Mixing can be accomplished by mechanical mixers, biogas recirculation, or by slurry recirculation. Mechanical mixers are reported to be most efficient in terms of power consumption (Brade and Noone, 1981). However, the internal fittings and equipment are not accessible for maintenance during digester operation, and long term reliability of operation is of paramount importance. In general, such reliability can be more readily attained with biogas or liquor recirculation systems, where there are no moving parts within the digester (Casey, 1986). Interestingly, in other literature sources it has been reported that biogas recirculation is the most efficient mode of mixing for anaerobic digesters (Morgan and Neuspiel, 1958; Kontandt and Roediger, 1977; Lee et al., 1995). Mixing of the anaerobic lab-scale digesters can vary between 20-100 rpm (Wu et al., 2010). In some studies, digesters were manually shaken once a day (El-Mashad and Zhang, 2010). Mixing in high rpms is difficult to be obtained in full scale digesters. Mixing in full scale digesters can also be performed by intermittent and minimal mixing which refer to mixing for 10 minutes prior to feeding and withholding mixing for 2 h prior to feeding, respectively (Kaparaju et al., 2008). Schreding is also an important application in biogas digesters. Decreasing the particle size by implementing a macerating unit with knives was found to increase biogas production in digesters operating with manure (Hartman et al., 2000).

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 use 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 lingo-cellulosic 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 slows 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 hydrolysing 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 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).

For the growth and survival of the existing groups of microorganisms in anaerobic digesters, certain macro and micro nutrients are essential. Micronutrients which are also called as trace elements such as iron, nickel, cobalt, selenium, molybdenum, and tungsten and macronutrients such as carbon, nitrogen and phosphorus are other important factors in anaerobic digestions due to microbial growth and survival (Speece and Parkin, 1983). For methanogenesis, iron, nickel, magnesium, calcium, sodium, barium, tungstate, molybdate, selenium and cobalt are considerably important. Selenium, tungsten and nickel are required for the enzyme systems of acetogenesis and methanogenesis (Henze and Harremoes, 1983). Although the micronutrients in low amount are sufficient, Preißler et al. (2009) showed that additional micronutrients always improve the performance of anaerobic digestion processes. The addition of macronutrients also provides positive impact on anaerobic digestion process and so biogas potential.

Light metal ions and heavy metals are also required to growth microorganisms and provide specific growth rate like any other nutrients in anaerobic digesters (Chen et al., 2008). Sodium, potassium, magnesium and calcium as the most significant light metal ions stimulate microbial growth in anaerobic systems, however, excess quantity of them cause to decelerate the growth and also can cause severe inhibition or toxicity (Soto et al., 1993). Heavy metals such as chromium, iron, cobalt, copper, zinc, cadmium, and nickel can be also found in important concentrations in anaerobic digesters. However, they can induce toxic effect on anaerobic processes because they cannot be biodegraded and they can accumulate on the system (Jin et al., 1998). Thus, heavy metals should be present at trace amount for microbial activity and avoiding potential toxicity (Chen et al., 2008).

Other inhibitory substances are also important because they cause severely failures of anaerobic digestion processes. These materials lead to change in the microbial population or inhibition of bacterial growth. Fundamental indicators for inhibition are accumulation of organic acids and decreasing of rate of biogas production (Chen et al., 2008). Although it was known that the inhibition can impact all groups of microorganisms in the anaerobic

digestion processes, such as, bacteria, archaea and anaerobic fungi, methanogens are the most susceptive groups to inhibitory or toxic material (Speece and Parkin, 1983).

Ammonia which is produced by the break down of nitrogenous compounds is one of the important inhibitory factors affecting anaerobic digestion performance. Although ammonia inhibition was observed to start at concentrations of 1500–2500 mg-N/litre, adaptation of the biogas process to ammonia, tolerance to 4 g-N/litre total ammonia was showed (Hashimoto, 1986). However, 3000 mg/L of ammonia may have inhibitory effects on methanogens because they are the least resistant microorganisms to ammonia inhibition (Chen et al., 2008). On the other hand, it was generally asserted that 50-200 mg/L of ammonia is beneficial, 200-1000 mg/L of ammonia is no adverse effect, 1500-3000 mg/L of ammonia is inhibitor for pH > 7.4 to 7.6 and above 3000 mg/L of ammonia is toxic on anaerobic processes (McCarty, 1964). Inorganic nitrogen is found in the forms of ammonuim (NH_4^+) and free ammonia (NH3) in anaerobic digesters. The free ammonia concentration depends mostly on the total ammonia concentration, temperature and pH. It was showed that thermophilic temperatures can more easily inhibit the methane fermentation of high ammonia-containing anaerobic digesters (Angelidaki and Ahring, 1994).

Another inhibitory parameter that affects the anaerobic digestion process is sulfate because of H_2S which is the toxic form of sulfide. It is reduced to sulfide by the sulfate reducing bacteria (SRB) in anaerobic digesters and H_2S leads to penetrate into cells. (Hilton and Oleszkiewicz, 1988). Therefore, sulfate can inhibit metanogenesis owing to the competition for acetate and hydrogen by SRBs. In addition, sulfides can be produced by sulfur containing inorganic compounds during the biological production in the anaerobic digestion. If concentration of soluble sulfide is less than 100 mg/L, it can be tolerated. However, Stronach et al. (1986) demonstrated that higher than 200 mg/L of sulfate directly cause inhibitory impact on anaerobic digestion systems.

Another example for the inhibition in anaerobic digestion systems is organic chemicals. Because they cannot be sufficiently dissolved in the water, they are absorbed by surfaces of solids. Thus, organic chemicals accumulate and cause the membranes of bacteria to swell and leak, disrupting ion gradients and finally providing cell lysis (Heipieper et al., 1994; Sikkema et al., 1994). Concentration of toxic materials, concentration of biomass, toxicant

exposure time, sludge age, feeding, acclimation and temperature are the most significant parameters influencing the inhibition of organic chemicals (Lay et al., 2003).

The concentration of VFA which are produced from complex organic material by acidogenic microorganisms in anaerobic digesters is another significant consideration for efficient performance of digesters (Wang et al., 1999). Because VFAs are considerably related to the changes in pH, alkalinity, and the activity of methanogens, they are one of the most sensitive indicators in order to measure the performance of anaerobic digesters. While acetic acid/acetate, propionic acid/propionate, butyric acid/butyrate, valeric acid/valerate, caproic acid/caproate, and enanthic acid/enanthate are main groups of VFAs, acetate and propionate are the predominant VFAs (Labatut and Gooch, 2012). Acetate, hydrogen and carbon dioxide, which are used methanogens for the generation of methane, are produced as a result of oxidation of the VFAs (Öztürk et al., 1993). Thus, VFAs are considerably significant intermediary products in the metabolic pathway of methane production. If they can cause inhibitory effect on anaerobic digestion processes, system failure can arise and biogas production can be negatively affected (Labatut and Gooch, 2012). Accumulation of VFA production can lead to inhibit the methane production. Moreover, microbial activity balance in anaerobic digesters can be readily disturbed by increasing of VFA and decreasing of methane production (Ahring and Westermann, 1983). Especially, it was reported that 35 mg/L of acetic acid, higher than 3000 mg/L of propionic acid and 1000 mg/L of butyrate concentration inhibit the microbial growth by Ianotti and Fischer (1983). Labatut and Gooch (2012) also reported that biogas production can be limited at VFA concentrations over 1500 – 2000 mg/L.

In addition to above mentioned parameters, some operational factors such as mixing and types of digester are also considerably important for anaerobic digestion performance (Brade and Noone, 1981). Because mixing provides the complete contact between the reactor contents and the biomass, it is particularly significant for anaerobic digesters operating with particulate substrates. The possible inhibitory impacts of local VFA accumulations and other digestion products can be also reduced by mixing. Mechanical mixers, biogas recirculation, or slurry recirculation can be used to accomplish the mixing. While mixing can vary between 20-100 rpm in lab-scale anaerobic digesters, mixing in high rpms is difficult to be obtained in full scale digesters (Wu et al., 2010).

The design of a digester is other conspicuous operational factor because of composition, homogeneity and the dry matter content of the anaerobic digester system. For animal manure and algal biomass which is rich in terms of solid materials, the high-rate reactors are not quite suitable due to granule formation causing coagulation. Anaerobic digestion systems can be performed in batch-wise, semi-continuous or continuous mode. While there isn't any addition of wastes during anaerobic digestion process in a batch system, quantities of waste are periodically added and removed to a digester leading to a de facto semi-continuous system. The raw waste is fed regularly into a digester, displacing an equal volume of digested material in the continuous-flow tank reactor systems (Wu et al., 2010).

2.2. Concept of Anaerobic Digestion of Agro-Industry Wastes

2.2.1. Feedstocks for biogas production

Each year, millions of tons of wastes are generated from agricultural, municipal and industrial sources. Animal manure, generated from livestock industries and agricultural activities, have been identified as a major source of environmental pollution. In the EU-27 alone, more than 1500 million tons of animal manure is produced every year and European agriculture handles more than 65% of livestock manure as slurry, liquid mixture of feces, urine, water and bedding material (Menzi, 2002). These large amounts of animal manure and slurries produced today represent a great pollution risk with a potential negative impact on the environment, if they are not managed properly (Holm-Nielsen et al., 2009).

Any type of organic waste can be used as a substrate for the anaerobic digestion process as long as it contains carbohydrates, fats and lipids. The composition of the biogas and methane yield depends on the type of the feedstock, retention time and digestion system (Braun, 2007). The theoretical gas yield happens to change with the varying amount of carbohydrates, proteins, and fats. Only strong lignified organic substances, e.g., wood, are not suitable due to the slowly anaerobic decomposition. The real methane content in practice is generally higher than the theoretical values for a part of CO_2 is solubilized in the digestate (Weiland, 2003).

Animal manure becomes a major source of air and water pollution, when untreated or poorly managed. Some of the major problems include nutrient leaching, mainly nitrogen and

phosphorous, ammonia evaporation and pathogen contamination. The animal production sector causes 18% of the overall greenhouse gas emissions, measured in CO_2 equivalent and for 37% of the anthropogenic methane, which has 23 times the global warming potential of CO_2. Furthermore, 64% of anthropogenic ammonia emission and 65% of anthropogenic nitrous oxide originates from the animal production sector all over the world (Steinfeld et al., 2006).

2.2.2. Process technology of biogas production

Biomass can be converted into biogas production which is useful form of energy using a number of different processes, but, these processes can be generally classified as wet and dry fermentation (McKendry, 2002). While wet digestion processes are performed with TS concentration less than 10% providing the application of completely stirred digesters, TS concentration between 15% and 35% is required to operate dry digestion processes. Although dry digestion processes are operated both batch and continuously, the wet digestion processes are operated only continuously (Weiland, 2010).

A lot of different kinds of biogas plants are applied in anaerobic process technology. Vertical continuously stirred tank fermenter is the most widespread wet fermentation reactor. Generally, the fermenter's roof is covered with a membrane layer in order to store the gas before utilization. Mechanical, hydraulic or pneumatic mixing can be used so as to provide the stirring in the reactors. Thus, microorganisms can contact with the substrate, facilitate the up-flow of gas bubbles and obtain stable temperature conditions in the reactors. In order to provide them, mechanical stirring equipment is usually used in biogas plants (Gemmeke et al., 2009).

Another type of wet fermentation reactor using anaerobic digestion of biogas is horizontal digesters. Horizontal digesters are plug-flow systems which are equipped with a low rotating horizontal paddle mixer. Because they can be operated at higher total solids concentrations of the input, paddle mixer are used for the first stage of two-stage reactor configurations. In addition, reactor volume is limited to 700 m^3 because of economical and technical reasons (Weiland, 2010).

Wet fermenters are usually operated at mesophilic temperatures up to 42 ºC. Despite of the higher temperatures, the degradation rate is faster and system can be operated at smaller HRTs with smaller reactor volumes (Weiland, 2010).

Batch reactors are used without mechanical mixing for dry fermentation processes. The substrate is loaded in the reactor and is mixed with inoculum. Because the necessary share of solid inoculums can be different each other, it should be determined individually for each substrate (Bonwin, 1998). The gas yields of dry fermenter are almost the same with the wet fermenters. Moisture content and the temperature through the digestion process are controlled by water spread on the substrate to accelerate start up and inoculation (Heiermann et al., 2007).

Continuous dry fermentation can be operated for substrates which have more than 25% of TS. Horizontal mechanically mixed fermenter or vertical plug flow fermenter can be used for continuous dry fermentation (De Baere and Mattheeuws, 2008).

The anaerobic digestion process can be generally performed in a single or multi step process. While the steps of anaerobic degradation are conducted in a single reactor in single phase digestion systems, hydrolysis and acidogenesis steps are seperated from the others in two phase digestion systems. This system provides the better acclimation of biomass to the substrate. Thus, the stability and rate of degredation can be improved in two phase digestion systems. However, control of operation and process parameters are difficult in these systems (Vieitez and Gosh, 1999). In addition, methane and hydrogen, generated from improper hydrolysis stage, can be formed in extent amount and they lead energy losses. Thus, the climate can be adversely affected by the gaseous emitted to the atmosphere (Oechsner and Lemmer, 2009).

Biogas produced as a consequence of anaerobic digestion process is composed of the mixture of methane (CH_4; 50%-85% by volume), carbondioxide (CO_2; 15%-50% by volume) and trace gases such as hydrogen sulfide (H_2S), carbonmonoxide (CO) or hydrogen (H_2). Before the utilization of biogas, biogas is saturated with water vapor which is called dewatering and the gases should be eliminated from all gas contaminants and the upgraded gas must have a methane content of more than 95% in order to apply the quality requirements

of the different gas equipments. In addition, H_2S concentration should be decreased to at least 250 ppm by biological desulfurization (Weiland et al., 2010).

Although studies showed that production of methane rich biogas can be provided by anaerobic digestion processes, lignocellulosic compounds can cause rate limitation in hydrolysis which is the first step of anaerobic digestion, negative effect on the performance of anaerobic digesters and reduction in yield during energy production from animal manure under anaerobic conditions. In order to eliminate these major problems, additional studies are required (Bayane and Guoit, 2011).

Animal manure as an important biomass source can contain cellulosic compounds due to herbal nutrition which cannot be adequately digested. Therefore, lignocellulosic biomass in animal manure is actually considered as a potential energy resource for biogas production (Bayane and Guoit, 2011).

Rumen fluid of animal ruminant was used as inoculums to increase biogas production rate from cattle manure at mesophilic condition. In this study Budiyono et al used 400 ml biodigester and also they said this is optimum batch operation mod. Optimum temperature is the room temperature. The results showed that the rumen fluid inoculated to biodigester significantly effected the biogas production. Rumen fluid inoculums caused biogas production rate and efficiency increase more than two times in compare to manure substrate without rumen fluid inoculums (Budiyono, 2014).

Rabiu at al, used fresh cattle manure (M) was assigned to each biodigester and mixed with rumen fluid (R) and distilled water (W) into three different M:W:R ratio; 1:1:0; 1:0.5:0.5; and 1:0:1 respectively. All the treatments were prepared in triplicates. The pH of the slurry was recorded before and after the biogas production was determined. The best performance biogas production was observed if the rumen fluid used between the ranges of 25–50% of rumen fluid. Cattle manure collected after 12 h of defecation recorded with the highest biogas production compared to 0 h and 24 h of cattle post-defecation (Rabiu, 2014).

Budinyo et al, mentioned that fresh cattle manure, rumen fluid and tap water ratio and best performance of biogas production temperature. M:W:R ratio contents i.e. 1:1:0;

1:0.75:0.25; 1:0.5:0.5; 1:0.25:0.75; and 1:0:1 (correspond to 0; 12.5; 25, 37.5; 50, and 100 % rumen, respectively). The research showed that, either in roomtemperature as well as in 38.5^{o}C, the best performance of biogas production was obtained with rumen fluid in the range of 25-50 %. Increasing rumen content will also increase biogas production (Budinyo, 2009).

According to Onakughotor, the substrate used is cattle manure and the inoculum used is rumen fluid. The chemical oxygen demand (COD) of the cow manure was determined to be 9200 mg/L. The results showed that biogas production increase with increased in the amount of inocula (Onakoughotor, 2015).

Oladeji et al., project work was on generation of biogas using cow dung and rumen fluid as co-substrate. A biogas digester with a capacity of 105 L was used to produce the gas. The substrate (cow dung and rumen fluid) was mixed in the ratio 3:2 and water to substrate ratio of 2:1 was used. Result of this study showed that methane has the highest percentage and generally cow dung with rumen fluid easily lent itself to process of anaerobic digestion (Oladeji, 2016).

2.2.3. Limitations due to inefficient hydrolysis of bio solids

Biogas is a product of anaerobic degradation of organicsubstrates, which is one of the oldest processesused for the treatment of industrial wastes and stabilization of sludges (Yadvika et al., 2004). Decomposition of organic material by anaerobic decomposers takes place in four stages called hydrolysis, acidogenesis, acetogenesis and methanogenesis in which different microorganisms show syntrophic relationship (Khanal, 2008). The most important problem in energy production from animal manure under anaerobic conditions is cellulosic compounds leading to hydrolysis as rate-limiting step. These substances have a negative impact on the performance of anaerobic digesters and also they reduce the yield. In the literature, there are some physical pretreatment methods such as pyrolysis, mechanical comminution and chemical pretreatment methods like ozonolysis, acid hydrolysis (Kumar et al., 2009). However, biological pretreatment method which contains enzymes and microorganisms that naturally digest lignocellulosic compounds in their natural environment is a remarkable alternative so as to improve the biogas potential of anaerobic digester

(Nkemka et al., 2015). Thus, rumen microorganisms are considered as one of the biological pretreatment options for enhancing of anaerobic digestion in recent years. Rumen bacteria in herbivorous animals' digestive track are evaluated as an alternative solution to overcome this problem. Herbivorous animals use cellulosic materials as an energy source by transforming to volatile fatty acids via fermentation as a result of symbiotic relationship of microbial population in digestive tract. High cellulolytic activity of rumen bacteria is an advantage in treatment process of lignocellulosic wastes in anaerobic digesters. There are some studies in literature on potential applications of these microorganisms on anaerobic digesters (Guiot et al, 2010; Özel et al, 2009). The practice of the rumen-based microbial fermentation technique into industrial anaerobic digestion systems was also considered by researchers in order to decrease and stabilize lignocellulosic compounds with recovery of biogas as renewable energy (Barnes and Keller, 2003).

2.2.4. Improvment of biomethanation using bio agents

To date, many alternatives have been applied to treat and dispose animal manure. Pond systems (Wang et al., 1996), composting (Tiqua and Tam, 1998; Guerra Rodriquez et al., 2001), land application (Sommer and Hutchings, 2001; Araji et al., 2001), constructed wetlands (Knight et al., 2000; Clark and Baldwin, 2002), anaerobic treatment (Lo and Liao, 1984; Wen et al., 2007; Alvarez and Giden, 2009) are examples of these techniques. The researches show that anaerobic digestion offers the best solution in terms of pollution reduction and energy production, which also improves the fertilize value of the manure.

The ruminant differs from other mammals in that its food is subjected to microbial fermentation in the rumen before it passes on to the true stomach and intestinal tract where normal mammalian digestion occurs. Processes taking place in the rumen due to microbial activity include the degradation of carbonhydrates such as cellulose that can not be utilized unless digested by microorganisms and those such as starch and certain sugars that can be utilized by the animal without microbial action (Weimer 1996; Miron 2001). Proteins, organic acids, and many other feed constituents are also attacked. The principal products are volatile fatty acids, carbon dioxide, methane, ammonia, and microbial cells. The fatty acids and many constituents of the microbial cells such as vitamins and protein are utilized by the animal. The ruminal fermentation has a considerable effect on metabolic processes of the

animal and the functions of the microorganisms are intimately associated with certain metabolic disorders of ruminants. It has been increasingly realized that more fundamental knowledge of the ruminal microorganisms is needed to obtain more efficient rations and better control of metabolic disorders (Moran, 2005; Bryant, 1997; Alataş et al., 2011).

Herbivore animals can convert the plant materials containing cellulose to volatile fatty acids as a result of the simbitoic relationship of microbial populations found in their alimentary canal (Forsberg et al., 1997). The stomach of these animals comprise of 4 compartments called Rumen, reticulum, omasum and abomasum. Digestion in the first there parts rely on microbial activity. Over 60% of digestive activities take place in reticulorumen (Özel and Sarıçiçek, 2009). This symbiotic microbiota comprising of bacteria, fungi and protozoa enables the animal to digest plant fibers (Koike et al., 2003). Rumen's pH varies between 5.5- 7.0 and temperature varies between 39-41ºC (Özel and Sarıçiçek, 2009). The methanogens also operate within three temperature ranges namely; Psychrophilic temperature (< 25°C), mesophilic (25-40°C) and thermophilic (45-60°C). Potentially, all organic waste materials contain adequate quantities of the nutrient essential for the growth and metabolism of anaerobic bacteria in biogas production. The most important representatives of Rumen microorganisms were identified in previous studies. However, microorganisms that were cultured were only one minority of this ecosystem (Rosero et al., 2012). *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* ve *Ruminococcus* are recognised as the representatives of cellulotic bacteria found in the rumen. In the literature (Tajima et al., 1999; 2001; Koike et al., 2003), rumen bacteria were cultivated but only 20% of the present population were cultured. Therefore, in order to clarify the digestion process in the rumen completely, bacteria that have not been investigated before should be studied together with the bacteria that were studied. Cellulotic activities of rumen bacteria cause a benefit in the treatment of lignocellulotic wastes by anaerobic decomposers. There are studies about the potential application of these microorganisms in anaerobic decomposers. In the studies carried out, it was found out that the yield of hydrolysis increases when ruminal fluid was used as a vaccine in anaerobic systems in which cellulose rich substrates were refined (Gijzen et al., 1987; 1988; Camp et al., 1989; Yue et al., 2007; Hu et al., 2005; Hu et al., 2008; Hu et al., 2012; Zhou et al., 2012; Quintero et al., 2012).

Rumen is primarily due to the activities of the ruminal cellulolytic bacteria (RCB) , in particular three predominant species: *Fibrobacter* (formerly *Bacteroides) succinogenes, Ruminococcus flavefaciens,* and *Rumiizococcus albus.* These three species have common characteristics that set them apart from other ruminal bacteria [including secondary cellulolytic species, such as *Butyrivibrio fibrisolvens*, *Clostridiurn longisporium,* and *Clostridium locheadii* and from cellulolytic bacteria from habitats other than the intestine.

One of the more obvious characteristics of the predominant RCB is their nutritional specialization. Most ruminal bacteria that ferment carbohydrates are capable of using numerous monosaccharides and disaccharides as growth substrates, and even those species with limited capability for digesting cellulose can utilize at least a few of these sugars. By contrast, *F. succinogenes* and the ruminococci are nearly restricted to cellulose and its hydrolytic products as growth substrates. The consequence of this nutritional specialization is that the primary means by which these species gain selective advantage in the rumen is by optimizing only two catabolic activities: cellulose hydrolysis (depolymerization) and efficient utilization of the hydrolytic products (cellodextrins) (Weimer, 1996).

2.2.5. Digestate production and application

The residues coming from the anaerobic digestion process are called digestate. Digestate is more balanced in C/N ratio than raw manure and can be used as organic fertilizer on farmlands (Holm-Nielsen et al., 2009; Rico et al., 2011). After digestion effective ratio of slurry increases significantly which increases short-term N fertilization (Monnet, 2003; Weiland, 2010; Masse et al., 2011). Use of digestate as fertilizer decreases use of mineral fertilizer (de Vries et al., 2010). Digestate is also less odorous and hygenic than raw manure. Soil penetration capacity of digestate is high, so loss of nitrogen to ammonia is low. These attributes makes digestate highly suitable for usage as organic fertilizer (Weiland, 2010).

2.2.6. Biogas as a renewable energy source

Until recently, fossil fuels have been used as the main energy source. As a result of this, greenhouse gases accumulated in the atmosphere have caused a change in the climate.

Carbon dioxide in the atmosphere increased to 375 ppm in the last 50 years from 313 ppm. The increase in carbon emission can be seen in Figure 2.5 (Rohde, 2007).

Recently, the governments have been heading towards alternative energy sources which have less carbon emission, to decrease the greenhouse gases in the atmosphere and prevent the climate change. In the Kyoto protocol, research on renewable energy sources was suggested (United Nations, 1998).

Biomass energy is much useful than other renewable energy sources. Biomass can be found almost everywhere and can be stored. Because of this, biomass is a non-stop energy source comparing to other sources such as wind energy. Another advantage of biomass is its availability for both electricity and heat (Karaosmanoğlu, 2004). Biogas systems can be used as heating source by small services such as farms. In larger services, a biogas facility can be used as electricity generator. Thus, biogas can be used as household, commercial and also industrial applications.

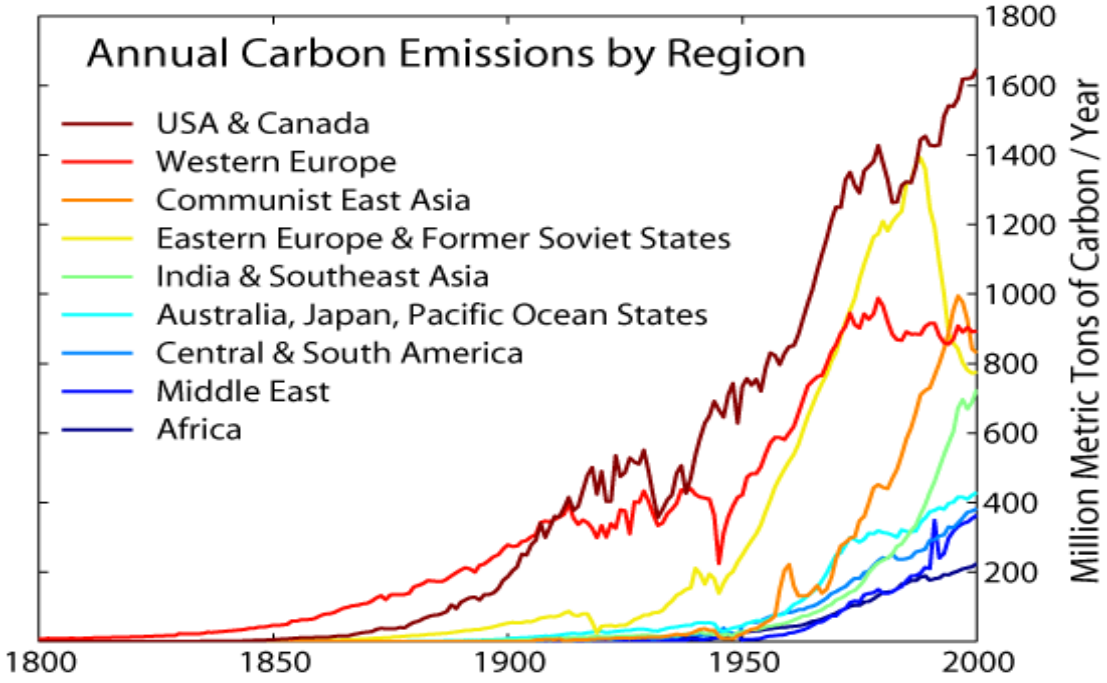

Figure 2.5. Annual carbon emissions by region (1800-2000) (Rohde, 2007).

One of the sources of biomass to be used for biogas production is the animal manure. Animal manure is a very rich source by means of organic matter. AD is animal manure (cow, pig, poultry, fish, etc.). The biomass of manure, a very rich source, fades in the environment

away when it is not managed with the anaerobic digestion. Anaerobic digestion of the manure converts organic matter to biogas, a usable energy. AD does not only recycle animal manure and organic waste but also meets energy demand by generating biogas (Figure 2.6). 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 (Martinez and Burton, 2003).

Table 2.2. 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

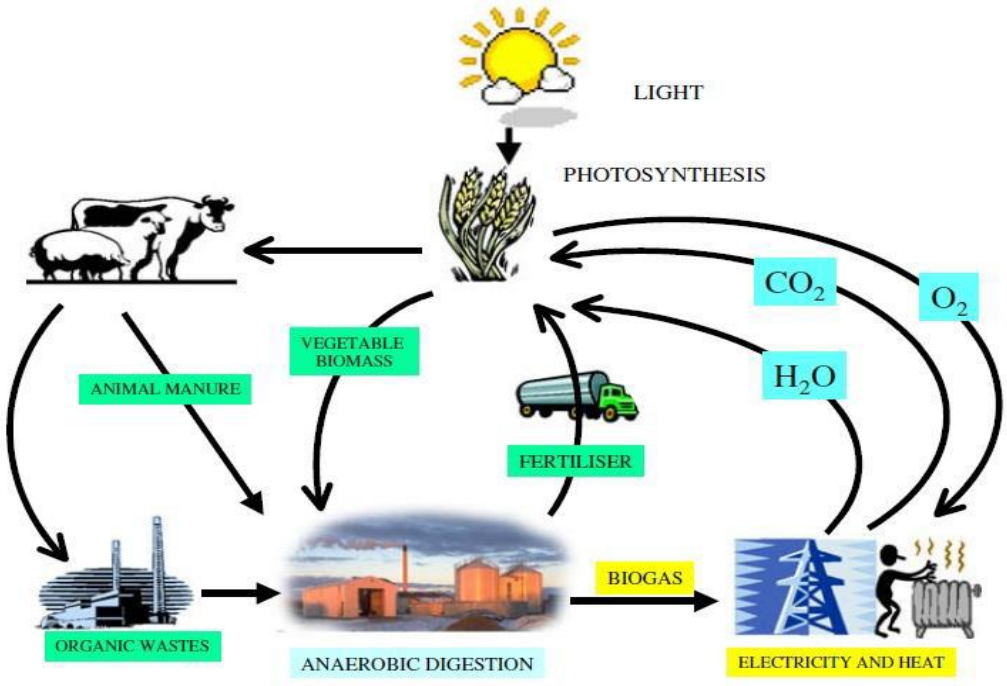

Figure 2.6. General scheme of the sustainable cycle of anaerobic co-digestion process (Al Seadi, 2002).

Utilization of biogas is not only cost effective, but environmnetally friendly and sustainable (Weiland, 2010). Biogas can be used in many ways such as; for the production of heat, steam, electricity, and hydrogen and for the utilization as a vehicle fuel. Many

sources, such as crops, grasses, leaves, manure, fruit, and vegetable wastes or algae can be used, and the process can be applied in small and large scales, which allows the production of biogas at any place in the world.

2.3. Characterization of Microbial Communities using Molecular Tools

Classical microbiology techniques used in identification of environmental microorganisms are mostly based on cultivation dependent methods on selective growth media. These methods have certain limits which prevent an efficient identification of the community. Since there are many groups of microorganism difficult to grow, this technique is not able to address whole microorganisms.

In early years of modern microbiology, the most common method for identification of microorganisms was cultivation dependent method. The main limitation of this method was cultivability of a small fraction of all microorganisms. Microorganisms living in anaerobic environment are hard to grow because of low growth rates, syntrophic interactions and unknown growth requirements. Also cultivation dependent methods cause cultivation shift by favoring a normally not favorable microorganisms by changing competitions. Therefore a microbial community cannot be cultured as whole and cultured microorganisms do not reflect microbial community. The cultivable microorganisms make up 0.1%-10% of all microorganisms on earth (Amann et al., 1995; Hugenholtz et al., 1998; Muyzer et al., 1993; Muyzer, 1999; Lim et al., 1999; Gouillou et al., 1999).

Despite the developments in the microscopy, direct microscopic analyses have many limitations in identifying microorganisms. The small size of prokaryotic organisms, the absence of distinguishing phenotypic characters, and the fact that most of these organisms cannot be cultured are the most important factors that limit the evaluation of the biodiversity (Pace, 1997). In the last 20 years, a significant number of studies dealing with microbial biodiversity involve the use of molecular tools and have often focused on investigating the dynamics of the composition and structure of microbial populations and communities in defined environments, and the impact of specific factors, such as pollution by xenobiotics on microbial diversity (Morris et al., 2002; Ranjard et al., 2000).

Molecular biology tools increased understanding of composition, dynamics and interactions within microbial ecosystems. Molecular phylogeny has provided a new basis for the direct identification and quantification of microorganisms (Olsen and Woese, 1993). Nucleic acids are biomarchaears and hereditary molecules most probably because of their important role in protein synthesis, making them one of the earliest evolutionary functions in all cellular life-forms (Woose, 1987).

Particularly, 16s rRNA, and its encoding genes are ideal biomarchaears. 16S rRNA is found in all prokaryotes and has conserved and variable sequence regions. (Woose, 1987). It is possible to design general and specific primers and probes for the study of evolution to species level (Amann et al., 1995). The rRNA is highly conserved in nucleotide sequence as well as in secondary structure since its function remains same through years of evolution. Random changes in the variable regions occur time to time and reflect to evolutionary relationship of organisms. There are several molecular biology approaches in the studies on microbial ecology of the anaerobic reactors, a summary is given in Figure 2.6 and 2.7 and Table 2.6.

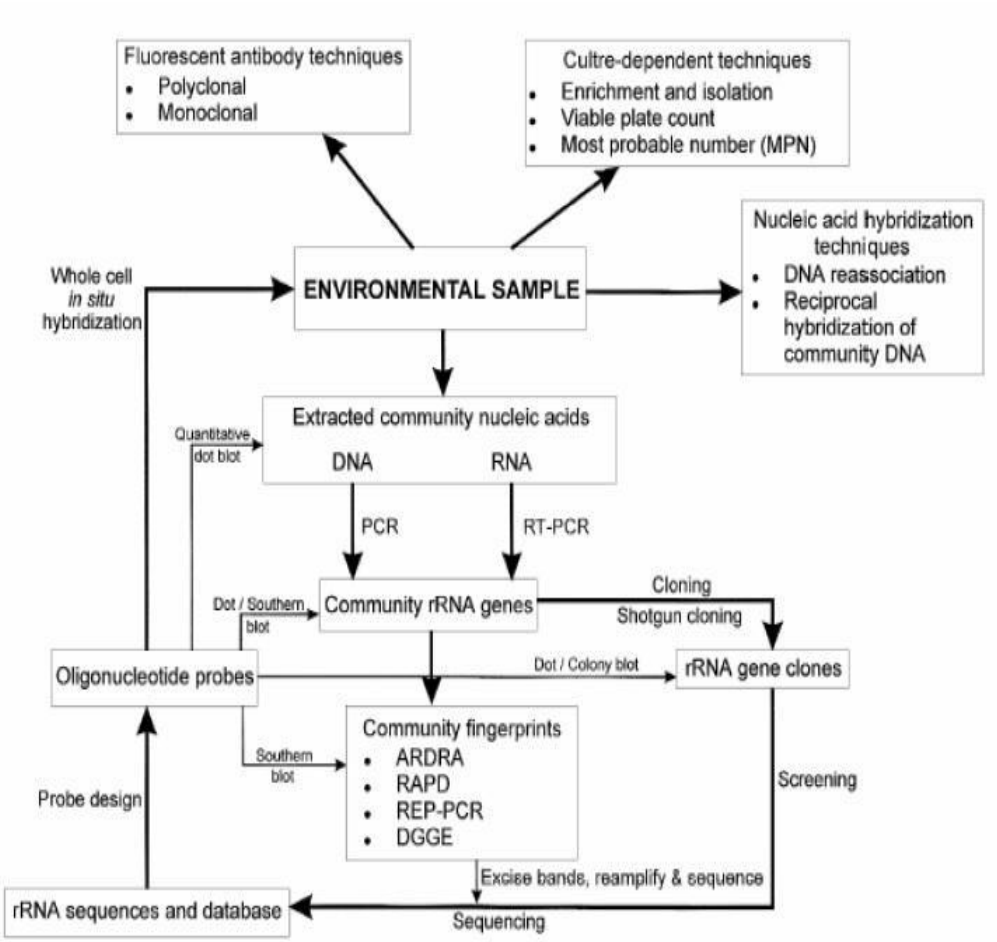

Figure 2.7. Summary of common molecular approaches used in microbial ecology (Theron and Cloete, 2000).

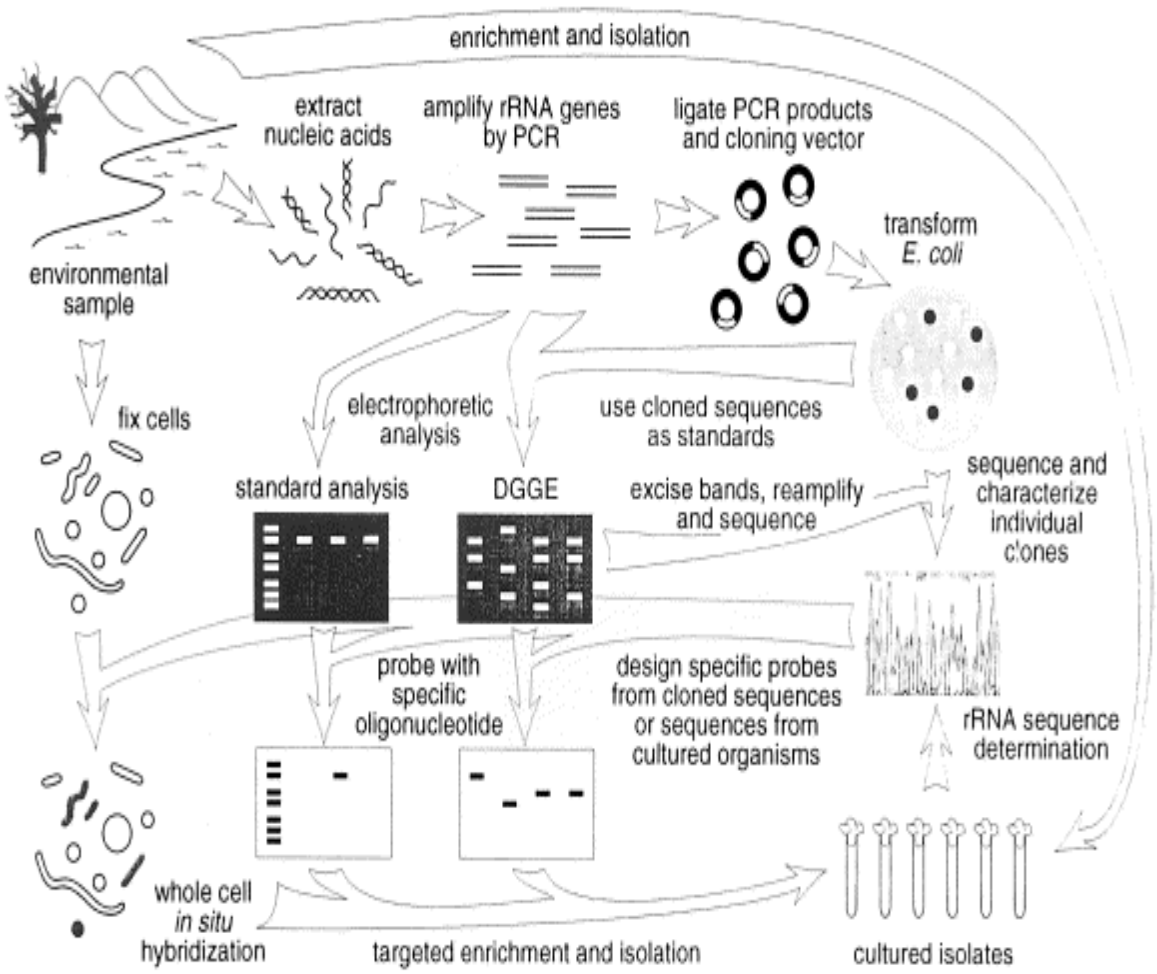

Figure 2.8. Summary of phylogenetic methodologies used in microbial ecology (Scow et al., 2004).

Table 2.3. Molecular biology applications in microbial ecology (Giraffa & Neviani, 2001).

Taxonomic resolution		Applications to microbial ecology
Genetic fingerprinting of microbial communities		
DGGE/TGGE	Community members (genus/species level)	Dynamics between microbial populations in different natural environments
SSCP	Community members (genus/species level)	Mutation analysis; dynamics between microbial populations in different natural environments
T-RFLP	Community and population members (genus/species/strain level)	Strain identification; dynamics between and within microbial populations in soils, activated sludge, aquifer sand, termite gut
LH-PCR	Community members (genus/species level)	Dynamics between microbial populations in aquatic and soil microbial environments
PCR-ARDRA	Community members (species level)	Automated assessment of microbial diversity within communities of isolated microorganisms

RISA/ARISA-PCR	Community members (species level)	Estimation of microbial diversity and community composition in freshwater environments
AP-PCR	Population members (strain level)	Automated estimation of microbial diversity (typing) within lactic acid bacteria populations
AFLP	Community and population members (genus/species/strain level)	Automated estimation of microbial diversity within communities (species composition) and populations (typing) of various Gram positive and Gram negative bacteria
Competitive PCR	Community members (species level)	Detection of microbial cells into the VNC state in freshwater samples
Fluorescence in situ techniques		
Fluorescence in situ hybridization	Community members (species level)	Detection of viable cells within bacterial communities from environmental samples or food ecosystems
Fluorescence in situ PCR	Community members (species level)	Detection of viable, slow growing cells within bacterial communities, particularly pathogens in clinical specimens

2.4. Polymerase Chain Reaction (PCR)

Amplification of DNA segments using thermostable DNA polymerase was a total breakthrough in molecular biology and opens wide range of alternatives of usage DNA in many fields including environmental microbiology (Saiki et al., 1985). PCR is used amplify a specific region of a double stranded DNA. This is actually replication of natural occurring process. DNA replication needs certain ingredients to perform. A reaction need to contain a template DNA, a heat-stable DNA polymerase enzyme, dNTPs, buffer containing magnesium and two oligonucleotide fragments called primers. At the end of reaction a single copy of DNA template amplified a billion times so fragments are available enough for experimenting on. PCR based on repetition of three steps called Denaturation, Annealing and Extension (Elongation). In denaturation, high temperature is applied to melt double stranded DNA. In annealing, temperature decreases to a point where primers can bind single stranded DNA template. In extension, temperature raised again for thermo stable polymerase to work. Enzyme adds then free dNTPs to end of primer, elongating second strand. These three phases repeated 30-40 times where original template amplified as 2^n where n is the repeat number. Then end product monitored for its correct size and amount on an agarose gel electrophoresis. PCR should be done with great care and cleanliness since as it can be seen a foreign DNA fragment can be amplified to a number which may affect downstream analyses. Therefore nearly all the time a negative control without a DNA template was also included to the reaction. In some reactions, a positive control was also added to monitor reactions performance. Although the general steps and ingredients are well defined, steps and ingredients can be manipulated to meet demands.

PCR also itself a technique to analyze microbial communities but also is used as beginning of many other techniques. Most of fingerprinting techniques such as DGGE, SSCP and TRFLP depend on PCR for their performance. PCR also used in cloning which allows identification of community members in an environment. (Hofman-Bang et al., 2003).

2.5. Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE) are also sensitive methods used to detect organisms which make up less than 1% of the total microbial community (Muyzer et al., 1993). Usually only the most dominant bacteria will be presented in the profiles when specific primers are used for especially regarding group-specific primers. But, minor microbial groups can also be monitored by DGGE analysis.

Some studies required the long-term storage of samples. In these situations, DGGE is critical as cloning is not an option. DGGE makes it possible to combine samples which were extracted at different times within one gel and, as such, it is an extremely effective tool for assessing the ways where microbial communities change over a given period of time (Petersen and Dahllöf, 2005).

DGGE is a form of electrophoresis where nucleic acids migrate in a chemical gradient according to their GC-content. PCR products are run in denaturing gradient polyacrylamide gel and separated according to melting domain and sequence in DGGE (Myers et al., 1987). DGGE, or the currently less used TGGE, is based on the melting behavior of double stranded DNA fragments. The melting behavior is mostly described with the melting temperature. Because of increasing denaturant concentration, double stranded DNA melts in melting point which is sequence specific and it has different specific melting temperature. Normally, a urea and a formamide gradient are applied in DGGE and a temperature gradient is used in TGGE (Mühling et al., 2008).

DGGE fingerprinting is an excellent and effective method for monitoring spatial and temporal changes in microbial communities. Additionally, DGGE is valuable for supervising complex communities, focusing on phylotypes that are affected by any environmental change for availability and relative frequency (Fromin et al., 2002). By comparison of across sample, dominant changes in population dynamics can be analyzed in details. The individual bands' intensity is a half-quantitative measure for the relative frequency of a species in the

community (Vaughan et al. 2000). DGGE is also one of the most frequently used techniques to screen clone libraries. Quick and reliable results decrease the amount of samples needed to perform clone libraries.

2.6. Fluorescence in Situ Hybridization (FISH)

FISH is based on the microscopic analysis of already defined (at least its SSU rRNA gene sequence) groups of bacteria by a fluorogenic oligonucleotide (or probe) targeting SSU rRNA molecules inside cells (Giovannoni et al., 1988; Amann et al., 1990). First, microbial cells are first fixed with appropriate chemical fixatives and then hybridised under optimal conditions on a glass slide or in solution with oligonucleotide probes. These probes are generally 15–25 nucleotides in length and are labelled covalently at the 5‘end with a fluorescent dye. After washing steps, specifically stained cells are detected by epifluorescence microscopy or flowcytometry. The determination of composition and number of bacteria can be achieved by rRNA-targeted oligonucleotide probes without cultivation, directly in their natural environment. rRNA gene fragments were used as phylogenetic stains firstly in 1989 (De Long et al., 1989). Since the pioneering study of De Long, fluorescence in situ hybridization technique has become a common tool for identification of microorganisms in environmental samples (Amann et al., 2001). Several hundred rRNA-targeted oligonucleotide probes suitable for FISH have been described, together with a large online database providing an encompassing overview of over 700 published probes and their characteristics (Loy et al., 2003). Such probes can be readily developed and tested to detect lineages of uncultured microbes in environmental samples (Pernthaler et al., 1997; Ravenschlag et al., 2001). The signal intensity of cells hybridized with oligonucleotide probes is directly related to the cellular rRNA content. This allows a quantification of rRNA concentrations both in single cells and in the environment (Poulsen et al., 1993). Raskin et al. (1994a) evaluated the methanogenic group composition in anaerobic digesters by oligonucleotide probe hybridization. Several studies (Merkel et al., 1999; Imachi et al., 2000; Tagawa et al., 2000; Upton et al., 2000; Wu et al., 2001) include FISH results using these same oligonucleotides but experimental conditions are variable. These probes are still reasonably accurate to target most of the defined phylogenetic groups of methanogenic *Archaea.*

The main advantage of FISH is that it does not require any DNA or RNA amplification and allows microscopic inspection of intact cells in the samples. The other important advantages of FISH technique are; it is an easy and fast technique and it allows direct visualization of organisms without cultivation. Tt is generally quantative and it allows possible to detect active microorganisms in the sample (Sanz and Kohling, 2006).

Despite the advantages above, FISH technique has its own limitations. The most significant one is that; not all bacterial and archaeal cells can be permeabilisied by oligonucleotide probes using standart fixation protocols (Amann et al., 1995).

The other disadvantages of FISH are; priori knowledge of the studied ecosystem and the microorganisms to be detected is necessary, meaning combining with other techniques is obligate. Also, in case a particular microorganism is to be detected and quantified, the rRNA sequence of the microorganism must be known (in case corresponding probe has not yet been published). In addition, it is not always possible to design a specific probe for a certain group of microorganism, especially if metabolic criteria are applied. Finally, the design and assessing optimum conditions for hybridization for a new probe is a difficult dedication and quantification of microorganisms can be tedious and subjective (manual counting) or complex (image analysis).

2.7. 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). This method makes DNA amplification and determination of DNA products in a single tube possible. With this method altering the analysis of gene expression, the conventional PCR method and gene analysis have been coupled. 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 identifying 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 product being obtained 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).

2.8. Next Generation Sequencing (NGS)

Parallel sequencing, a new tool in the field of molecular biology, has great potential for the development of environmental analysis. The first automated sequencing process, developed from Sanger sequencing, produces 550-900 bp read lengths but its sequencing capacity is just 96 reads per run; the process can also be costly and fraught with errors (Sanapareddy et al., 2009). However, sequencing has become more financially manageable with the development of next-generation technologies meaning that many smaller organizations and research groups have more access to these extremely powerful sequencing tools (Shendure and Ji, 2008).

Molecular diversity among microbial communities can be characterized thanks to Next-generation sequencing Technologies. In addition, all functional analyses of microbial communities can be done and various geneomic analyses can be perfformed (Cardenas et al., 2008; Prest et al., 2014). Nevertheless, these newer deep-sequencing-based methods require detailed bioinformatics databases and sophisticated software for data processing. Consequently, technical developments are needed as much as improvements to sequencing tools.

Although various approaches can be used to generate next-generation sequencing, the Illumina has arisen as the most effective method of deciphering DNA sequences in recent years (Hayes et al., 2013). High-throughput sequencing method is counducted by Illumina Hiseq 2000 sequencing system. Especially, identification of the whole communities in the environment studies is analyzed by using Illumina. Using this approach, during each respective cycle of the sequencing process each of the four nucleotides is labeled with an allocated dye and are then simultaneously bound to the flow cell. Each nucleotide incorporates a chemically blocked 3'-OH group, meaning that only one nucleotide is incorporated per sequencing cycle; the unbound nucleotides are washed away so that the

incorporated nucleotides can be identified in an imaging step and the next round of sequencing can commence (Metzker, 2010).

3. AIM OF THE STUDY

Environmentally-friendly and low coast energy production has gained importance in our country and in the world. Thus, many countries have tended to use renewable energy in recent years. Utilization of biogas from biomass, which is one of the most important alternatives in renewable energy sources, became an emerging application around the world. While animal manure is one of the most common renewable energy sources. Conspicuous benefit of anaerobic digestion is the production of methane rich biogas. However, major problem in energy production from animal manure is cellulosic compounds causing rate limitation in hydrolysis, negative effect on the performance of anaerobic digesters and reduction in methane yield. Therefore, various pretreatment techniques have attracted the attention in order to improve the biogas potential of anaerobic digester systems. In order to overcome the limitation of manure feedstocks, rumen bacteria can be used to produce biomethane. From this point of view, the aim of the thesis is to enhance anaerobic digester performance by using cow manure and cow rumen fluid at different mixing ratios in order to determine the maximum performance of biogas production in varying temperatures.

4. MATERIALS AND METHODS

4.1. Sampling and Characterization Studies

The use of ruminant animals, involving husbandry and experimental procedures, and collection of the rumen samples were approved by the Animal Ethics Committee of Veterinary Faculty of Istanbul University. Fresh cow manure and ruminal fluid were supplied from Veterinary Faculty of Istanbul University, Istanbul. Samples of all rumen content consisting of fluid and solids were taken via rumen fistulae from a cow (live weight 400-450 kg) by veterinaries. A cow was older than two years old and fed with alfalfa hay, barely grass, legumes, silage and soybean meal during the summer and winter periods. The cow rumen fluid was flushed with N_{2gas} to provide anaerobic conditions after loading and sealing. It was used in the anaerobic digestion test set up on the same day. Some of the samples of rumen fluid were stored at -20 ^{o}C in order to extract DNA for investigation of metagenomic survey of rumen fluid. Characteristics of these materials are shown in Table 4.1.

Table 4.1. Characteristics of the substrates and the inocula.

Parameter	**Cow manure**	**Cow rumen fluid**
pH	7.65 ± 0.4	6.80 ± 0.2
TS (g TS/g fresh sample, %)	15.1 ± 0.08	1.8 ± 0.02
VS (g VS/g fresh sample, %)	13.1 ± 0.08	1.44 ± 0.03
sCOD (mg/L)	13250 ± 310	9420 ± 290
Alkalinity (mg $CaCO_3$ /L)	2750	3500
TKN (mg/kg)	1355 ± 105	50 ± 2

4.2. Experimental Set-ups

In order to determine the optimum mixing ratio, in which the VFA and biogas productions are at the highest level, the rumen fluid and the animal cow manure were used in different mixing ratios as R1 0%, R2 10%, R3 20%, R4 30%, R5 40%, R6 50%, R7 100% (table 4.2.) and each mixture was diluted to a final volume of 80 mL with tap water. pH of

the digesters was adjusted by using 1M HCl or KOH. 3000 mg $NaCO_3$/L alkalinity were added externally to each digesters in order to maintain excess buffering capacity. The headspaces were sealed with rubber septums and screw caps, and covered with parafilm to prevent oxygen intake and escape of gasses. All serum bottles were placed on a mechanical shaker which was continously stirred at 120 rpm. Each digestion set was operated for 40 days at 36 ^oC, 41 ^oC, 43 ^oC and 45 ^oC.

Table 4.2. Operational conditions in anaerobic digesters fed with rumen.

Experiment Sets	Mixing persentage (% rumen fluid)	Animal cow manure amount (g)	Rumen fluid volume (ml)
R1 (Control)	0%	40	-
R2	10%	36	4
R3	20%	32	8
R4	30%	28	12
R5	40%	24	16
R6	50%	20	20
R7 (Control)	100%	-	40

Figure.4.1. Serum bottles with cow manure and cow rumen fluid.

4.3. Analytical Measurements

On days 0, 2, 5, 10, 20, 30, 40 samples were taken from the digesters for analytical and molecular analyses. While samples were stored at +4 ^{o}C, except for DNA samples which were stored at -20 ^{o}C. The analysis for alkalinity, total solids (TS), and volatile solids (VS) were carried out appropriately with standard methods (APHA, 2005). Every day gas pressure of serum bottles were measured via 7000 mBar x 5mBar manometer Lutron PM-9107. Gas compositions were measured using HP Agilent 6850 gas chromatograph (GC) with a thermal conductivity detector (HP Plot Q column 30 m x 530 μm) at days 10, 20, 30 and 40 for all digester sets. 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. 2 ml of gas was taken from the digesters and 0.5 mL of it was injected to GC for the analysis. Methane production values were provided by multiplying methane percentages of biogas with gas pressures of the digesters. Biogas and methane productions were calculated as volume in ambient conditions. pH was measured and adjusted using HANNA HI 221 Microprocessor pH meter. Gas chromatography with a flame ionization detector (Perichrom, France and Agilent Technologies 6890N, USA, respectively) and Elite-FFAP column (30 m X 0.32 mm) was utilized to measure the gas compositions and the volatile fatty acids (VFA) concentrations. The set point of the oven was 100 ^{o}C and the maximum temperature of the inlet was 240 ^{o}C. In addition, helium gas was utilized as a carrier gas at a rate of 0.8 mL/min.

4.4. Microbiological Techniques

The standard phases of bacterial culture growth (lag, log, stationary, and death) are well documented, with the log phase recognized as the point where bacteria divide as rapidly as possible. Using a spectrophotometer to measure the optical density at 600 nm (OD_{600}) of a bacterial culture to monitor bacterial growth has always been a central technique in microbiology (Matlock et al., 2011).

To isolate microorganisms producing cellulases, rumen fluid of cattle taken in Veterinary Faculty of Istanbul University. The cellulose-hydrolytic bacteria were isolated by using Bushnell Haas medium (BHM) amended with carboxymethyl cellulose (CMC) as

the sole carbon source. The CMC-amended BHM medium consisted of (gl^{-1}): CMC, 10; $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 1; KH_2PO_4, 1; NH_4NO_3, 1.0; $FeCl_3 \cdot 6H_2O$, 0.05; $CaCl_2$, 0.02. For isolation, 2 mL rumen fluid transferred to the fresh 80ml BHM medium containing CMC as the sole carbon source in 100 mL sealed bottles for incubation (Lo et al., 2009). The optical density in the nanodrop spectrophotometer was measured by the 600 method.

4.5. Molecular Techniques

4.5.1. 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.

4.5.2. Next-generation sequencing

In this study, 16S universal Eubacterial primers Bac515F and Bac806R were used. These primers sequences used in the PCR amplifications can be seen in Table 4.3. 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).

Table 4.3. Used primer sequences for PCR amplifications in NGS assays.

Primer Type	**Target Molecular Unit**	**Sequences**
Bacterial	Bac515F	GTGCCAGCMGCCGCGGTAA
	Bac806r	GGACTACVSGGGTATCTAAT

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.

4.5.3. 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_2 O, 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 4.4. 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 4.4. 16S rDNA specific primers used for PCR amplifications in qPCR analysis.

Target microorganism	Target gene	Target Molecular Unit	Sequence of the primer (5'-3')	Annealing (^{o}C)	References
Bacteria	16S rDNA	Bac519f	CAGCMGCCGCGGGTAANWC	53	Lane, 1991
		Bac907r	CCGTCAATTCMTTTRAGTT		
Archaea	16S rDNA	Arc349f	GYGCASCAGKCGMGAAW	60	Takai and Horikoshi, 2000
		Arc806r	GGACTACVSGGGTATCTAAT		
Ruminococcus flavefaciens	V3-V4	Rf154f-K	TCT GGA AAC GGA TGG TA	55	Zhou et al., 2011
		Rf425r-K	CCT TTA AGA CAG GAG TTT ACA A		
Fibrobacter succinogenes	V3-V4	Fs-f	GGT ATG GGA TGA GCT TGC	3	Zhou et al., 2011
		Fs-r	GCC TGC CCC TGA ACT ATC		
Ruminococcus albus	V3-V4	Ra1281f	CCC TAA AAG CAG TCT TAG TTC G	55	Zhou et al., 2011
		Ra1439r	CCT CCT TGC GGT TAG AAC A		

5. RESULTS AND DISCUSSION

In this study, seven different anaerobic digesters operated under different operational parameters to better understand of impacts of parameters on biogas and methane production. Thus, anaerobic digesters fed with cow manure and rumen fluid operated under different temperature (mesophilic) and different inoculum to substrate. 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 PGMTM) and Q-PCR methods.

5.1. Performance of Batch Digesters

5.1.2. Volatile fatty acid production

On days 2, 5, 10, 20, 30 and 40 of the digestion, VFAs concentrations were measured. While major VFAs found in the digester were acetic and propionic acid; isobutyric, butyric, isovaleric, valeric, isocopric, caproic and heptonic acid were the minor VFAs found in the digesters. The total VFA concentrations as equivalent of acetic acid in set 1 (36 ^{o}C), set 2 (41 ^{o}C) and set 3 (43 ^{o}C) during 40 days are depicted in Fig. 5.1. First 10 days, an increase in total VFA concentrations was observed. Total VFA was produced most in Set 2 as 7135 mg acetic acid /L (Figure 5.2.). Set 1 had the second highest VFA production as 6850 mg acetic acid/L (Figure 5.1.). The amount of total VFAs reached up 5281 mg acetic acid/L in Set 3(Figure 5.3.). After 10th day, VFA concentrations decreased substantially in all sets. It can be clearly seen that VFAs were consumed efficiently and VFAs accumulation was not detected.

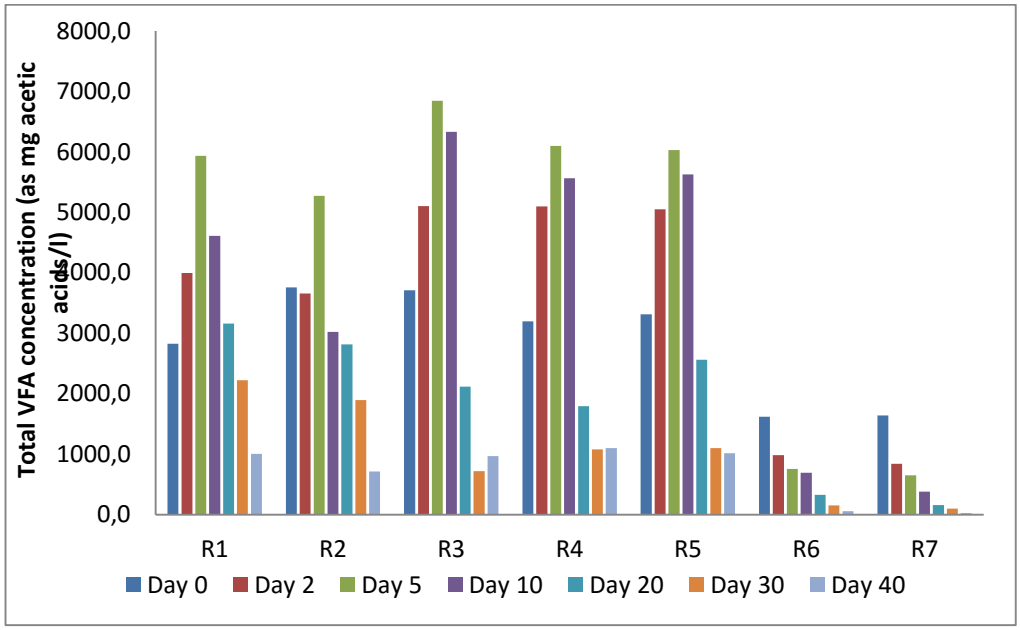

Figure 5.1. Total VFA concentrations of set 1 (36 ^{o}C) during anaerobic digestion.

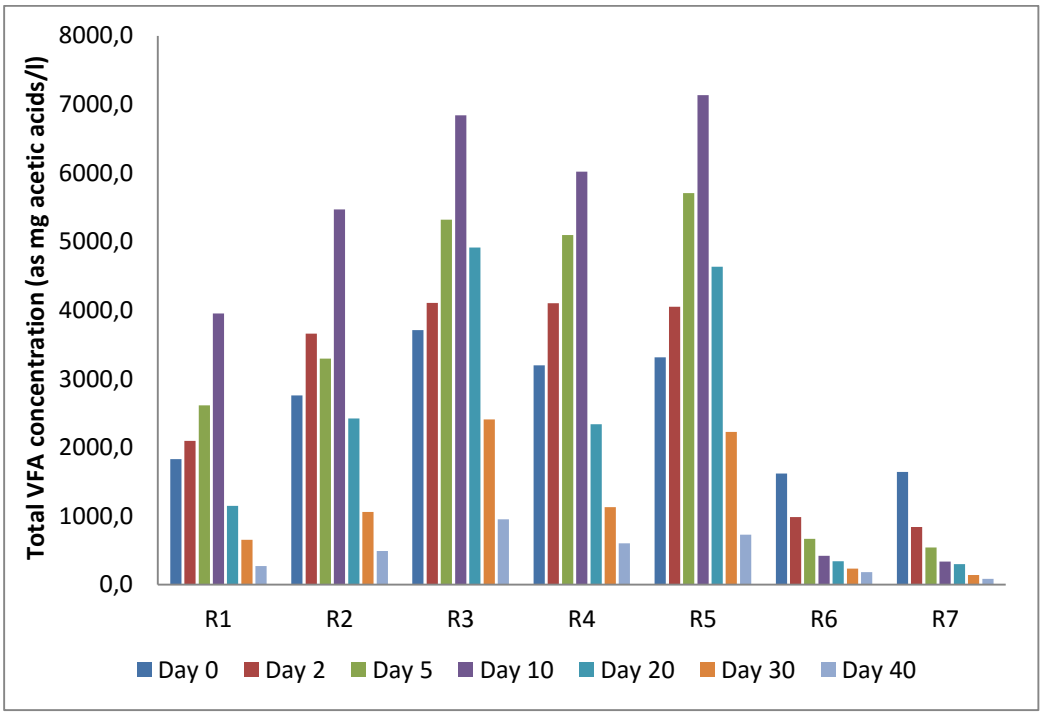

Figure 5.2. Total VFA concentrations of set 2 (41 ^{o}C) during anaerobic digestion.

In all sets; propionic acid concentrations reached up to 4000-5000 mg/L on 10th day, but most of it was consumed on 20th day. As can be seen, there was no VFA accumulation at the end of the process. During the start-up phase, R3(20%), R4(30%) and R5(40%) had the higher amount of acetic, propionic and butyric acid in a considerable amount when

compared the other serum bottles. This can be attributed to the increasing amount of the ruminal fluid in these bottles.

All sets adjusted to optimum operational conditions. The solid content of the sets range between (R1) 7.5 ± 0.05 %, (R2) 6.8 ± 0.04 %, (R6) 4.2 ± 0.08% and (R7) 1.2 ± 0.03 %, respectively. On the one hand, R3, R4 and R5 showed best performance.

The initial pH was 7.3 ± 0.2 in all bottles. During the incubation, the pH decreased in all bottles due to VFA production. However, the pH slightly increased after day 14. This observation could be attributed to the consumption of VFA in the bottles and correlated with the higher methane production. At the last day of incubation, the pH was measured as 6.2 to 6.6 in the inoculated bottles and it stayed around 7.2 ± 0.1 in the controls.

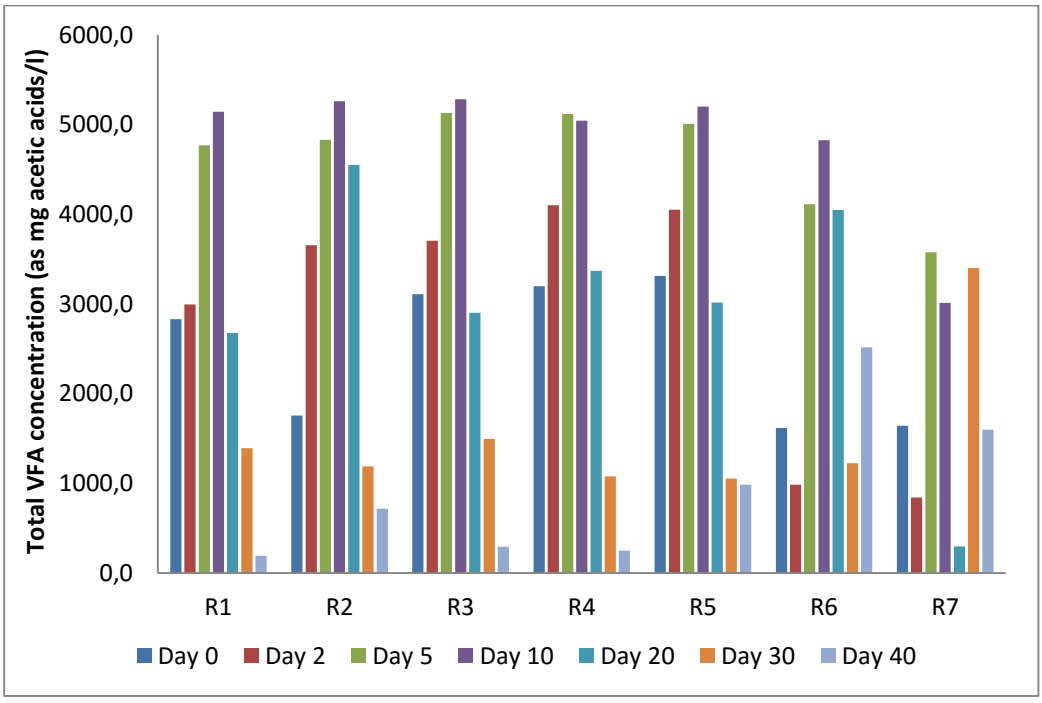

Figure 5.3. Total VFA concentrations of set 3 (43 ºC) during anaerobic digestion.

Pertiwiningrum et al. reported that during the process of fermentation, biogas production occurs as the principal volatile fatty acids formed (acetic, butyric and propionic acids). Acetic acid is usually the dominant volatile fatty acid. Wang et al. (2009) observed that acetic acid concentrations up to 2400 mg/L did not cause a failure in the digester 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., 2009). 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 (Ahring et al., 1993, Franke-Whittle et al., 2014).

5.1.2. Biogas and Methane Production

Cumulative biogas production in the anaerobic digesters for 40 days at 36 ºC, 41 ºC and 43 ºC are given in Figure 5.2., 5.2.1 and 5.2.2., respectively. Lag phase at each digestion temperature lasted for approximately 10 days. Between the days 8 and 35, biogas production sigificantly increased due to exponential growth of microorganisms (Budiyono et al., 2014). Jha et al., adding that it happened due to high VFAs production, a decrease in pH value and lack of methanogens. However, the control takes a longer time, so if calculated economically, the treatment with the addition of rumen fluid is more likely to encourage selectivity towards the methane formation with higher concentration in a shorter time (Pertiwiningrum et al., 2017). In this study, the results of R3, R4 and R5 (20%, 30% and 40%) are close to each other, in all sets at end of a 40 days' digestion period, biogas production stopped in all anaerobic digesters.

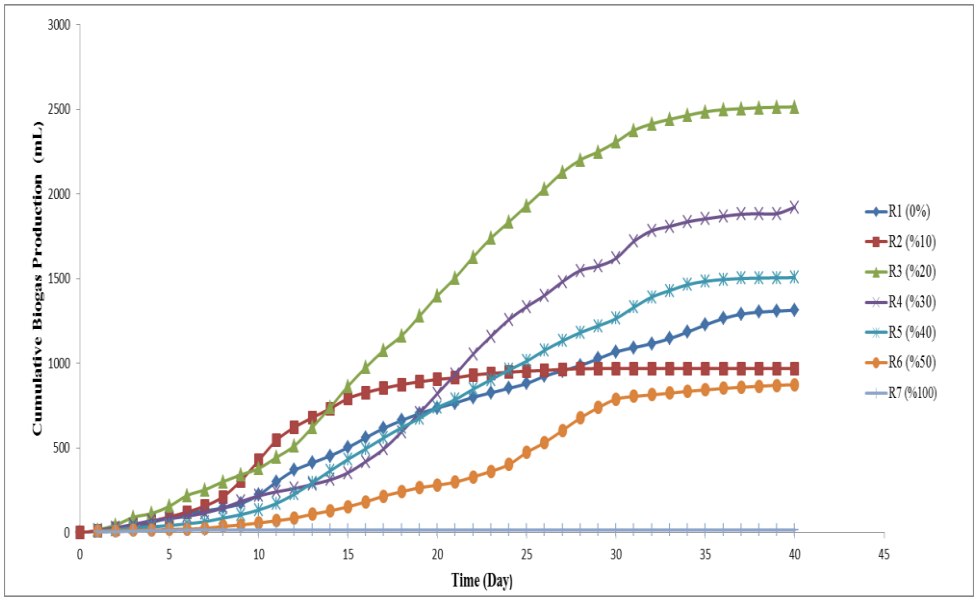

Figure 5.4. Cumulative biogas production in of set 1 (36 ^{o}C) during anaerobic digestion.

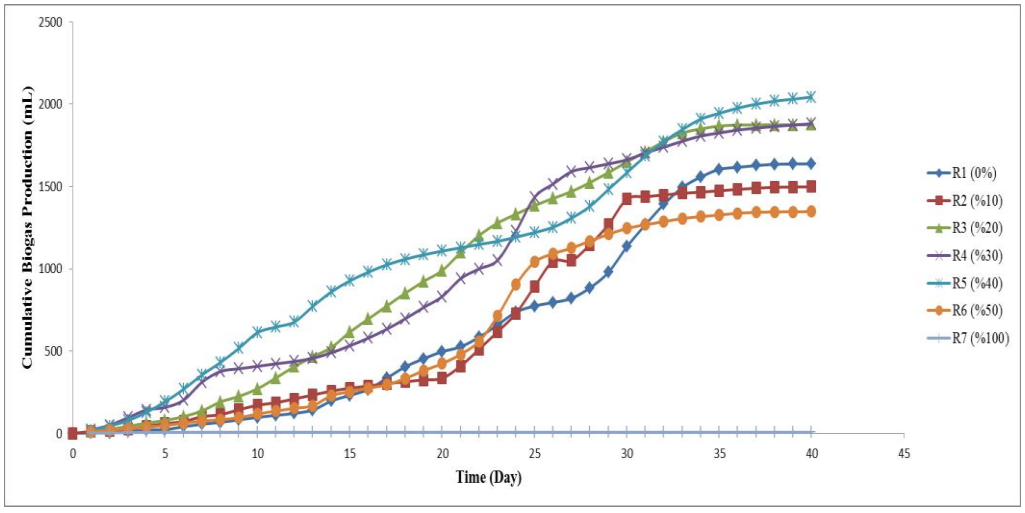

Figure 5.5. Cumulative biogas production in of set 2 (41^{o}C) during anaerobic digestion.

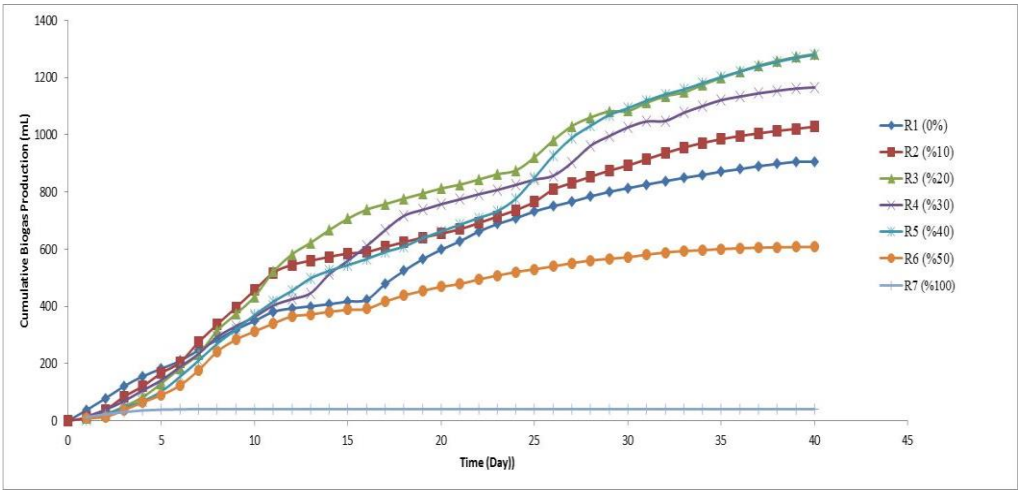

Figure 5.6. Cumulative biogas production in of set 3 (43 ^{o}C) during anaerobic digestion.

However, on 20th day, the addition of rumen fluid (20, 30 and 40%) caused to produce a higher concentration of methane compared to the digesters without rumen fluid. And when compared with the volume of formed biogas, the addition of rumen fluid showed a difference in the volume of methane than the treatment without rumen fluid addition (0% of rumen fluid).

It can be seen that rumen neat (100 % of rumen fluid) do not contribute to the biogas production. Hence, all of biogas produced during the digestion test were originated only from substrate contained by manure. This indicated that the addition of liquid rumen to feed will increase biogas production rate in compare to feed without liquid rumen.

In all the serum bottles, the percantage of CH_4 was found to be %40-50, while:

- at 36 ^{o}C

  The highest biomethane content was 47.6% and the best biomethane production was achieved in R3 (20%) with 230 ml/g VS_{added}.
- at 41 ^{o}C

  The highest biomethane content was 52.7% and the best biomethane production was achieved in R5 (40%) with 262 ml/g VS_{added}.
- at 43 ^{o}C

  The highest biomethane content was 40.5% and the best biomethane production was achieved in R5 (40%) with 201 ml/g VS_{added}.

Budiyono et al., the temperature of 38.5 ºC was selected due to the fact that the rumen condition on animal ruminants is +38.5 ºC. Either at room temperature as well as at 38.5 ºC, substrate contains rumen fluid (MR11) consistently exhibits higher biogas production rate than substrate without rumen fluid (MW11). In addition, either substrate with and without rumen fluid, anaerobic digestion at 38.5 ºC exhibit higher biogas production rate than at room temperature. In this study, increasing the temperature from 36 ºC to 41 ºC caused a higher biogas production rate from 230 ml/g VS to 262 ml/gVS respectively.

5.1.3. Results of microbiological analyses

Figure 5 shows the OD_{600} values at the temperatures of 36, 41 and 43 ºC. The highest increase in the curve has been observed in the sets of 36 ºC. In two different sets of 36 and 41 ºC, there is an approximatly in the increase of curves. The growth phase was completed with the increase that had been observed for ten days, which was followed by the death phase. OD_{600} results have shown that 36 and 41 ºC are suitable temperatures for rumen bacteria. The increase rate of the curve in the 43 ºC was quite low. However, reactors were used in order to improve the efficiency of the bacteria with substrate. The temperature of 45 ºC has not growth phase, thus reactor was not installed.

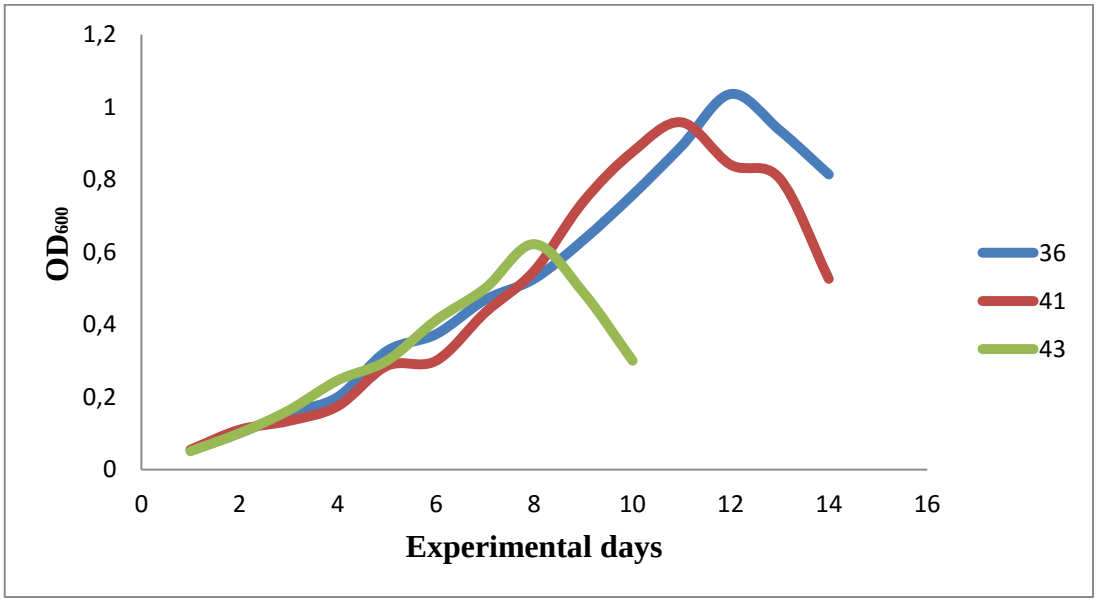

Figure 5.7. The OD_{600} results depending on selective temperatures and sampling days.

5.2. Results of Molecular Analyses

5.2.1. Next -generation sequencing (NGS) results

Figure 5.8. presents the bacterial community composition of the samples at the phylum, class, order and family levels. it was identified that *Bacteroidetes* (57%) was the most abundant phylum. *Firmicutes* (22%) was the second most dominant phylum as well. *Proteobacteria* (5%), *Lentisphaerae* (4%), *Fibrobacteres* (3%) and *Spirochaetes* (2%) 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%). Further evaluation of the two major classes showed that Clostridia were mainly composed of the families *Ruminococcaceae* and *Lachnospiraceae* (39% and 15% on average of the total sequences, respectively), belonging to the *Clostridiales* order. Predominant bacterial families in the *Bacteroidia* class were *Prevotellaceae* (39% and 5.9% on average of the total sequences), belonging to the *Bacteroidales* order. *Prevotella* is in accordance with literature showing that *Prevotella* was the major ruminal genus, representing up to 39% of total bacteria of rumen content. The Prevotella genus comprises a wide range of species who have important roles in the utilization of polysaccharides of plant origin, including xylans, pectins, and starch (Zened et al., 2013). Fibrobacter is favoured in the bovine rumen and, given that it is cellulose degrader, may play an essential role in the degradation of plant fibre in cattle (Hendersen et al., 2015). The abundance of the family *Victivallaceae* (phylum *Lentisphaerae*) was found 4%. Members of this family were reported to play a role in the fermentation of sugars such as cellobiose and glucose and to produce acetate and hydrogen in the presence of methanogens. The genus *Ruminococcus* was reported to be involved in cellulose decomposition. Some members of the *Lachnospiraceae* have the ability to degrade xylan and some have cellulolytic activity. It is also known that members of the *Lachnospiraceae* play a key role in butyric acid production. Moreover, some members have the capability to degrade plant polymers in gut environments. Besides their function in plant polymer degradation, members of the *Lachnospiraceae* and *Ruminococcaceae* have been described to play a role in reductive acetogenesis, which is an alternative hydrogen sink in rumen systems (Ozbayram et al., 2017).

In the manure bacterial phylum composition can be seen in Figure 5.8. it was identified that *Firmicutes* (37%) was the most abundant phylum. *Bacteroidetes* (22%) was the second most dominant phylum as well. *Proteobacteria* (12%), Actinobacteria (7%), *Lentisphaerae* (6%), *Spirochaetes* (4%) and *Fibrobacteres* (2%) were also found in a significant amount. Within the *Firmicutes*, sequences belonging to the classes *Bacilli* and *Clostridia* dominated. Some species of the order Clostridiales are known tocreate cellulosomes, which are intensively involved in the anaerobic digestion of recalcitrant cellulose, supporting acetogens and methanogens with compounds necessary fortheir growth. Apart from their role in hydrolysis and acidogenesis, members of the class *Clostridia* are also involved in acetogenesis and syntrophic acetate oxidation (Ziganshin et al., 2013). *Actinomycetes* form an important part of the microbial community responsible for nutrient recycling in natural substrates. Their species play a significant role to lignocellulose degradation (McCarthy, 1987).

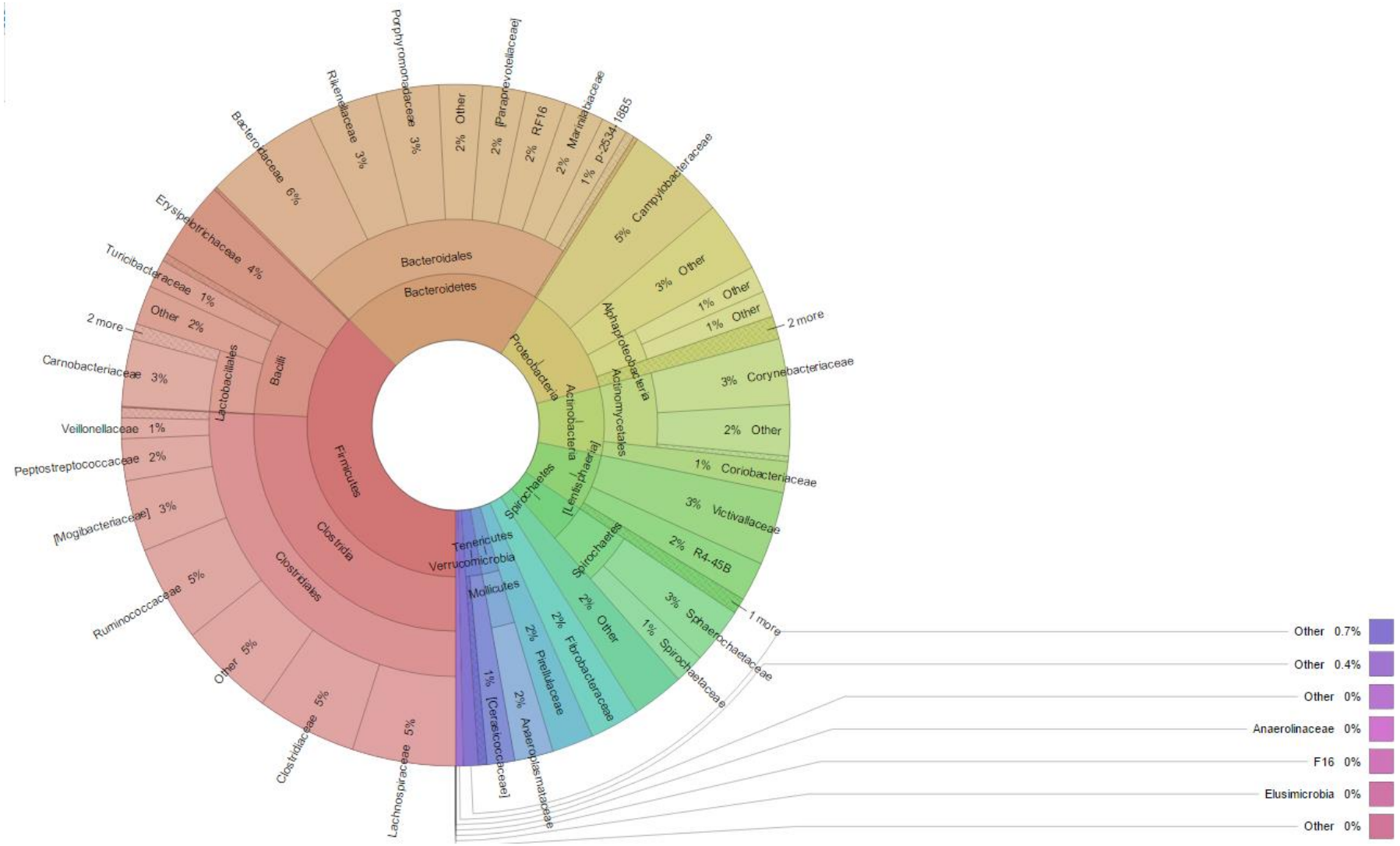

Figure 5.8. Bacterial phylum of manure

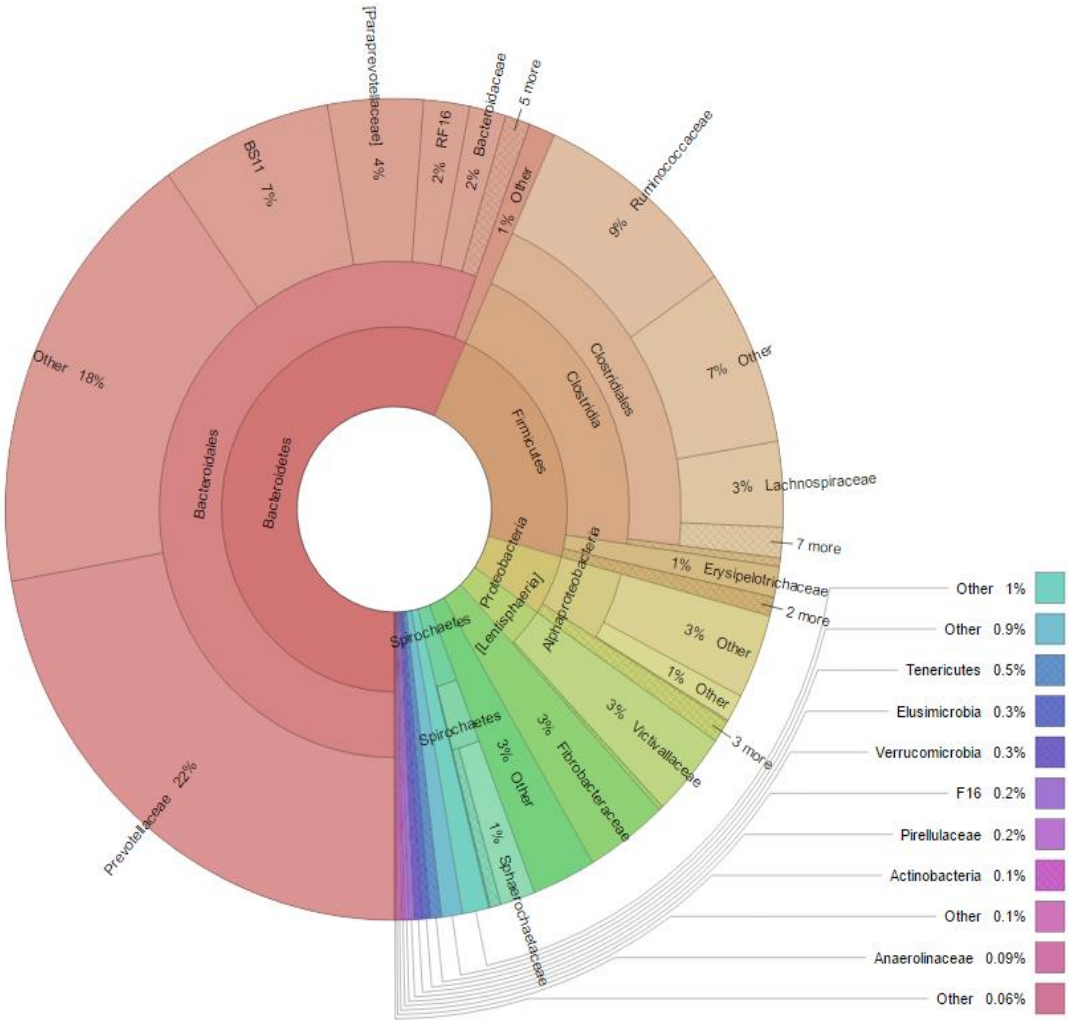

Figure 5.9. Bacterial phylum of ruminal fluid.

5.2.2. 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 5.9 and 5.10.

As indicated before, Q-PCR analysis were conducted in set1 at 36 ^{o}C R3(%20), set2 at 41^{o}C R5(%40), set3 at 43 ^{o}C R5(%40) in accordance with the mixtures that yield the highest rates of biogas and biomethane. The total number of bacteria showed that set1 had the highest copy number in the first five days and total bacteria were detected to be 4.2×10^{10} copies/ml

while the copy number in set2 was the highest after the 5th day on the 10th total bacteria were determined to be 6.3 × 10^{9} copies/ml. The reason for the increase in set2 after the 10th day might be that the bacteria completed the lag phase with the excess amount of rumen fluid in this set. The copy number of archaea in set1 and set2 increased between the 5th and 10th days total archea were detected to be 4.3 × 10^{7} and 4.5 × 10^{7} copies/ml, respectively. However, no significant difference between the two sets was observed. As already expected, the copy number of archaea in set3 was quite low. The results from Q-PCR showed that set3, with a comparatively higher heat at 43 ^{o}C, could not set favorable conditions for the rumen bacteria.

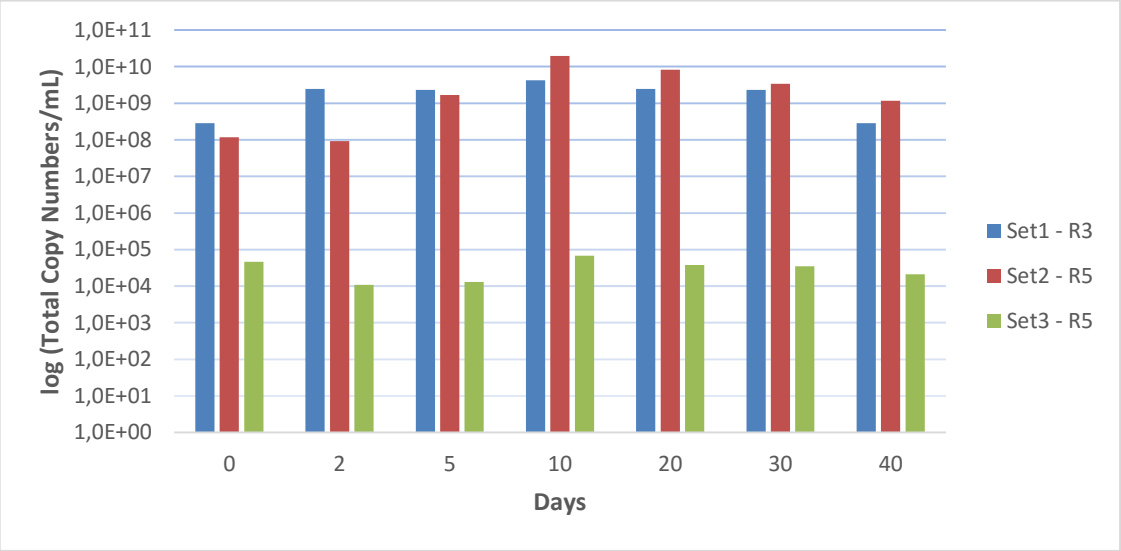

Figure 5.10. Total 16S rDNA copy number of bacteria.

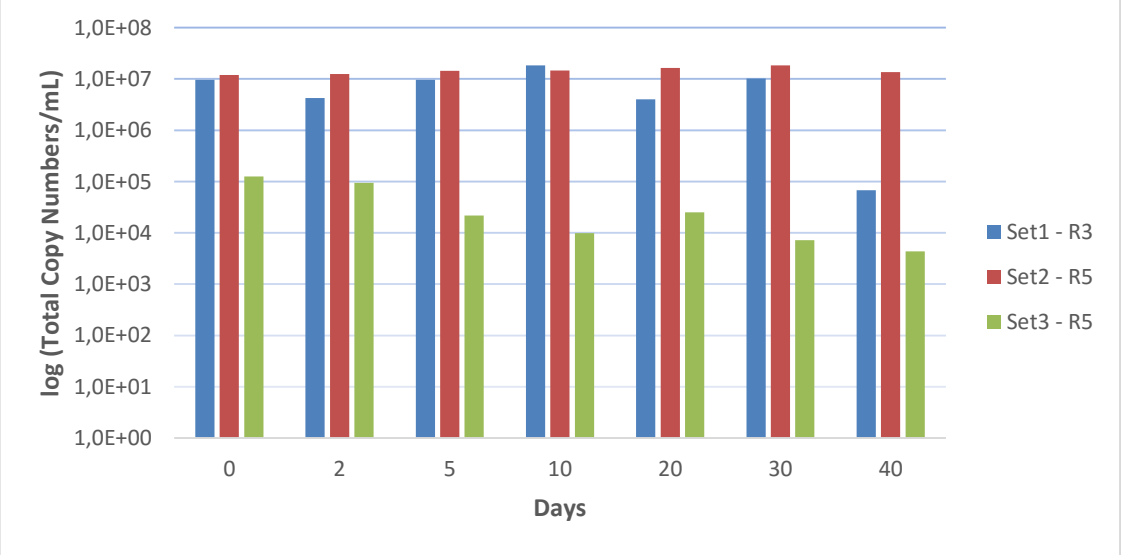

Figure 5.11. Total 16S rDNA copy number of archaea.

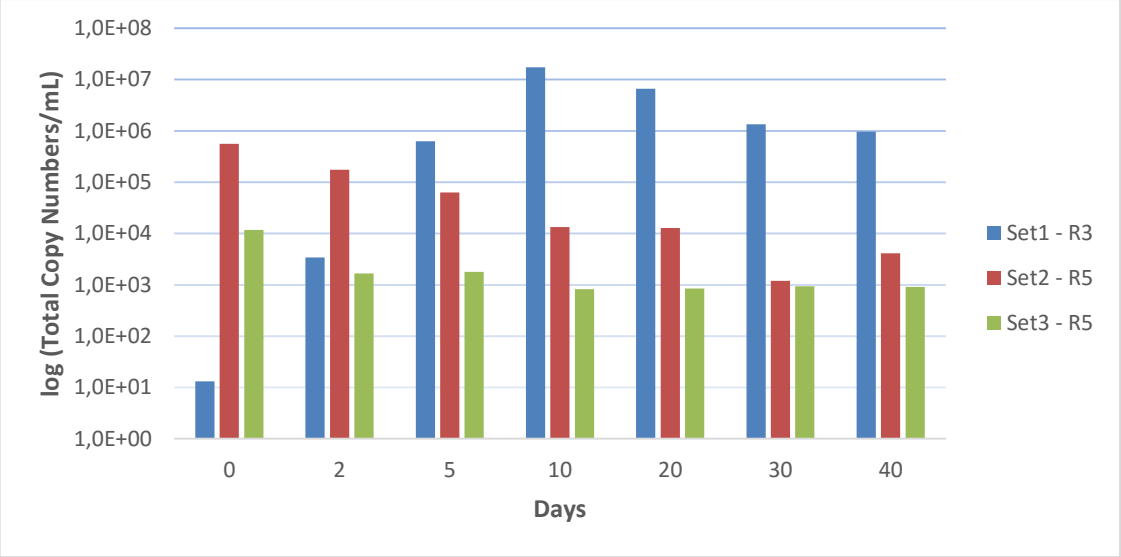

Figure 5.12. Total 16S rDNA copy number of *Ruminococcus albus*.

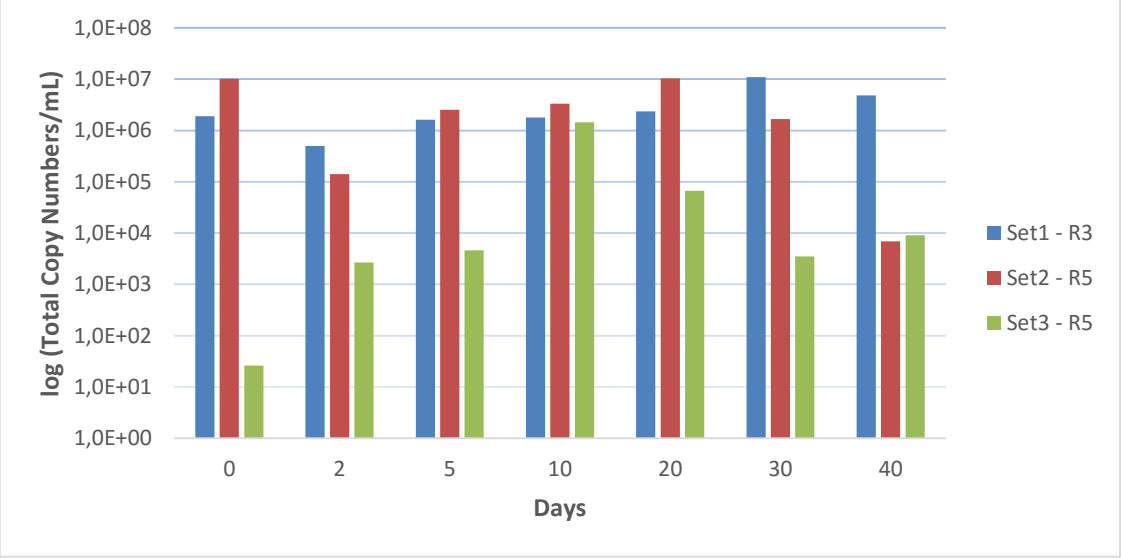

Figure 5.13. Total 16S rDNA copy number of *Fibrobacter succinogenes*.

Fibrobacter succinogenes (*FS*), *Ruminococcus albus* (*RA*), and *Ruminococcus flavefaciens* (*RF*) are presently recognized as the major cellulolytic bacterial and species found in the rumen and these bacteria groups were determined in every set. The results showed that the all sets harbored *R.albus* and *F. succinogenes* at high levels (*R.albus* > *F. succinogenes*), with both species outnumbering *R. flavefaciens*. The number of RA copy was the highest in set1 compared to the oter sets. It increased in the 5th and 30th days and it quantified to be highest 2.58 × 10^{7} copies/ml. Meanwhile, the bacteria detected

to be highest 2.17 × 10^6 copies/ml and the number of *R.albus* copy in set2 showed a decrease everyday. The reson for the decrease might be the inability of *R.albus* bacteria to adopt to the heat conditions. The number of *F. succinogenes* copy did not exhibit a significant difference in set1 and set2 (between 1.11 × 10^7 and 1.33 × 10^6 copies/ml) while there was an increase in set3 in the tenth day and it was measured 1.22 × 10^6 copies/ml. The reson for this might be the positive adaptation of *F. succinogenes* bacteria to the heat conditions. These results were almost the same for *R. Flavefaciens* and the copy number in set1 and set2 increased between the 5^{th} and 10^{th} days. The higest measured values were 2.56 × 10^6 and 1.31 × 10^5, respectively.

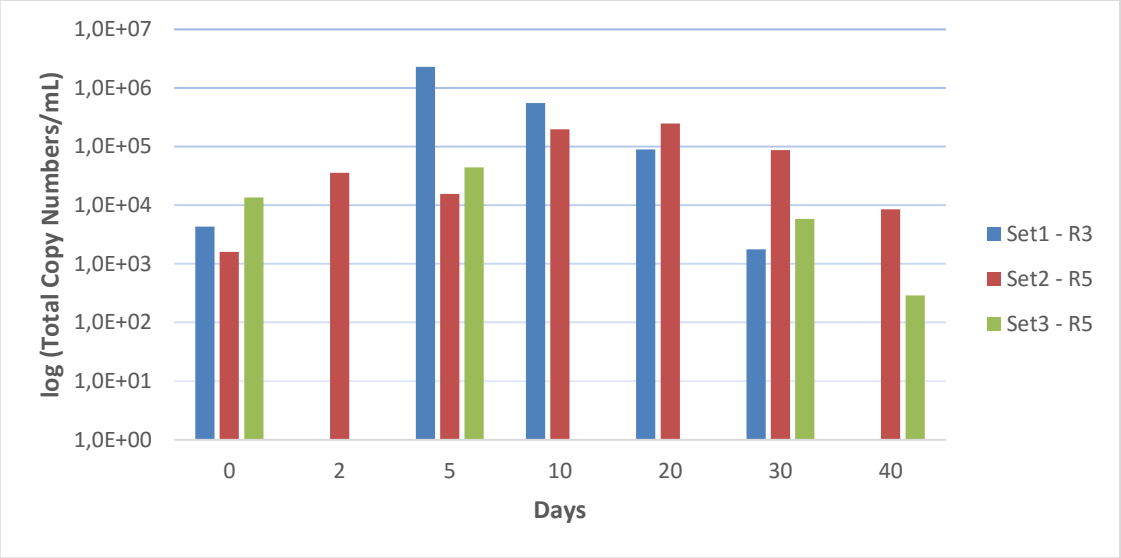

Figure 5.14. Total 16S rDNA copy number of *Ruminococcus flavefaciens*.

6. CONCLUSIONS

In this study, effect of rumen fluid addition on biogas production and microbial dynamics in batch-wise operated serum bottles with cow manure was investigated. Three experiment sets were conducted, namely Set 1, Set 2 and Set 3 and operated at 36 ^{o}C, 41 ^{o}C and 43 ^{o}C, respectively for 40 days.

According to the findings of the study, the highest specific methane yield was found in the Set 2 41 ^{o}C, digester containing 40% as a supportive inocula. Addition of rumen fluid to inoculum effectively improved the bio-methane yield especially in 20 days, resulting in a methane yield of 262 mL CH_4/g VS, in which methane content of the biogas was around 52%. The next highest specific methane yields were obtained in the Set 1 36^{o}C, digester containing 20% as a supportive inocula with a biomethane content of 47%, resulted in a methane yield of 230 mL CH_4/g VS. In the Set 3 43 ^{o}C, the methane yield was found as 201 mL CH_4/g VS and biomethane content was 40%. 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.

Bacterial and methanogenic profiles among rumen fluid and manure were detected through NGS-based metagenomics analysis applied using Ion PGM^{TM} platform. The phylum *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were determined as the most dominant three bacterial phyla. The most abundant classes were *Clostridia* (Phylum: *Firmicutes*), *Bacteroidia* (Phylum: *Bacteroidetes*) and *Bacilli* (Phylum: *Firmicutes*).

According to Q-PCR results, the total number of bacteria showed that set1 had the highest copy number in the first five days while the copy number in set2 was the highest after the fifth day. The number of archaea copy in set1 and set2 was observed to increase between the fifth and thirtieth days. As already expected, the number of archaea kopya in set3 was quite low. The results from Q-PCR showed that set3, with a high heat of 43 ^{o}C, could not set favorable conditions for the rumen bacteria.

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