EFFECT OF RUMEN FUNGI ON POTENTIAL OF BIOGAS PRODUCTION IN ANAEROBIC DIGESTERS FED WITH DIFFERENT LIGNOCELLULOSIC COMPOUNDS

by

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In memory of my lovely grandmother,

Elif Yıldırım

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Although lignocellulosic biomass are considered as an alternative biogas source, they also cause rate limitation in hydrolysis step of anaerobic digestion process, negative effect on the performance of anaerobic digesters and reduction in yield. Thus, they can be also defined as a significant problem in energy production from animal manure, microalgae and macroalgae. Anaerobic rumen fungi can be seen as a potential treatment method to improve the biogas production because they produce pretty much hydrolyze enzymes.

In this study, effects of anaerobic rumen fungi on the performance of anaerobic digesters and biogas production from different lignocelluloosic compounds and also microbial community dynamics during anaerobic digestion process were investigated.

In the different three sets, it was examined that effects of bioaugmentation with anaerobic rumen fungi at various ratios of inoculums on biogas production of anaerobic digesters fed with animal manure, microalgae and macroalgae respectively. The highest biogas productions reached 5500 ml in digester $R_{2,}$ 6250 ml in digester F_5 and 3500 ml in digester A_1. It was also found that the highest methane productions are 60% for animal manure, 57% for microalgae *H. pluvialis* and 54,7% for macroalgae *Ulva lactuca* due to addition of anaerobic rumen fungi. Changes in quantity and diversity of bacterial and archaeal communities were detected by lllumina MiSeq sequencing technology. Changes in the number of total cells of fungi were analysed by qPCR to show effect of anaerobic rumen fungi on biogas production from different lignocellulosic compounds.

RUMEN FUNGUSLARININ FARKLI LİGNOSELÜLOZİK BİLEŞİKLERLE BESLENEN ANAEROBİK ÇÜRÜTÜCÜLERDEKİ BİYOGAZ ÜRETİM POTANSİYELİNE ETKİSİ

Lignoselülozlu biyokütle alternatif bir biyogaz kaynağı olarak düşünülmesine rağmen, aynı zamanda bu maddeler oksijensiz arıtma işleminin hidroliz basamağında hız kısıtlanmasına, oksijensiz çürütücü performansının olumsuz etkilenmesine ve verimde düşüşe neden olurlar. Bu yüzden, lignoselülozlu bileşenler aynı zamanda hayvan gübresinden, mikroalglerden ve makrolalglerden enerji üretiminde önemli bir problem olarak tanımlanabilir. Oksijensiz rumen mantarları oldukça fazla miktarda hidroliz enzimleri ürettikleri için, biyogaz üretimini geliştirmek amacıyla olası bir iyileştirme metodu olarak görülmektedir.

Bu çalışmada, anaerobik rumen mantarlarının havasız çürütücü performansına ve farklı lignoselülozlu bileşiklerden biyogaz üretimine etkileri ve ayrıca oksijensiz çürütme basamakları boyunca mikrobiyal komünitelerdeki değişimler incelenmiştir.

Üç farklı sette, farklı miktarlardaki anaerobik rumen mantarlarının biyoagumentasyonunun, sırasıyla hayvan gübresi, mikroalg ve makroalg ile beslenen havasız çürütücülerdeki biyogaz üretimine etkisi incelendi. En yüksek biyogaz üretimleri R_2 çürütücüsünde 5500 ml'ye, F_5 çürütücüsünde 6250 ml'ye ve A_1 çürütücüsünde 3500 ml'ye ulaştı. En yüksek metan üretimleri ise, eklenen anaerobik rumen mantarları sayesinde hayvan gübresi için %60, mikroalg *H. pluvialis* için %57 ve makroalg *Ulva lactuca* %54,7 olarak bulundu. Bakteri ve arke komünitelerinin miktar ve çeşitliliğindeki değişimler lllumina MiSeq sekanslama teknolojisiyle belirlendi. Mantarların toplam hücre sayısındaki değişim, oksijensiz rumen mantarlarının, farklı lignoselülozlu bileşiklerden biyogaz üretimindeki etkisini göstermek için qPCR ile analiz edildi.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Units used
ASBR	Anaerobic Sequencing Batch Reactor	
AD	Anaerobic Digestion	
ATP	Adenosine Triphosphate	
COD	Chemical Oxygen Demand	(mg L^{-1})
DGGE	Denaturing Gradient Gel Electrophoresis	
DNA	Deoxyribonucleic Acid	
dNTP	deoxynucleoside triphosphate	
EtBr	Ethidium Bromide	
FISH	Fluorescent in situ Hybridization	
FOG	Fats, Oils and Grease	
GC	Gas Chromatography	
gDNA	Genomic DNA	
GHGs	Greenhouse gases	
HGT	Horizantal Gene Transfer	
HRT	Hydraulic Retention Time	(d)
ITS	Internal Transcribed Spacer	
MSW	Municipal Solid Waste	
NGS	Next Generation Sequencing	
NRB	Nitrate Reducing Bacteria	
OLR	Organic Loading Rate	(g TVS L^{-1}-day)
OHPA	Obligate Hydrogen Producing Bacteria	
PCR	Polymerase Chain Reaction	
RNA	Ribonucleic Acid	
rDNA	Ribosomal DNA	
rRNA	Ribosomal RNA	
SRB	Sulphate Reducing Bacteria	
SRT	Sludge Retention Time	(day)
Q-PCR	Real Time PCR (Quantitative PCR)	
TGGE	Thermal Gradient Gel Electrophoresis	

TS	Total Solid	(mg L^{-1})
TVS	Total Volatile Solid	(mg L^{-1})
VFA	Volatile Fatty Acids	(mg L^{-1})

1. INTRODUCTION

Environmentally-friendly and low coast energy production gains importance every day in our country and the world due to overpopulation and fast-growing industry. Because the main purpose of renewable energy is to reduce poverty and allow sustainable development, many countries tend to use renewable energy in recent years (Goldemberg and Teixeira Coelho, 2003). Another reason why renewable energy has become prominent is reduction in reserves of non-renewable energy resources and causing of these resources to climate change. Concordantly, biogas which is produced by processing various renewable energy sources plays an important role for succession of fossil fuels causing to increase greenhouse gases (GHGs), global warming and climate change effects, in power and heat production (Weiland, 2010). Alternative sources of energy such as solar, geothermal, wave, biomass, hydraulic are considered as possible renewable energy resources (Ellabban et al., 2014).

Biomass which is one of the most important alternatives in renewable energy sources is described as an organic matter originated from photosynthetic capture of solar energy and stocked as chemical energy. (Gunaseelan, 1997). Thus, biomass which is an efficient biological material can be used as fuel and it provides power in terms of renewable and sustainable energy. The process of breaking down the biological materials in an anaerobic environment generates biogas. After, biomethane is procured owing to purification of biogas (Chum and Overend, 2001).

Although there are many sources of biomass such as agricultural crop wastes and residue, municipal solid waste, sewage, forestry crops and residue, industrial residue and animal residue, sources of biomass including lignocellulosic compounds are more favorable for production of biogas. Biomass resources which contain plant dry materials are defined as lignocellulosic biomass and it is the most widespread bio-renewable biomass on earth (Isıkgor and Becer, 2015).

Animal manure has high potential of lignocellolosic compounds owing to herbal nutrition which cannot be adequately digested, thus, it is defined as a primary source of

biomass. Moreover, biogas produced from animal manure is the most common renewable bio-fuel source (Thien Thu *et al*., 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.

In addition to animal manure, algae are considered as a feasible substrate for high value biogas production in recent years. Because terrestrial crops for biogas have a negligible contribution to net greenhouse gas emissions, aquatic and marine production for biogas gradually gain importance (Hughes et al., 2012).

Microalgae provide a lot of specific qualities making them favourable as a source of biogas and for use in renewable energy production. These properties contain high photosynthetic efficiencies, ability to function without an external organic carbon supply and high growth rates (Yen and Brune, 2007). Production of biogas comprising hydrogen or methane from anaerobic digestion of algae as a source of biomass is conspicuous technology because they have capacity of energy conservation and environmentally friendly feature (Ward et al., 2014).

Macroalgae have also high potential for biogas production because they contain negligible or little amount of lignin and cellulose. Thus, it is provided that macroalgae can be easily degraded in comparison to terrestrial biomass crops and they can be subjected to more efficient hydrolysis process (Montingelli et al., 2015). In addition to low amount of lignin and cellulose, they gain advantageous over the conventional energy crops thanks to high growth rates and no competition with human foods (Saqib et al., 2013).

Recovery of biomass from animal manure and micro-macroalge is the most significant function of anaerobic digesters where microorganisms break down

biodegradable compounds in the oxygen-free environment. Organic wastes can be treated and recovered in the form of biogas thanks to anaerobic digestion (Clemens et al., 2006). Methane, carbon dioxide and some trace gases are released as a consequence of anaerobic digestion phases. Especially methane is quite important biogas because it provides a renewable alternative for utilization of heat and power (Lusk and Moner, 1996).

Although conspicuous benefits of anaerobic digestion is the production of methane rich biogas, major problem in energy production from animal manure, microalgae and macroalgae under anaerobic conditions is cellulosic compounds causing rate limitation in hydrolysis which is the first step of anaerobic digestion, negative effect on the performance of anaerobic digesters and reduction in yield. Cellulose and hemicelluloses which are the cellulosic compounds are linked with lignin and together called lignocelluloses (Bayane and Guoit, 2011). Cellulose, hemicelluloses and lignin are chemically bonded by non-covalent forces and by covalent cross-linkages (Perez et. al, 2002).

Especially algae have high potential of cellulosic compounds because lignocelluloses are the basic structural polysaccharides of them. Moreover, they have rigid cell walls which can limit degredation and, therefore, negatively affect bioenergy production. As well as algae, animal manure can contain cellulosic compounds due to herbal nutrition which cannot be adequately digested. Therefore, lignocellulosic biomass in animal manure, microalgae and macroalgae is actually considered as a potential energy resource for biogas production (Bayane and Guoit 2011). Thus, some methods must be found in order to prevent the adverse effects of these cellulosic compounds and rate limitation on anaerobic digestion. At this point, various pretreatment techniques have attracted the attention so as to improve the biogas potential of anaerobic digestion process. The basic aim of pretreatment methods is to enhance hydrolysis rates owing to change of chemical and physical structure of the lignocellulosic materials.

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 microorganisms such as bacteria, archaea and anaerobic fungi in herbivorous' digestive system are able to effectively digest lignocellulosic compounds (Hobson, 1989). Thus, all herbivorous mammals can have energy from the lignocellulosic components thanks to symbiotic associations with rumen microorganisms (Flint, 1997). Rumen microorganism has higher hydrolytic and acidogenic activity than other microbial in columns when using lignocellulosic biomass as substrates (Bo Yue et al., 2013). 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).

In addition, bioagumentation which is a method for enrichment of specific microorganisms is used in anaerobic digester in order to enhance yield of hydrolysis, nutrient recovery, biogas production.

Although there are some studies in literature on potential applications of rumen microorganisms on anaerobic digesters, it is found that when ruminal fluid or rumen bacteria are used as a seed, the hydrolysis yield of cellulose-rich substrate is increased. However, there is no comprehensive study about rumen fungi for neither pretreatment of anaerobic digester nor bioagumentation of anaerobic digestion processes in the literature (Nkemka et al., 2015).

Because anaerobic fungi produce pretty much plant carbohydrate hydrolyzing, cellulolytic, hemicellulolytic, glycolytic, and proteolytic enzymes, they have quite important role in the digestive system of herbivorous. These enzymes consist of extracellular multi-enzyme complexes which is called cellulosomes. Cellulolytic efficiency of anaerobic fungi is originated from the cellulosomes because they can degrade both amorphous and crystalline cellulose. In addition, these enzyme systems hydrolyze complex lignocelluloses feed stock to soluble sugars and volatile organic compounds (Dollhofer et

al., 2015). Moreover, anaerobic fungi provide degradable plant polymers for the use of other microorganisms in the rumen thanks to extracellular enzymes. As a consequence, anaerobic fungi can provide improvement of biogas production thanks to the enzymatic degradation (Liggenstoffer et al., 2014).

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, microalgae and macroalgae. 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 bioagumentation method with anaerobic fungi for enhancement biogas potential of anaerobic digesters. Since there is no detailed study in this concept in the literature, the comprehensive study will be contributed to the literature and it will shed light on other studies for improvement of biogas production from different lignocellulosic compounds with anaerobic rumen fungi as well as determining microbial community dynamics by Illumina Miseq and control of anaerobic rumen fungi changes by qPCR in the digestion process.

2. THEORETICAL BACKGROUND

2.1. Fundamentals of Anaerobic Digestion

Anaerobic digestion is a stream of biological process providing degradation and stabilization of organic materials under anaerobic conditions by microbial organisms. The formation of biogas like carbon dioxide and methane as a renewable energy and microbial biomass are obtained thanks to anaerobic digestion (Chen at al., 2008). Thereby, biomass is converted to energy. Animal manure, municipal solid waste, food waste, industrial wastewater and residuals, fats, oils and grease (FOG), and various other organic waste streams are converted into biogas through a range of anaerobic digestion technologies. Because this biogas is environment friendly and economically beneficial, it is considered that the production of biogas during anaerobic digestion is one of the most efficient methods of producing renewable energy production (Dupla et al., 2004).

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 involves four phases consisting of hydrolysis, acidogenesis, acetogenesis and methanogenesis. During hydrolysis, insoluble organic matters are converted into soluble organic matters like amino acid, fatty acid and glucose by the fermentative microorganisms. Thus, the complex polymeric matters are hydrolyzed to monomers. Because hydrolysis step is considered as a rate limiting step in the digesters operated with lignocellosic compounds, it is considerably important process (Pavlostathis and Giraldo Gomez, 1991). In the acidogenesis step, the products of hydrolysis are converted to simple organic acids, such as volatile fatty acids and alcohols. The products of acidogenesis are converted into acetate, hydrogen and carbon dioxide by acetogenetic

microorganisms in the third step of anaerobic digestion which is called acetogenesis. Finally, methane and carbon dioxide are produced during methanogenesis in two ways. One of them is reduction of carbon dioxide with hydrogen. The other one is decaboxylation of acetic acid. Methane, carbon dioxide and some trace gases are released as a consequence of anaerobic digestion (Ostrem, 2004). These stages are shown on Figure 2.1.

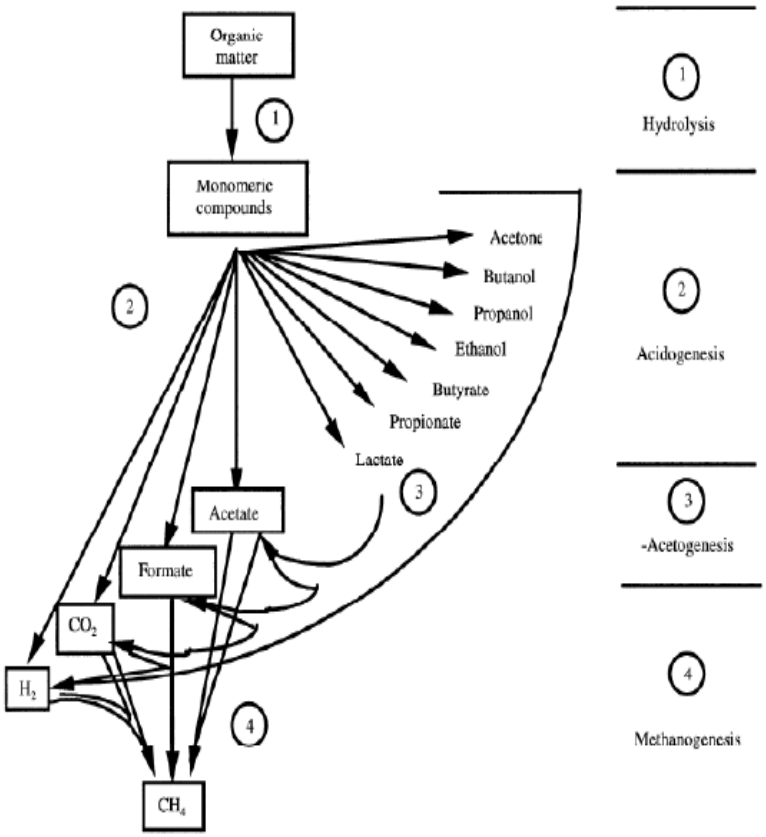

Figure 2.1. Schematic diagram showing anaerobic degradation of organic matter (Garcia et al., 2000).

In order to explain these biochemical steps in anaerobic digestion in detail, some models such as Three-stage model (Gerardi, 2003), Six-stage model (Lester et al., 1986) and Nine-stage model (Harper and Pohland, 1986) have also been developed. The nine-step model is described on Figure 2.2.

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.

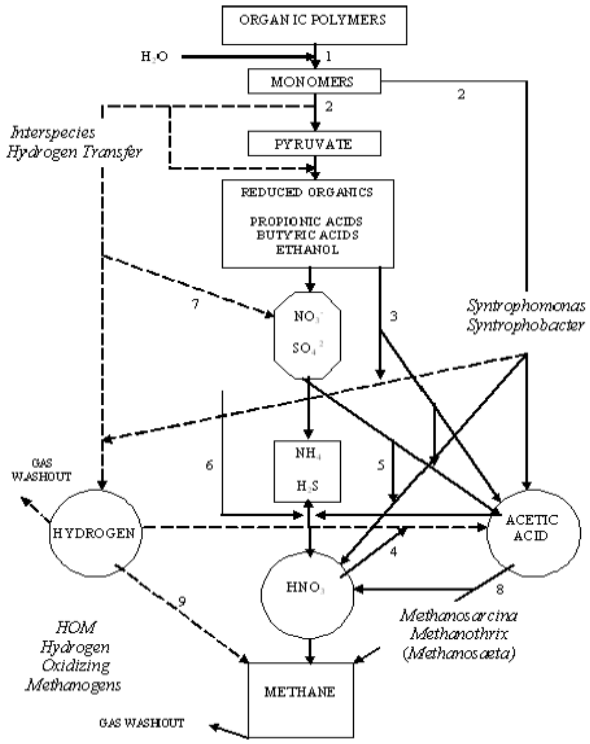

Figure 2.2. Substrate conversion patterns associated with the anaerobic digestion (Harper and Pohland, 1986).

2.1.2. Process Microbiology

Several groups of facultative and anaerobic microorganisms take part in the steps of anaerobic digestion process in order to degrade organic material. Synergetic community of microorganisms is found in an anaerobic digester so as to conduct the process of fermenting organic matter into methane. Anaerobic digestion is mediated during the

process of hydrolysis, acidogenesis, acetogenesis and methanogenesis by these microorganisms. These microorganisms are described as the following (Chernicharo, 2007).

1. Hydrolytic fermentative bacteria
2. Acidogenic (acid forming) bacteria
3. Hydrogen-producing acetogenic bacteria
4. Hydrogen-utilizing acetogenic bacteria
5. Carbon dioxide-reducing methanogens
6. Acetoclastic methanogens
7. Anaerobic fungi

Firstly, it is required that complex particulate materials such as lipids, proteins and carbohydrates are hydrolyzed to soluble organic matter that can be absorbed by microbial cells. This hydrolysis step is performed by specific extracellular enzymes which are produced by hydrolytic fermentative bacteria under anaerobic conditions in anaerobic digesters. pH, temperature, substrate composition, cell residence time, the by-products produced by the hydrolytic bacteria are important factors affected the reaction rates of extracellular enzymes in hydrolysis (Gerardi, 20013). The microbial community taking place in the hydrolysis stage is considerably heterogenic. It was found that the compounds containing cellulose are degraded by *Clostridium spp.*, but, *Bacillus spp*. is responsible for the degradation of protein and fats (Noike et al., 1985; Lema et al., 1991). The most widespread hydrolytic microorganisms are classified as cellulytic (Clostridium thermocellum), proteoytic (*Clostridium bifermentas, Peptococcus spp*.), lipolytic (genera of *Clostridia* and *Micrococci)* and aminolytic (*Clostridium butyricum, Bacillus subtilis*) bacteria (Payton and Haddock, 1986). In addition, it was found that a number of anaerobic fungi also can degrade the cellulose and hemicelluloses (Pearce and Bauchop, 1985). The hydrolytic microorganisms are also capable of degradation of some intermediate products to simple volatile fatty acids (VFAs), lactic acid, carbon dioxide, hydrogen, ethanol, ammonia and hydrogen sulfide (Eastman and Ferguson, 1981).

After hydrolysis step, the soluble monomers generated in consequence of hydrolysis are converted to short chain organic acids, alcohols, hydrogen and carbon dioxide by

facultative and obligatory anaerobic bacteria in the second step which is defined as acidogenesis. The concentration of hydrogen ions is important to determine the type of end products (Ren et al., 2007). It is required that the partial pressure of hydrogen is high in order to form acetate. Acidogenic or fermentative bacteria can metabolize amino acids and sugars to intermediary products like acetate and hydrogen. While single amino acids are produced by *Clostridia, Mycoplasmas* and *Streptococci*, butanol, butyric acid, acetone and iso-propanol are usually produced by *Clostridum sp.* Butyrate is produced by *Butyribacterium*, acetone and butanol are produced *by Clostridium acetobutylicum*. *Clostridium butylicum* also produces butanol, hydrogen, carbondioxide and iso-propanol (Macy et al, 1978).

During these reactions, different pathways are used. In the degradation of carbohydrates, propionic acid is formed by succinate pathway and the acrylic pathway. Butyric acid and Fatty acids are degraded by the beta oxidation reaction. Proteins are degraded by the Stickland reaction and if cysteine is degraded, hydrogen sulfide can be formed (Chernicharo, 2007).

In the third step of anaerobic digestion, acetogenesis, the product of acidogenesis are converted into acetate, hydrogen and carbon dioxide by acetogenic bacteria from which methane can be obtained. Because acetate is the most common and significant precursor of methane production, acetogenic bacteria have an important role for methanogenic microorganisms. Hydrogen producing or hydrogen consuming acetogenetic bacteria plays a role in the conversation acidogenesis end products to acetate. There are two different types of acetogenic mechanisms; acetogenic hydrogenation and acetogenic dehydrogenation. Acetogenic hydrogenation includes production of acetate from fermentation hexoses or from CO_2 and H_2. Acetogenic dehydrogenation refers to the anaerobic oxidation of long and short chain volatile fatty acids by obligate proton reducing or obligate hydrogen producing bacteria (OHPA) (Gavala et al., 2003). Acetic acid producing bacteria are *Methanobacterium bryantii, Desulfovibrio Syntrophobacter wolinii, Syntrophomonas wofei and Syntrophus buswellii* are the most common acetic acid producing bacteria (Gujer et al., 1983).

Methanogenesis which is also called as methane fermentation is the final step in anaerobic digestion where products of acetogenesis are converted to methane and carbon dioxide by methanogenic Archaea which is consisted of strictly anaerobic microorganisms belonging to *Euryarchaeota* (Woese et al., 1977). Phylogenetically, methanogens are classified in domain Archaea. Archaeal cells have unique properties separating them from the other two domains of Bacteria and Eukaryota. Phylogenetic tree of 3 domains based on rRNA analysis is given in Figure 2.3. (Woese et al, 1990).

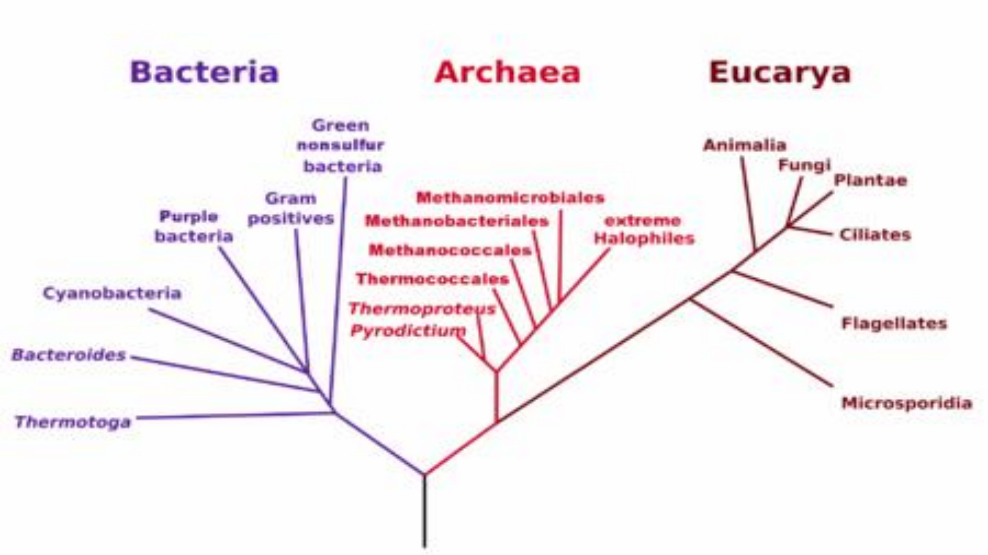

Figure 2.3. Universal phylogenetic tree (Woese et al, 1990).

Methanogens are grouped into five orders within kingdom Archaeobacteria: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales and Methanopyrales* (Figure 2.4). Most methanogenic archaea can produce methane (CH_4) and carbon dioxide from carbon monoxide, formate and a few alcohols (Thauer et al., 2008). Methyl groups can also be reduced to methane by methanogens. Only limited number of substrates can be used by methanogens in order to produce methane. They can be classified into 3 main groups according to their affinity for different substrates (Table 2.1.).

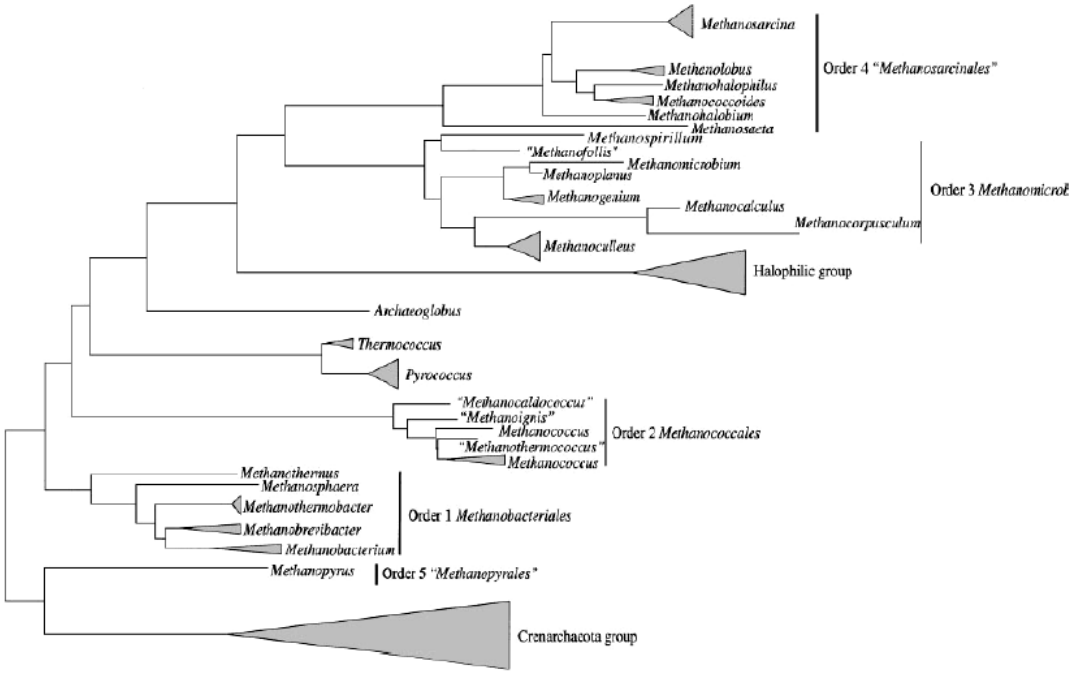

Figure 2.4. Phylogeny of methanogens, domain Archaea (Garcia et al., 2000).

In the CO_2 type of substrate, methane is produced from hydrogen and carbon dioxide by hydrogenotrophic methanogens including *Methanobacteriales, Methanomicrobiales, Methanococcales* and *Methanosarcinaceae* (Chernicharo, 2007). The general equation for this conversion is shown in Eq 2.1.

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (2.1)$$

In the methyl group of substrate, methane is produced from methyl compounds by methylotrophic methanogens via two different pathways. While methane is formed by reducing methyl group substances with an external electron donor such as H_2 (Equation 2.2) in the first mechanism, methyl compounds are converted to methane without H_2 in the second mechanisms (Equation 2.3) (Singh et al.,2005).

$$CH_3OH+H_2 \rightarrow CH_4+H_2O \quad (2.2)$$

$$4CH_3OH \rightarrow 3CH_4+CO_2+2H_2O \quad (2.3)$$

Table 2 1. Substrates converted to methane by various methanogenic archaea (Madigan et al., 2002).

Substrates and Reaction	**Organisms**
I. CO_2-type substrates (Carbon dioxide with electrons derived from H_2, certain alcohols, or pyruvate; Formate, Carbon monoxide)	**Hydrogenotrophic Metanogens**
$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	Most methanogens
$4\ HCOOH \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$	Many hydrogenotrophic methanogens
CO_2+4isopropanol$\rightarrow CH_4$ + 4acetone + $2H_2O$	Some hydrogenotrophic methanogens
$4\ CO + 2H_2O \rightarrow CH_4 + 3\ CO_2$	*Methanothermobacter* and *Methanosarcina*
II. Methylated C1 compounds (Methanol, Methylamine, Dimethylamine, Trimethylamine, Methylmercaptan, Dimethylsulfide)	**Methylotrophic Methanogens**
CH3OH→3 CH4 + CO2 + 2 H2O	*Methanosarcina* and other methylotrophic methanogens
CH3OH + H2 →CH4 + H2O	*Methanomicrococcus blatticola* and *Methanosphaera*
2 (CH3)2-S + 2 H2O→3 CH4 + CO2 + 2 H2S	Some methylotrophic methanogens
4 CH3-NH2 + 2 H2O→3 CH4 + CO2 + 4 NH3	Some methylotrophic methanogens
2(CH3)2-NH + 2 H2O→3 CH4 + CO2 + 2 NH3	Some methylotrophic methanogens
4 (CH3)3-N+ 6 H2O→9 CH4 + 3 CO2 + 4 NH3	Some methylotrophic methanogens
4CH3NH3Cl + 2H2O→3CH4 +CO2 + 4 NH4Cl	Some methylotrophic methanogens
III. Acetate	**Acetoclastic methanogens**
$CH_3COOH \rightarrow CH_4 + CO_2$	*Methanosarcina* and *Methanosaeta*

In the acetate type of substrate, methane is produced from acetate by Acetoclastic methanogens containing *Methanosarcina* and *Methanosaeta* (Equation 2.4.). Because acetate is the major product of fermentation, they are generally dominant in the anaerobic digester systems. Almost 70% of all methane production is provided by acetoclastic methanogens. *Methanosarcina* have higher methane yield in comparisiton to *Methanosaeta* (Schmidt and Arhing, 1996).

$$CH_3COOH \rightarrow CH_4+H_2O \quad (2.4)$$

In addition to bacteria and methanogens, recent studies showed that anaerobic fungi have also important role in anaerobic digestion systems. In nature, anaerobic fungi are principally known from the digestive tracts of larger mammalian herbivores, where they have significant role for ingestion of forages (Liggenstoffer et al., 2010). Anaerobic fungi are actually a member of rumen microorganisms and they are represented 6 genera (*Neocallimastix, Piromyces, Caecomyces, Anaeromyces, Orpinomyces* and *Cyllamyces)* and 20 species. Because of anaerobic life style and life cycle with two stages, classification of anaerobic fungi was a matter of debate in the past, but, they are classified in a new phylum *Neocallimastigomycota* according to recent studies (Hibbett et al., 2007). It was showed that anaerobic fungi which belong to the phylum *Neocallimastigomycota*, are the most fundamental lineage of the kingdom Fungi (Figure 2.5).

It was found that there is a close relationship of anaerobic fungi with methanogens, especially (Cheng et al., 2009). Anaerobic fungi can effectively degrade lignocellulosic plant materials and they can produce several en-products which contain acetate, formate, lactate ethanol, H_2 and CO_2. After production of these end-products, they can be utilized by methanogenic Archaea in order to generate methane. Thus, anaerobic fungi and methanogens are also referred to as methane producing co-cultures (Dollhofer, 2015). Inter-species hydrogen transfer provides methane production and more effective re-generation of oxidized nucleotides (e.g., NAD^+, $NADP^+$). Performance of anaerobic fungal fermentation is affected by inter-species hydrogen transfer in these co-cultures. Hence, it changes catabolism pathways and specific enzyme profiles, altering fungal product formation away from more oxidized end-products (lactate and ethanol) and towards

production of more reduced products (formate and acetate). Acetate and formate are used as growth substrates for methanogens (Nakashimada et al., 2000).

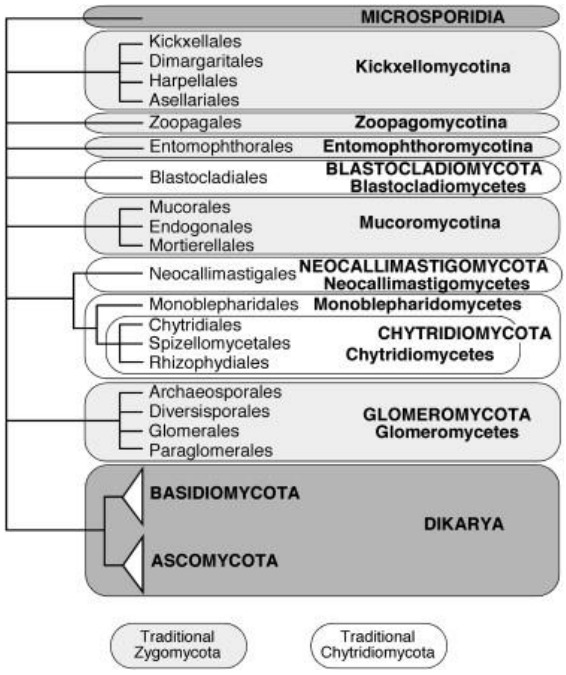

Figure 2.5. Fungus Taxonomy (Hibbett et al., 2007).

2.2. Environmental and Operational Factors Affecting Anaerobic Digestion

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 significant factors affecting the performance of anaerobic digestion in terms of ionization equilibrium, solubility of substrates, substrate removal rate, specific growth rate, decay biomass yield, and half saturation constant. Stover et al. (1994) demonstrated that anaerobic digestion processes, especially conversation of methane from acetate, are relatively sensitive to the temperature variations. Biogas production can be influenced adversely by temperature fluctuations. Anaerobic digestion can actually proceed under psychrophilic (<25 °C), mesophilic (25–40 °C) and thermophilic (>45 °C) conditions, however, mesophilic (25ºC - 40ºC) and thermophilic (45 ºC - 60 ºC) conditions are more favorable (El-Mashad et al., 2004). Thermophilic conditions provide many advantages such as higher metabolic rates, higher specific growth

rates, so, process can be faster and more effective. On the other hand, lower stability and energy requirement are the important downside of thermophilic conditions (Duran and Speece, 1997). Thus, anaerobic digestion is usually processed under mesophilic conditions, mostly between 35-42^{o}C for lower stability and higher susceptibility to changes in environmental and operational conditions of thermophilic conditions. Nevertheless, temperature mostly depends on the type of microorganisms in anaerobic digestion processes. Methanogenesis can be carried out both in the mesophilic and the thermophilic temperature conditions. Although bacteria are not usually sensitive to temperature fluctuations until the metanogenesis step, methanogenic Archaea is highly sensitive to even small temperature changes (Noike et al., 1985). On the other hand, anaerobic fungi can be able to tolerate temperature changes in mesophilic temperature conditions (Lowe et al., 1987).

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. 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. Although anaerobic fungi can tolerate a wide range of pH, the optimum pH conditions are acidic (Orphin and Joblin, 1997). 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 sufficiently high, organic acids producing by acidogenic bacteria lead to decrease 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 unfavourable environmental conditions, acidity can lead inhibitory effect on methanogens. Yet, acidogenetic bacteria and anaerobic fungi are more resistant to acidity (Malina and Pohland, 1992).

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. Yen and Brune (2007) were proven that the addition of carbon-rich waste papers as a macronutrient into algal biomass digester leads to enhance methane yield owing to ensuring balance between carbon and nitrogen in feed.

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 breakdown 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 (NH_3) 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 volatile fatty acids (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 1,500 – 2,000 mg/L.

In addition to abovementioned 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).

2.3. Biogas from Anaerobic Digestion System

Use of renewable energy sources instead of fossil fuels has increased in recent times due to pressures on the global environment generating greenhouse gases accumulated in the atmosphere and caused climate change. Recently, in order to decrease the greenhouse gases in the atmosphere and prevent the climate change, alternative renewable energy sources were suggested in Kyoto protocol (United Nations, 1998). Biomass is one of the most important potential sources of renewable energy because it can be found almost everywhere and can be stored. Thus, it can be said that biomass is a non-stop energy source comparing to other sources (McKendry, 2002). Although the initial applications of anaerobic digester were for stabilization and treatment of waste sludge, anaerobic digestion systems are also source of renewable energy and the most substantial part for production of biogas (Yu and Schanbacher, 2010). They are naturally occurring phenomenon in which organic matter is converted by various microorganisms in an oxygen-free environment in order to produce biogas including methane (CH_4) and carbon dioxide (CO_2) (Frigon and Guiot, 2010).

Biogas is utilized by main conversion processes of anaerobic digesters. Organic materials like manure, food scraps, crop residue, micro-macoalgae or wastewater sludge are fed into the digester and stirred for 30-60 days, slowly producing a combination of methane, carbon dioxide and other gases which are known as biogas. The biogas can then be used for power generation, heating, electricity and cooling needs or piped into the natural gas grid. After completing of biogas production, high-quality fertilizer which is called digestate is produced from the wastes and all processes starts all over again (Weiland, 2010). Therefore, biogas can be used for various purposes such as household, commercial and industrial applications.

2.3.1. Feedstocks for Biogas Production

Biomass materials supply feedstocks for several biogases, end-products and end-uses. Although all kinds of organic waste containing carbohydrates, fats, lipids, cellulose and hemicelluloses may be used as a substrate for the anaerobic digestion process, the majority of biomass for biogas feedstocks is classified in three main sources, such as, forests, agriculture, and waste. In addition, non-forest lands including grasslands, savannahs and algaculture are also potential sources of biogas feedstocks. On the other hand, biomass sources can be classified into two extensive groups: woody and non-woody (Figure 2.6.). While forests provide just woody materials, agriculture sources provide both woody and non-woody biomass for biogas production (Figure 2.7.). Alongside of them, types of biomass were classified as terrestrial or aquatic by Gunaseelan et al. (2007) (Figure 2.8.). In addition to type of feedstocks, retention time and digestion system are also important for the composition of the biogas and methane yield. As for the theoretical biogas yield, it depends on the carbohydrate, protein and fat content of the substrate (Braun, 2007).

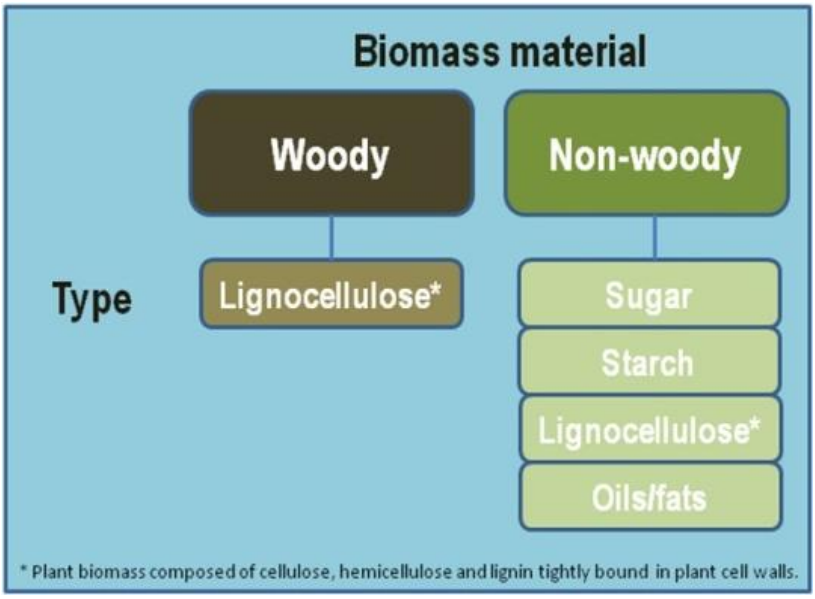

Figure 2.6. Two broad categories of biomass materials, and four composition types (CL Williams, 2011).

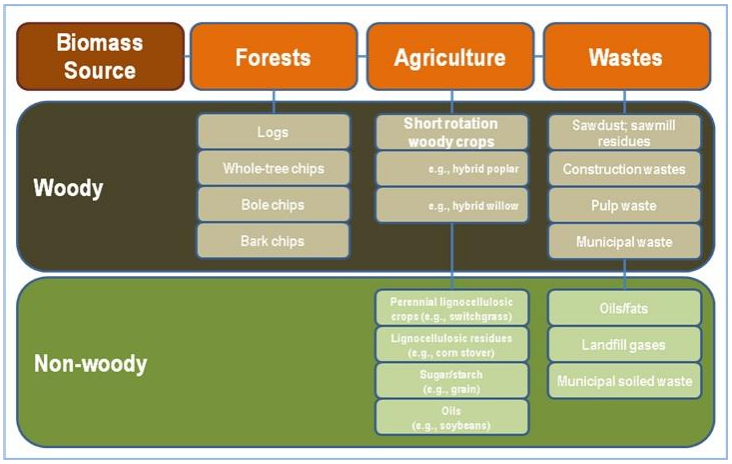

Figure 2.7. Sources and types of biomass materials for conversion into biogas (CL Williams, 2011).

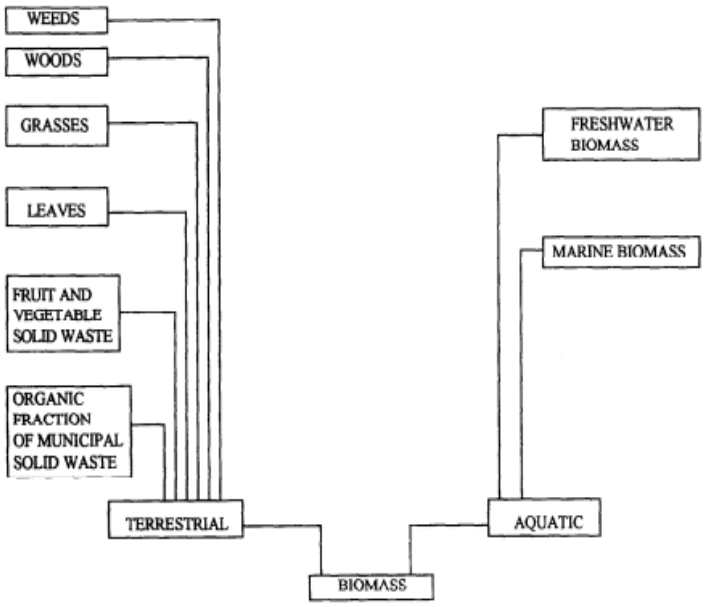

Figure 2.8. Selected types of methane yielding biomass (Gunaseelan et al., 2007).

Until today, agricultural crop wastes and residue, municipal solid waste, sewage, forestry crops and residue, industrial residue and animal residue have been mostly used as a biomass source in anaerobic digestion processes. Moreover, these substrates can be used with the additional cosubstrates so as to increase the substances of organic material for efficient biogas production. Biogas yield values of these several substrates are shown on Table 2.2. (Weiland, 2010).

Table 2.2. Biogas yield values of several substrates (Weiland, 2010).

Substrate	DM[a] (%)	ODM[b] (% DM)	C/N	Biogas yield (m^3CH_4/kg ODM)
Chicken manure	15	77	7	0.2–0.4
Pig manure	5–7	77–85	5–10	0.2–0.3
Raw glycerol (biodiesel)	> 98	90–93	–	0.69–0.72
Rotten potatoes	25	79	25	0.5–0.6
Clover	20	80	12	0.4–0.5
Apple slops	25	86	30	0.3–0.4
Spent grain	20–22	87–90	10	0.6–0.7
Bread (waste)	90	96–98	42	0.7–0.75
Cacao peels	95	91	2.8	20–22
Molasses	80	95	14–27	0.3
Whey	95	–	27	0.5–0.6
Rape seed slops	92	97	9–12	0.58–0.62
Green waste	60–75	30–70	40–80	0.2–0.6
Flotating sludges (fat)	5–24	83–98	–	0.6–0.8
Feces (intestinal)	12–15	80–84	17–21	0.2–0.3
Rumen (pressed)	20–45	90	11–20	0.6–0.7
Animal meal	8–25	90	–	0.5–0.8
Fat (separators)	35–70	96	–	0.7 (1.0)
Grass	21–23	76–80	22–24	0.45–0.5

[a] dry matter
[b] organic dry matter

After treatment of municipal wastewater, large amount of sewage sludge is produced. Sewage sludge which is also called as wastewater sludge contains the dry matters consisting of nontoxic organic compounds and microbiological sludge. Therefore, use of sewage in anaerobic digesters is one of the most significant options for biogas production (Müller et al., 2004). Wastewater sludge can be stabilized and also converted to the volatile compounds in to biogas by anaerobic digestion, so, the biogas can be applied as an energy resource at wastewater treatment plants or some other place. Production of biogas from sewage sludge is already carried out worldwide on small, medium, and large scales (Rulkens, 2007).

Industrial wastes such as pharmaceuticals, personal care products, steroid hormones, surfactants, some industrial chemicals, pesticides, bio-pesticides can be also considered as another important feedstock for biogas production. Because industrial wastes have wide range of organic matters and high chemical oxygen demand (COD) levels, they are considerably favourable alternative for anaerobic biogas production. Moreover, anaerobic digestion is a very useful process for high COD containing wastewaters (Carballa et al., 2007). Because most of these compounds are resistant, they cannot be completely removed by conventional wastewater treatment plants (Hug et al., 2014). Thus, use of industrial

waste in anaerobic digesters as a feedstock is comparatively important to induce undesirable ecological effects on water quality and environment (Eggen et al., 2014).

Another most common source of biomass for biogas production is forestry crops and residues. There are many sources of crop and forest residues and they have different amount of biomass remaining after harvest (Pimentel et al., 1981). Small trees, branches, tops and unmerchantable wood are usually considered as main sources of forest residues. They can be considered as a feasible substrate for biogas which is one of the high value products. After timber harvesting, they can be collected and used for energy production (Ravindran and Jaiswal, 2015). Conversion of forestry residues which is an important lignocellulosic biomass to biogas is quite important for environment. Since biogases are cleaner-burning in comparison with fossil fuels, they can enhance energy security and reduce greenhouse emission (Stöcker, 2008).

Energy crops, which are defined as a plant grown as a cost-efficient and low-maintenance harvest used for biogas or combusted for bioenergy, are the most common cosubstrates. Because they require less maintenance and fewer inputs, they are cheaper and more sustainable to produce. The use of them is considerably interesting alternative to cofermentation. The most commonly used energy crops are maize, corn, sunflower and grass. Weiland (2013) investigated that high methane yields can be achieved by them if energy crops are harvested before lignification.

Food crops and ligno-cellulosic plant biomass have been usually studied as an alternative feedstock for biogas production, however, the competition of fuel with food caused to lose the popularity of these feedstocks (Saqib et al., 2013).

In addition to abovementioned biomass sources, animal manure is usually defined as a primary source of biomass and biogas produced from animal manure is considered as the most common renewable bio-fuel source (Thien Thu *et al*., 2012). Animal manure is produced by husbandry and agriculture. It is a major source of environmental pollution in many countries which have generally agricultural and livestock breeding activities such as Turkey, because animal manure contains harmful pathogens and noxious gases which are toxic and harmful to living organisms (Sorathiya et al., 2014). Despite the fact that there

are some alternatives in order to treat and dispose animal manure, it was revealed that anaerobic digestion provides the effective solving for pollution reduction and energy production also improving the fertilize value of the manure by Alvarez and Giden (2009). The main aim of the anaerobic digestion of animal manure is to convert organic residues into biogas. After that, it may be also used to generate heat, electricity or as vehicle fuel and fertilizer in agriculture (Holm-Nielsen et al., 2009). El-Mashad and Zhang (2010) demonstrated that about 90% of the final biogas yield can be obtained from animal manure after 20 days of digestion. Production of biomethane from animal manure wastes as an alternative source of energy was also revealed by Obiukwu and Nwafor (2014). This study showed that biogas which contains the methane content of 65% was produced during anaerobic digestion process.

Although algae were not considered as a biomass source for biogas production in the past, it is known that algae have considerably high potential for anaerobic digestion processes in order to produce biogas, nowadays. Moreover, they are considered as a promising biomass source for biogas production. Because algae have high growth rates and the possibility of cultivation on non-arable land areas or in lakes or the ocean, they have got many potential advantages in comparison with higher plants (Rittmann, 2008). Anaerobic digestion of algae can be divided into two principal components: biogas derived from macroalgae (seaweed) and biogas derived from microalgae (single cell plants) (Brennan and Owende, 2010).

Microalgae, which are found in freshwater and marine systems living in both the water column and sediment, are one of the most conspicuous types of algae. Because microalgae have high lipid content, high photosynthetic performance, advanced growth rate and characteristics of not needed external organic carbon source, they can be an alternative to terrestrial energy crops for biofuel and biogas production (Passos et al., 2013). Production of biogas comprising hydrogen or methane from anaerobic digestion of microaalgae as a source of biomass is conspicuous technology because they have capacity of energy conservation and environmentally friendly feature. In addition to environmental conditions promoting the microbial activity, degradation of substrate is quite important parameter through anaerobic process. Anaerobic digestion can be used directly on algae which newly collected or microalgal wastes after lipid extraction (Mahdy et al., 2015).

Mussgnug et al. (2010) investigated that algae species can be favorable substrates for biogas production and that anaerobic fermentation can seriously be considered as the last step in future microalgae-based biorefinery concepts. *Haematococcus sp.* and *Chlorella sp.* are most common species used in studies of biogas production of anaerobic digestion processes.

Macroalgae, which are also defined as seaweed and members of the huge group of aquatic plants, are another alternative biomass source for biogas production in anaerobic digesters in recent times. Since accumulation of macroalgae as a consequence of eutrophication in coastal waters makes an important pollution problem, it can be considered that the accumulation of macroalgae can provide the exploitation of macroalgal biomass as feedstock for biogas production (Migliore et al., 2012). Despite the fact that the production of macroalgae for biofuels in the marine environment was first experienced in the late 1960's, they have become popular nowadays. Although macroalgae are generally used to produce transport fuels like biodiesel referring to vegetable oil or animal fat based diesel fuel consisting of long-chain alkyl esters, it was reported that methane has the higher heating value in comparison with biodiesel, bioethanol and biomethanol by Reijnders and Huijbregts (2009). Hughes et al. (2012) investigated that the energy conversion via anaerobic digestion is succeeding as the biochemical composition of macroalgae makes it an ideal feedstock. Because macrolgae have little cellulose and no lignin, they can easily complete hydrolysis of anaerobic digester process. They can be transformed into biogas by many processes involving thermal treatment and fermentation, however, the most direct route to provide biogas from macroalgae is via its anaerobic digestion (AD) to biogas (~ 60% methane) (Goh and Lee, 2010). After, heat and electricity can be also produced so as to use as a transport fuel. Macroalgae species of the genus Ulva (Chlorophyta) are usually preferred as biomass sources for food and feed purposes, owing to its high contents of vitamins, trace metals, and dietary fibers (Nielsen et al., 2012). Bruhn et al. (2011) proved that macroalgae Ulva lactuca can gain 20 times the production potential of conventional terrestrial energy crops.

2.3.2. 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, 2008).

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 Cº. 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 separated 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 degradation 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 (CO2; 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 (Schneider et al., 2002).

2.4. Improvement of Biogas Production on Anaerobic Digesters

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 and algal biomass under anaerobic conditions. In order to eliminate these major problems, additional studies are required (Bayane and Guoit, 2011).

Pretreatment methods are most commonly used techniques in order to improve biogas production in anaerobic digesters. Hydrolysis performance can be improved by pretreatment methods rates owing to change of chemical and physical structure of the lignocellulosic materials. Pretreatment, which is well-investigated process for biogas production from lignocellulosic materials, can be physical, chemical or biological. In general, all pretreatment methods pretreat waste materials, improve the bio-digestibility of the wastes for biogas production and accessibility of the enzymes to the materials. As a consequence of pretreatment, adverse effects of difficult biodegradable lignocellulosic compounds can be prevented and biogas production on anaerobic digesters can be improved. In addition to biogas, bioethanol production can be obtained during pretreatment process (Figure 2.9.) (Taherzadeh and Karimi 2008).

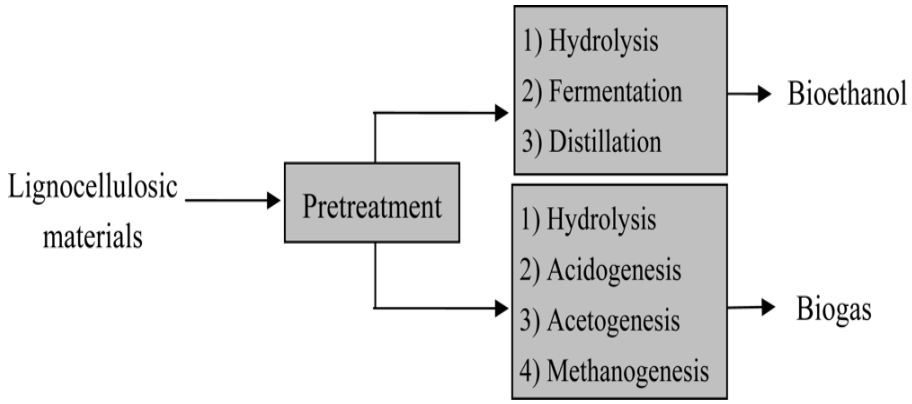

Figure 2.9. Pretreatment of lignocellulosic materials prior to bioethanol and biogas production (Taherzadeh and Karimi, 2008).

Lignocelluloses contain a large range of animal manures, municipal solid waste (MSW), crop residues, forest residues, energy crops and also micro and macroalgal biomass (Figure 2.10.). Hemicellulose, lignin, extractives, and several inorganic materials are found in lignocelluloses (Sims, 2003). Cellulose which is also called as β-1-4-glucan is a linear polysaccharide polymer of glucose made of cellobiose units. Hydrogen bond are found between the cellulose chain and this structure is defined as so-called 'elementary and microfibrils' (Ha et al., 1998). These fibrils are attached to each other by hemicelluloses, amorphous polymers of different sugars, pectin, and covered by lignin. This special and complicated structure makes cellulose resistant to conventional treatment methods (Persson et al. 2006).

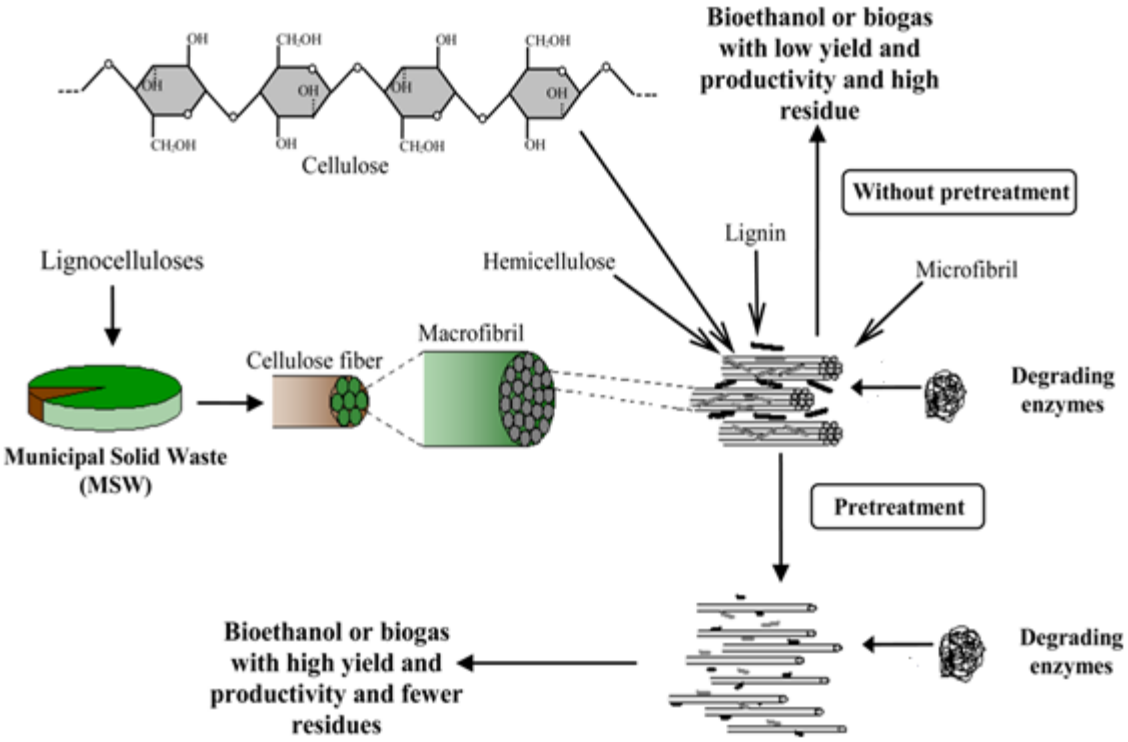

Figure 2.10. Effect of pretreatment on accessibility of degrading enzymes (Taherzadeh and Karimi 2008).

Biodegradation of lignin is particularly difficult because it is a considerably complex molecule made of phenylpropane units which are linked in a three-dimensional structure. If a molecule has the high proportion of lignin, this molecule is highly resistance to chemical and enzymatic degradation. Lignin, hemicellulose and cellulose are linked with chemical bonds which are quite difficult to break (Palmqvist et al., 2000).

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). However, the cellulosic compounds in manure, which lead the limitation in hydrolysis step, are the most important problem in energy production from animal manure under anaerobic conditions. Thus, it is required to find some treatment methods in order to improve biogas production.

Because microalgae have high photosynthetic performance, advanced growth rate and characteristics of not needed external organic carbon source, microalgae are considered as a high potential source of bioenergy and biofuel production. However, digestibility of microalgae for production of bioenergy can also be restricted by their difficult cell walls. Because cellulose causes to the recalcitrance of cell wall for the microalgae *Haematococcus pluvialis*, some treatment methods are required to eliminate the restriction of microalagaes' cell wall (Ward et al., 2014).

Macroalgae contain little amount of lignin and cellulose in comparison to other lignocellulosic compounds such as terrestrial plants, agricultural plants and forestry residues, but yet certain treatment methods are also required to improve biogas production from macroalgae *Ulva lactuca* (Nikolaisen et al., 2011). Lignocellulosic compounds in macroalgae can cause rate limitation in hydrolysis which is the first step of anaerobic digestion. This limitation in hydrolysis leads the negative effect on the performance of anaerobic digesters and reduction in yield (Nielsen and Heiske, 2011). Thus, further improvement in this conversion technology is desired to get rid of the adverse effects of these lignocellulosic compounds in macroalgae (Saqib et al., 2013).

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). Despite the fact that these pretreatment methods could improve biogas especially methane yield, the energy cost of these pretreatment technologies was quite high. Moreover, if the thermochemical pretreatment methods are used, it can be caused to a probable configuration of inhibitory substances (Carrere et al., 2015). However, biological pretreatment method which contains enzymes and

microorganisms that naturally digest lignocellulosic compounds in their natural habitat and under physiological conditions is a remarkable alternative so as to improve the biogas potential of anaerobic digester (Nkemka et al., 2015).

Bioaugmentation which is a method of addition of specific microorganisms, selected strains or mixed cultured is a considerably important biological pretreatment method. Bioaugmetation is used in anaerobic digester in order to enhance yield of hydrolysis, nutrient recovery and biogas production. It can be efficient alternative process to overcome troubles and improve performance of biogas production in anaerobic biological treatment (Herrero and Stuckey, 2014).

Angelidaki and Ahring (2010) investigated that biogas potential of the animal manure is increased by bioaugmentation with the hemicellulose degrading bacterium B4. It was showed that there is an increase of approximately 30% in methane potential in comparison with controls thanks to biological treatment of animal manure.

Aydin (2016a) investigated that how bioaugmentation with the bacterium *Clostridium thermocellum* at various inoculums ratios affects the CH_4 production from microalgae. This study showed that bioaugmentation with *Clostridium thermocellum* provided to increase the microalgal biomass and enhance 18–38% increase in methane production due to increased cell disruption. Although anaerobic digesters are bioaugmented with various kinds of microorganisms, rumen microorganisms are considered as one of the most important biological pretreatment options for improvement of biogas production in recent years.

Despite the fact that some studies have been conducted to improve biogas production from macroalgae in the literature, there is no study about biological pretreatment or bioaugmentation. Nielsen and Heiske (2011) researched the effects of mechanical pretreatment in the form of maceration on the methane yield of *Ulva lactuca.* Montingelli et al. (2015) demonstrated that improvement of methane yield in anaerobic digesters fed with macroalgae depends upon the type of pretreatment and algal species. Although this study showed the effects of physical pretreatment on anaerobic digestion, it is also revealed

that it is required to investigate the effects of different pretreatments under optimal AD parameters for enhancement methane production from macroalgae.

Rumen is a natural cellulose-degrading system in the mammalian animals. It is like a large fermentation room that microbial population helps to digest the herbivorous' diet. Rumen fluid or ruminal bacteria, ciliate protozoa and anaerobic fungi can be used in order to bioaugment the anaerobic digesters. Lignocellulosic compounds in animal manure, microalgae and macroalge can be effectively digested thanks to bioaugmentation of rumen microorganisms (Bo Yue et al., 2013). It was displayed that rumen microorganism has higher hydrolytic and acidogenic activity than other microbial in columns when using lignocellulosic biomass as substrate by Bo Yue et al. (2013).

Budiyono et al. (2014) studied with ruminal fluid of animal ruminant in order to improve biogas production rate from cattle manure at mesophilic condition. A series of laboratory experiments were carried out in batch digesters. As a consequence of the study, it was observed that the ruminal fluid inoculated to biodigester considerably affected the biogas production. When compared to manure substrate which was not bioaugmented with ruminal fluid, it was proven that ruminal fluid inoculums induce the biogas production rate and efficiency increase more than two times.

Jin et al. (2014) studied anaerobic fermentation of biogas liquid pretreated maize straw by rumen microorganisms in vitro. This work investigated that rumen microorganisms have feasible and efficient influence on anaerobic hydrolytic acidification of biogas liquid pretreated maize straw.

Some studies have evaluated for bioaugmentation of anaerobic digestion processes with rumen anaerobic bacteria. Cirne et al. (2006) indicated that bioagumentation with an anaerobic rumen bacterium on anaerobic digestion improved the hydrolysis of the lipid fraction. The bioaugmenting lipolytic bacterium strain *(Clostridium lundense)* was isolated from bovine rumen fluid. It was showed that anaerobic digesters which are bioaugmented with anaerobic rumen strain provide to increase in the methane production rate and accordingly, a reduction in the digestion period required to achieve the same methane yield as the control.

Rumen fluid or rumen bacteria were generally used in order to improve biogas production on anaerobic digesters by anaerobic rumen microorganisms in the literature. However, using of ruminal fluid on anaerobic digesters is considerably impractical because sampling from ruminants is very difficult and painful process. Thus, bioaugmentation with anaerobic rumen microorganisms, which can be isolated from rumen fluid and reproduced without the need for sampling frequently from ruminants, is more useful method for enhancement of biogas production on anaerobic digesters. Although rumen microorganisms contain bacteria, archaea and fungi, there is no comprehensive study about rumen fungi for neither pretreatment of anaerobic digester nor bioagumentation of anaerobic digestion processes in the literature. (Nkemka et al., 2015).

Anaerobic rumen fungi were first isolated from the rumen of a sheep and described by Orpin (1975). It was believed that there are only bacteria and protozoa in the microbial population of rumen until the discovery of anaerobic rumen fungi. After finding large numbers of fungi which are colonized fibrous plant materials in the rumen of herbivorous, the importance and possible role of rumen fungi in fiber digestion was noticed (Akin and Borneman, 1990). Because anaerobic fungi produce pretty much plant carbohydrate hydrolyzing, cellulolytic, hemicellulolytic, glycolytic, and proteolytic enzymes, they have quite important role in the digestive system of herbivorous. These enzymes consist of extracellular multi-enzyme complexes which is called cellulosomes. Cellulololytic efficiency of anaerobic fungi is originated from the cellulosomes because they can degrade both amorphous and crystalline cellulose. In addition, these enzyme systems hydrolyze complex lignocelluloses feed stock to soluble sugars and volatile organic compounds (Dollhofer et al., 2015). Moreover, anaerobic fungi provide degradable plant polymers for the use of other microorganisms in the rumen thanks to extracellular enzymes. Because they can effectively degrade lignocellulose-rich substrates as physical and enzymatic (Table 2.3.), they have the high potential to make the biogas production from lignocellulose-rich materials more effective and profitable. In addition, the ability of the anaerobic fungi to penetrate deeply into plant tissues that are not accessible to rumen bacteria hints that they have a special role in fiber digestion (Fliegerova et al., 2012). As a consequence, anaerobic fungi can provide improvement of biogas production thanks to the enzymatic degradation (Liggenstoffer et al., 2014). Akhtar et al. (1992) indicated that

biological fungal pretreatment resulted in up to 38% energy saving and safe alternative compared to other treatments.

Lignocellulosic and hemisellulosic compounds cause a physical barrier for enzymatic attack. However, anaerobic rumen fungi have the ability to open up the plant tissue thanks to rhizoidal growth and so produce enzyme complex (cellulosomes) in order to degrade the various lignocellulosic compounds in lignocellulosic biomass. These enzymes can synergistically hydrolyze the recalcitrant materials through three different enzymatic activities; endoglucanases (EC 3.2.1.4) exoglucanases (EC 3.2.1.176; EC 3.2.1.91) and cellobiohydrolases (EC 3.2.1.176; EC 3.2.1.91) (Dollhofer et al., 2015). These anaerobic fungal enzymes have the synergistic and more effective way in comparison with bacterial enzymes due to efficient degradation and also protection against the surrounding environment in anaerobic digester (Steenbakkers et al., 2002).

Exchange and stable integration of genetic material between different strains or species are defined as horizontal gene transfer (HGS) (Doolittle, 1999). One of the main reasons why anaerobic rumen fungi evolved the resistant and influential activity of cellulolytic hemicellulolytic and lignocellulosic capability is considered as horizontal gene transfer from bacteria. Cellulosomes were firstly identified in bacteria *Clostridiaceae*. On the other hand, anaerobic rumen fungi also have this enzyme system as only eukaryotic representatives. Because cellulosome in anaerobic rumen fungi shows similarity to bacterial cellulosome in terms of structure and phylogenetic, it was thought that fungal cellulosomes evolved from bacterial cellulosomes during HGT. Up till now, cellulosomes arising from horizontal gene transfer have been described for species of *Piromyces, Orpinomyces, Neocallimastix and Anaeromyces.*

Although there is not enough study with anaerobic rumen fungi to improve biogas production in literature, some studies have been contacted with several fungal species. Taniguchi et al. (2005) studied with biological pretreatment of rice straw using different white-rot fungi (*Phanerochaete chrysosporium, Trametes versicolor, Ceriporiopsis subvermispora, and Pleurotus ostreatus*). Results were showed that influence of enzymatic hydrolysis of rice straw and biogas production of rice straw increase due to biological pretreatment of white-rot fungi.

A comparative study has not been reported in literature so far on bioaugmentation of anaerobic rumen fungi in anaerobic digesters fed with manure, microalgae and macroalgae. However, some studies have performed with other substrates such as slurry and grass. Fliegerova et al. (2012) worked on potential influence of anaerobic fungi on biogas production. Batch, semicontinuous, and continuous reactors fed with anaerobic slurry, grass and maize silage were bioaugmented with anaerobic rumen fungi. This study showed that there is a positive effect of rumen anaerobic fungi on biogas amount and quality. The biogas amount was improved by anaerobic fungi by 9% up to 18 %; methane ratio in biogas was higher about 2.5% depending on the used substrate and species of rumen fungi. It was considered that efficient hydrolysis due to rumen fungi caused to improve the degradation of substrates, provide the highly effective biogas yield and quality.

Table 2.3. Examples for lignocellulosic residues degraded by anaerobic fungi (Dollhofer et al., 2015).

Lignocellulosic residue	Lignin content %	Organism
Wheat straw	16-21	*Neocallimastix frontalis*
Coastal Bermuda grass	6.4	*Piromyces* MC-1, *Orpinomyces* PC-1-3, *Neocallimastix* MC-2
Sugar cane bagasse	19-24	*Piromyces* strain E2
Hard wood	18-25	*Neocallimastix* sp.
Rice straw	18	*Piromyces* M014, *Orpinomyces* GSRI-001, *Neocallimastix* T010

2.5. Molecular Methods Used in Microbial Ecology of Anaerobic Digesters

Microbial ecology which is also defined as environmental microbiology is the ecology of microorganisms. Microbial ecology explores the microorganisms' relationship with one another and with their environment. Microbial activity and biodiversity are considerably significant parts in microbial ecology (Atlas and Bartha, 1986).

In the past, microbial analysis for identification of microorganisms was performed with culture dependent methods which are time consuming and lacked high sensitivity.

Culture-based methods are generally used to determine the microbial ecology of natural environments and those that have been anthropogenically changed by human activities. Because of the limitations of culture dependent methods, microbial communities cannot be totally cultured and so, a microbial community cannot be cultured as a whole and cultured microorganisms do not reflect microbial community can not reflect the all microbial community (Nagarajan and Loh, 2014). In addition, because some of the groups of microorganisms cannot be in a laboratory environment and microorganisms living in anaerobic environment cannot easily grow due to low growth rates and syntrophic interactions, these types of microorganisms can only be detected by using culture-independent methods (Abbasi-Guendouz et al. 2013). Thus, microecological studies gained importance after reorganization of DNA and RNA based culture independent tools (Muyzer et al., 1993). Moreover, the use of culture-independent molecular methods to analyses the microbial community in environmental research has become increasingly prevalent as a result of recent developments in genomics and sequencing technologies (Aydin, 2016b).

Molecular methods containing the isolation and assessment of DNA, RNA, proteins, metabolites and stable/radioactive isotopes from environmental samples have been successfully deployed and these can supply valuable insights into the structure and functional behavior of microbial communities as seen in Figure 2.11 (Vanwonterghem et al., 2014).

Not only whole genomes but also selected genes can be analyzed thanks to molecular phylogeny which is a culture-independent nucleic acid approach based on the comparative analyses of rRNA. New basis for the direct identification and quantification of microorganisms can be provided by nucleic acid sequences. Therefore, ribosomal RNA (rRNA) and ribosomal DNA (rDNA) are the most widespread used objective nucleic acids in microbial ecology. Because the rRNA is key elements of cells and it is homologous for all organisms, the rRNA is considerably important molecule for phylogenetic analyses (Hofman-Bang et al., 2003). Cellular life for three primary classifications: Eukarya, Bacteria and Archaea can be segmented according to analysis of these rRNA signatures (Nagarajan and Loh, 2014).

In recent times, molecular methods have been also used in order to analyze the microbial communities in anaerobic digesters. Thanks to molecular methods used in anaerobic digestion studies, it was investigated that microorganisms in anaerobic environments have highly diversity. It was also considered that microbial relationships and metabolic functions need to be clarified by the data obtained from molecular techniques (Talbot et al., 2000).

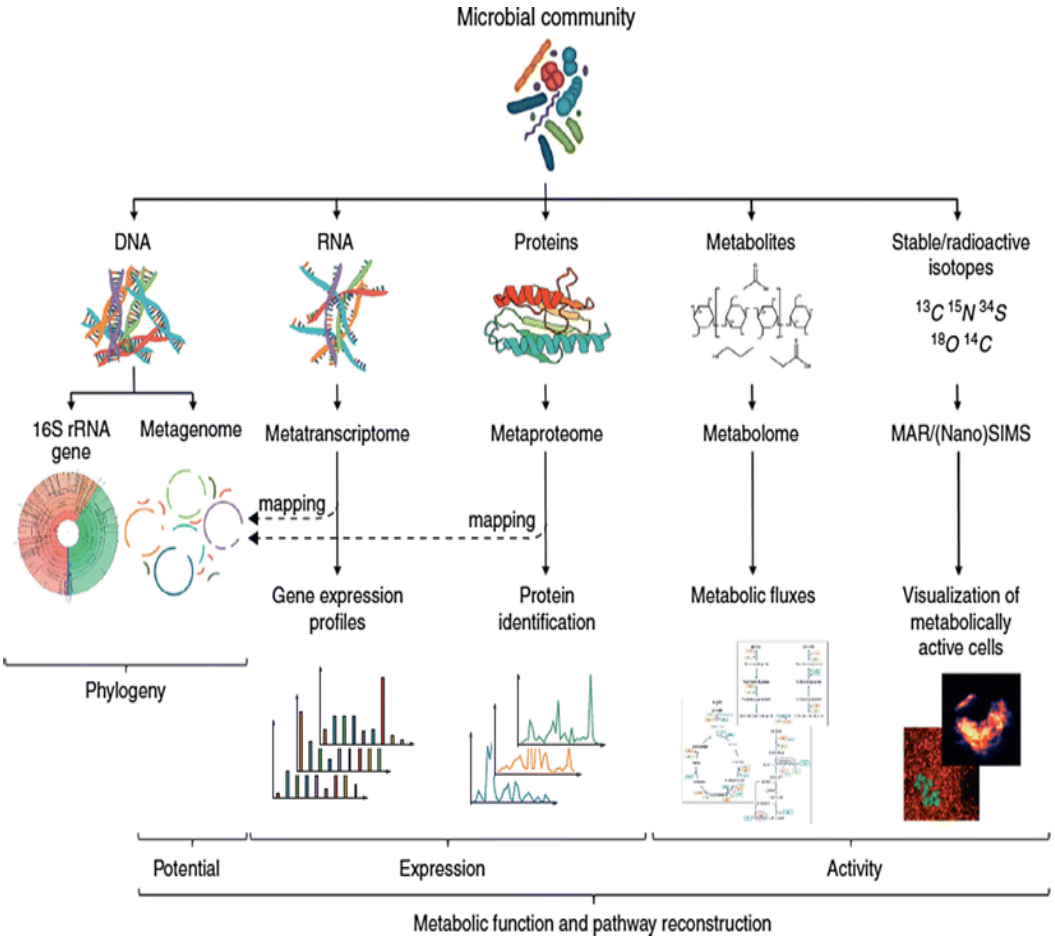

Figure 2.11. A combination of molecular methods from environmental samples (Vanwonterghem et al., 2014).

2.5.1. Polymerase Chain Reaction (PCR)

PCR is an amplification of DNA segments method developed by Kary Mullis in the 1980s. PCR is based on using the ability of DNA polymerase in order to synthesize new strand of DNA complementary to the offered template strand. It can be a single gene, just a

part of a gene or a non-coding sequence. PCR technology provided a wide range of alternatives of usage DNA in environmental microbiology (Saiki et al., 1992).

PCR process typically based on three steps: Denaturation, annealing, and extension. These steps have a serious of different number of cycles and different temperatures. In denaturation step which is the first regular cycling step, double stranded DNA templates are melted and separated the hydrogen bonds between complementary bases by high temperature like 94–98 °C. In annealing step, the reaction temperature is lowered so that the primers can attach to the single-stranded DNA template. In extension or elongation step, temperature is increased again to a high level (72 °C mostly) so that Taq polymerase enzyme can elongate the chain thanks to addition of nucleotides (dNTPs). Cycles of binding, elongation and then disassociation repeated 30-40 times in order to recover enough DNA segment of interest. The resulted product is run on an agarose gel so as to monitor efficiency of the PCR. Ethidium Bromide (EtBr) is usually used to stain DNA which renders DNA visible under UV light.

2.5.2. 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.

2.5.3. Fluorescence in situ Hybridization (FISH)

Fluorescence in situ hybridization (FISH) depend on the microscopic analysis of defined (at least its SSU rRNA gene sequence) groups of bacteria by a fluorogenic oligonucleotide (or probe) targeting SSU rRNA molecules inside cells (Amann et al., 2001). Firstly, microbial cells are fixed with appropriate chemical fixatives and they are hybridized on a glass slide or in a solution with oligonucleotide probes under optimal conditions. Fluorescent dye is used to label the oligonucleotide probes so that they can be observed under a fluorescence microscope (Giovannoni et al., 1988).

FISH is a relatively useful method that can be used to count and identify single cells. FISH-based approaches start by rapid fixation of samples after being extracted from the environment in order to preserve the cell morphology of the microbial communities involved (Poulsen et al., 1993). Once extracted, they are washed and hybridized using oligonucleotide probes that are specific to the gene sequences of the cells involved.

Since FISH is considerably easy and fast technique providing direct visualization of organisms without cultivation, it enables the possibility to detect active microorganisms in the sample. Because any DNA or RNA amplification is not required for FISH, it has an advantage over other molecular techniques (Sanz and Kohling, 2006).

FISH technique has also become a widespread tool for identification of microorganisms in environmental samples (Amann et al., 2001). Many rRNA-targeted oligonucleotide probes that are suitable for FISH have been described, together with an online database providing a compassing overview of over 700 published probes and their characteristics (Loy et al., 2003). Some probes can be readily developed and tested to detect lineages of uncultured microbes in environmental samples (Ravenschlag et al., 2001). The signal intensity of cells hybridized, which are with oligonucleotide probes, is related to the cellular rRNA content directly and this allows a quantification of rRNA concentrations both of the single cells and in the environment (Poulsen et al., 1993).

2.5.4. Real Time Quantitative PCR (Q-PCR)

qPCR is a highly reproducible and sensitive method to quantitatively track phylogenetic and functional gene changes under various environmental or experimental conditions across temporal and spatial scales. Variations in gene abundances and/or levels of gene expression in terms of transcript numbers can be compared with differences in abiotic or biotic features and/or biological activities and process rates through the use of any quantitative data that is produced through this process (Kim et al., 2003). The arrangement of qPCR data sets, which are described by the abundance of specific microorganisms or genes for completion of other quantitative environmental data sets, has significance in microbial ecology for understanding of the roles and contributions of particular microbial and functional groups within ecosystem.

Contrarily to the conventional PCR, quantification occurs during the exponential phase of amplification in qPCR. Therefore, the bias usually observed in the PCR template-to-product ratios can be largely avoided (Malinen et al., 2003).

It is commonly achieved through the use of fluorescence-based technologies containing SYBR green (a non-specific fluorogenic molecule which binds to double stranded DNA) and a dual-labeled TaqMan probe, which are two main methods for Q-PCR. In SYBR green based experiments, after each amplification cycle is quantified by the relative fluorescent intensity of each sample, the amount of DNA production is produced. In TaqMan, binding of target DNA and the dual labeled probe is expected along with the standard PCR amplification primers. For both of them, determination of cycle threshold (Ct) or a value called the critical as the relative cycle at which the fluorescence of a sample increased above background. By comparing the Ct values of samples with unknown amounts of initial target DNA to those of standards with known starting quantities of template DNA, it is possible to accurately quantify the abundance of a particular gene sequence in a mixed community DNA sample (Taniguchi et al., 2009).

2.5.5. Metagenomics

Metagenomics, which is also known as environmental genomics, community genomics or microbial ecogenomics, involves retrieving microbial genomes directly from environmental samples. It can be used to study genetic material without any existing understanding of the microbial communities or the requirement to cultivate samples. Metagenomics excludes any approaches that reply solely on the interrogation of PCR-amplified selected genes, as these methods are limited to developing an understanding of the genetic diversity of the genes under investigation (Schloss and Handelsman, 2013). The main fundamental principle of metagenomics is that it allows for the sequencing and analysis of the complete genetic composition of environmental microbial communities.

Until today, metagenomic approaches have been applied successfully to gain insights into the phylogenetic and functional diversity of uncultured microorganisms that are found in a range of different environments (including soil, the ocean and acid mine drainage) and it has become an extremely important means of developing an advanced understanding of the biochemical functions of uncultured microorganisms and their relationship with biotic and abiotic factors (Vanwonterghem et al., 2014).

In recent years, environmental metagenomic libraries have become increasingly popular as microbial enzymes and antibiotics resources and they have been used in a range of different industries, including biotechnology and medicine (Schloss and Handelsman, 2013). The construction of these libraries involves a number of steps. First, the DNA is isolated from the environmental sample. The random DNA fragments are cloned into an appropriate vector before the resulting clones are transformed into a host bacterium and screened for positive clones. As a direct result of the fact that metagenomic libraries contain small DNA fragments (2-3kb), they facilitate metagenome coverage of the environment that is superior to that on offer via larger fragments. According to some experts, at least 1011 genomic clones would be required to assess the genomes from rare members of microbial communities (Taupp et al., 2011). The phenotyoes encoded by single gene can be screened thanks to use of small-insert DNA libraries. Metagenoms can be also simply reconstructed for genotype analyses by small-insert DNA libraries. On the other hand, investigation of multigene biochemical pathways studies is generally analyzed by large-fragment metagenomic libraries (100–200 kb) (Aydın, 2016).

There are two methods of screening metagenomic libraries: sequence-driven metagenomic analysis involving massive high-throughput sequencing or functional screening of expressed phenotypes. There are several advantages associated with sequence-driven analysis. Massive whole-genome metagenomic sequencing provides detailed insights into a range of different genomic aspects, including the presence of redundant functions in a community, genomic organizations and the traits distinctly related to taxa acquired via horizontal gene transfers (Taupp et al., 2011).

2.5.6. Next Generation (Illumina Miseq)

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 genomic analyses can be performed (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 conducted 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).

2.6. Aim of the Study

Environmentally-friendly and low coast energy production has gained importance in our country and 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 emerging application around the world. While animal manure is most common renewable biogas source, algae are considered as a promising alternative for biogas production. Conspicuous benefit of anaerobic digestion is the production of methane rich biogas. However, major problem in energy production from animal manure and algae under anaerobic conditions is cellulosic compounds causing rate

limitation in hydrolysis, negative effect on the performance of anaerobic digesters and reduction in yield. Therefore, various pretreatment techniques have attracted the attention in order to improve the biogas potential of anaerobic digester systems. Although effects of ruminal fluid or rumen bacteria on biogas production have been studied, effects on anaerobic rumen fungi take place in the process is unknown. This study aims to investigate the effect of anaerobic rumen fungi on potential of biomethane production in anaerobic digesters fed with different substrates. Batch anaerobic reactor fed with cow manure, microalgae and macroalgae under optimum conditions are used in order to understand the effects of rumen fungi on biogas production. Thus, it is proposed to enhance the biogas potential of anaerobic digester thanks to rumen fungi, investigate the microbial community dynamics by Illumina Miseq and control the anaerobic rumen fungi changes by qPCR.

3. MATERIALS AND METHODS

3.1. Sampling and Characterization Studies

Table 3.1. Characteristics of manure, microalgae, macrolage and anaerobic seed sludge.

Samples	Total Solids (%)	Total Volatile Solids (%)	TVS/TS (%)
Manure	14,9	12,1	81
Microalgae	9,7	7,1	73
Macroalgae	9,1	6,8	75
Anaerobic Seed Sludge	19,1	15,6	82

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. Rumen samples which contain anaerobic rumen fungi were collected from Veterinary Faculty of Istanbul University. Samples of all rumen content consisting of fluid and solids were taken via rumen fistulae from a cow (live weight 400-450 kg) with using confidential techniques 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. All samples of ruminal fluid were flushed with nitrogen gas (N_2) in order to provide anaerobic conditions after loading and sealing. Some of the samples of rumen fluid were stored at -20 ˚C in order to extract DNA for investigation of metagenomic survey of rumen fluid. The animal manure which was used as a substrate to investigate the effect of rumen fungi on anaerobic digester was also provided from cows at Veterinary Faculty of İstanbul University. Microalgae *H. pluvialis* was commercially purchased from Scottish Marine institute, Scotland, United Kingdom. Macroalgae *Ulva lactuca* which is abounding in the aquatic ecosystems was obtained from The Sea of Marmara. Anaerobic seed sludge containing methane bacteria and archaea which are responsible for anaerobic digestion process, was obtained from Sütaş Company.

Characteristics of manure, microalgae, macrolage and anaerobic seed sludge are given Table 3.1.

3.2. Isolation and Cultivation of Anaerobic Rumen Fungi

Figure 3.1. Culture media for anaerobic rumen fungi growth.

Complex culture media was prepared by using previously described protocols in order to isolate the anaerobic rumen fungi from rumen fluid (Joblin, 1981). Two different salt solutions were prepared to use in media. While salt solution I involved (g/L) KH_2P0_4, 3.0; $(NH)_2S0$, 3.0; NaCl, 6.0; MgSO, 0.6; CaCl, 0.6, salt solution II involved K_2HP0_4, (3 g/L). Salt solution I 150 ml; salt solution II, 150 ml; centrifuged rumen fluid (Orpin, 1975), 200 ml; Bactocasitone (Difco), 10 g; yeast extract (Oxoid), 2.5 g; NaHCO, 6 g; L-cysteine. HC1, 1 g; fructose, 2 g; xylose, 2 g; cellobiose, 2 g; resazurin solution (0-1 %, w/v),8 g; trace elements solution, 10 ml; haemin solution, 10 ml; resazurin solution (0.1 %, w/v), 1 ml; deionized water to 900 ml were added. The media was then autoclaved for 20 min at 115 ˚C. After autoclaving the media, 0.1 % (v/v) two different vitamin solutions were added. Vitamin solution I contained (g/L): thiamin. HCl, 0.10; riboflavin, 0.20; calcium Dpantothenate, 0-60; nicotinic acid, 1.00; nicotinamide, 1.00; folic acid, 0.05; cyanocobalamin, 0.20; biotin, 0.20; pyridoxine. HCl, 0.10; paminobenzoic acid, 0.01. Vitamin solution II contained (mg/L): thiamin, HC1, 5; riboflavin, 5; calcium Dpantothenate, 5; nicotinic acid, 5; folic acid, 2; cyanocobalamin, 1; biotin, 1; pyridoxin.HC1, 10; paminobenzoic acid, 5. Antibiotics solution 0.1 % (v/v) containing penicillin (5 g/L), streptomycin (5 g/L), neomycin (5 g/L), and chloramphenicol (5 g/L),

was added to the isolation media to suppress bacterial growth. After preparing the media, all cultures were incubated under CO_2 at 39°C during a week in order to reproduce rumen fungi.

Figure 3.2. Culture conditions for microalgae.

2% of CO_2-enriched air was photoautotrophically used to cultivate microalgae *H. pluvialis* strain SCCAP 34/7 (Scottish Marine institute, Scotland, United Kingdom). Bold Basal Medium with 3-fold Nitrogen and Vitamins (3N-BBM+V; CCAP 2015) at 25 ^{o}C was utilized to growth microalgae cells in L photobioreactor system (Grofizz LLC, Austin, TX). 9” x 9” x 9” chamber at 8000-10000 lux LED lights was performed as a source of light. After incubation, the microalgal biomass was obtained. It was concentrated by centrifugation at 3600 × g for 15 min.

Figure 3.3. Culture conditions for macroalgae *Ulva lactuca*.

It was prepared a culture medium containing effluent water and salty water in order to growth the macroalgae *Ulva lactuca* in natural conditions. Culture media were renewed during several weeks so as to provide maximum growth.

3.3. Lab – Scale Anaerobic Digesters Set-up

Three different anaerobic sequencing batch reactor (ASBR) experiment sets containing different substrates (manure, microalgae and macrolagae) were operated in order to determine the effect of rumen fungi on biomethane production.

In the first set, different initial concentration of anaerobic rumen fungi and methanogenic sludge were used in the batch digesters fed with animal manure. The digesters containing animal manure were bioaugmented with isolated rumen fungi (Orpinomyces sp., Piromyces sp. Anaeromyces sp. and Anaeromyces sp.) at different inoculum ratios: 0% (R_0-control), R_1 (5%), R_2 (15%), R_3 (20%) (v/v). In addition, anaerobic granular seed sludge and water were added to provide optimal conditions. The buffer contained (per L): 1.0 g of NH_4Cl, 0.4 g of $K_2HPO_4.3H_2O$, 0.2 g of $MgCl_2.6H_2O$, 0.08 g of $CaCl_2.2H_2O$, 10 ml of trace element solution, and 10 ml of stock vitamin solution. A stock trace element and vitamin solution were prepared. All reactors were flushed with nitrogen gas (N_2) in order to provide anaerobic conditions after loading and sealing. The gas outputs were measured with milligas counters. pH was adjusted to 7-7.4 and alkalinity was added to maintain ph. All reactors with 900 ml volumes were carried out during 40 days at 40˚C.

Table 3.2. Operational conditions in anaerobic digesters fed with animal manure.

Reactors fed with animal manure	Inoculum Ratios of Rumen Fungi	Working Volume (ml)	T (˚C)	HRT (days)
R_0	0	900	40	40
R_1	5%	900	40	40
R_2	15%	900	40	40
R_3	20%	900	40	40

In the second set, *H. pluvialis* (2 g VS/L of the algal biomass) and 3 g VS/L of methanogenic sludge were used in batch experiments performed. The digesters containing microalgae were bioaugmented with isolated rumen fungi *Orpinomyces sp.*, *Piromyces sp.* and *Anaeromyces sp.*, *Neocallimastix frontalis* at different inoculum ratios: 0% (F_0), %1 (F_1), 5% (F_2), 10% (F_3), 15% (F_4) and 20% (F_5) (v/v). In addition, anaerobic granular seed sludge and water were added to provide optimal conditions. The buffer contained (per L): 1.0 g of NH_4Cl, 0.4 g of $K_2HPO_4.3H_2O$, 0.2 g of $MgCl_2.6H_2O$, 0.08 g of $CaCl_2.2H_2O$, 10 ml of trace element solution, and 10 ml of stock vitamin solution. A stock trace element and vitamin solution were prepared. The gas outputs were measured with milligas counters. pH was adjusted to 7-7.4 and alkalinity was added to maintain pH. All reactors with 2000 ml volumes were carried out during 40 days at 41˚C.

Table 3.3. Operational conditions in anaerobic digesters fed with microalgae.

Reactors fed with microalgae	Inoculum Ratios of Rumen Fungi	Working Volume (ml)	T (˚C)	HRT (days)
F_0	0%	2000	41	40
F_1	1%	2000	41	40
F_2	5%	2000	41	40
F_3	10%	2000	41	40
F_4	15%	2000	41	40
F_5	20%	2000	41	40

In the third set, different initial concentration of anaerobic rumen fungi and methanogenic sludge were used in the batch digesters fed with macroalgae *Ulva lactuca*. The digesters containing *Ulva lactuca* were bioaugmented with isolated rumen fungi (*Orpinomyces sp.*, *Piromyces sp. Anaeromyces sp. and Anaeromyces sp.*) at different inoculum ratios: 0% (A_0-control), A_1 (15%), A_2 (20%). In addition, anaerobic granular seed sludge and water were added to provide optimal conditions. The buffer contained (per L): 1.0 g of NH_4Cl, 0.4 g of $K_2HPO_4.3H_2O$, 0.2 g of $MgCl_2.6H_2O$, 0.08 g of $CaCl_2.2H_2O$, 10 ml of trace element solution, and 10 ml of stock vitamin solution. A stock trace element and vitamin solution were prepared. All reactors were flushed with nitrogen gas (N_2) in order to provide anaerobic conditions after loading and sealing. The gas outputs were

measured with milligas counters. pH was adjusted to 7-7.4 and alkalinity was added to maintain ph. All reactors with 900 ml volumes were carried out during 40 days at 37˚ C.

Table 3.4. Operational conditions in anaerobic digesters fed with macroalgae.

Reactors fed with macroalgae	Inoculum Ratios of Rumen Fungi	Working Volume (ml)	Temperature (˚C)	HRT (days)
A_0	0	900	37	40
A_1	15%	900	37	40
A_2	20 %	900	37	40

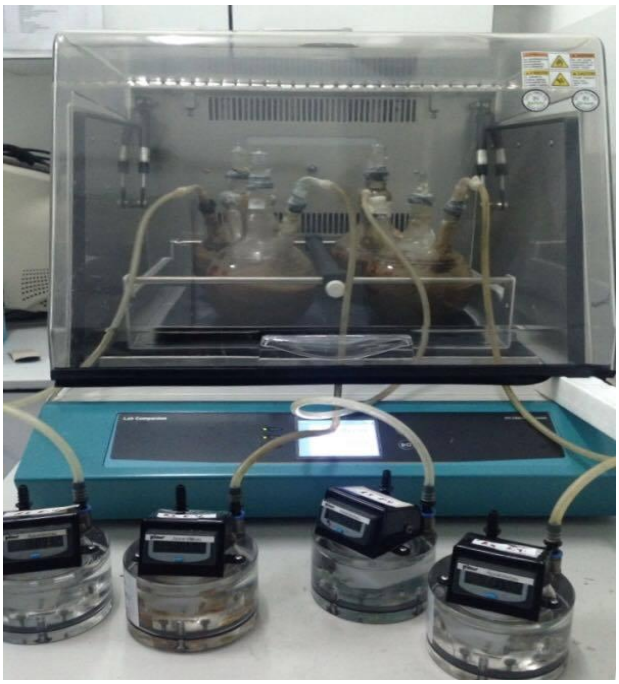

Figure 3.4. The digesters and milligas counters used in the study.

3.4. Analytical Measurements

Every 10 days, samples were taken from the digesters for analytical and molecular analyses of all three sets containing different kind of substrates (manure, microalgae and macroalgae). While analytical DNA samples were stored at +4 ˚C, DNA samples were stored at -20 ˚C. The analysis for alkalinity, total solids (TS), and volatile solids (VS) were

carried out appropriately with standard methods (APHA, 2005). The biogas production was measured by using Milligas counters (Ritter Digital Counter, U.S.A.) in all SBRs and noted every day cumulatively during 40 days operational period. 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 ˚C and the maximum temperature of the inlet was 240 ˚C. In addition, helium gas was utilized as a carrier gas at a rate of 0.8 mL/min. Concentration of VFA which is one of the most significant parameters for the accurate control of anaerobic digestion was used to evaluate and control of anaerobic digestion process.

3.5. Molecular Techniques

3.5.1. DNA Extraction

The total DNAs were isolated from isolated rumen fungi samples which were stored at -20 ˚C by using PureLink Genomic DNA extraction kits (Invitrogen, U.K.). In addition, the DNAs of the digesters were also isolated from the digester's samples taken regularly in order to investigate the change of microbial community dynamics. Concentration of the isolated DNAs was measured with NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The DNAs samples were stored at -20 °C until required for later investigation.

3.5.2. Next Generation Sequencing - Metagenomics Analysis

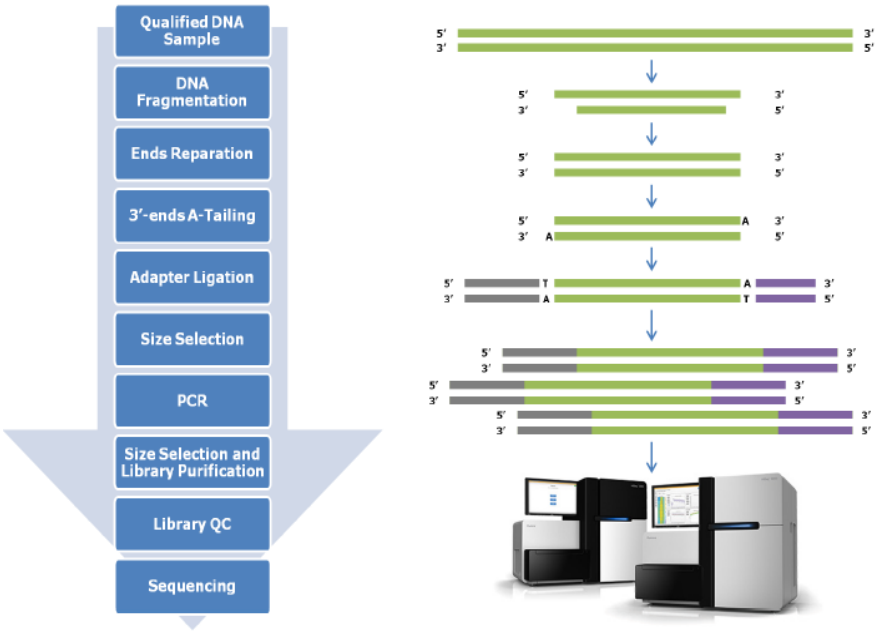

Figure 3.5. Experimental Workflow of Metagenomic survey.

Metagenomics which is also called as environmental genomics was used for determination of the abundance and identity of rumen fungi in a sample as a next generation sequencing technology. Thus, metagenomic survey originated from total purified DNA was comprehensively investigated in species of rumen fungi classification, gene function analysis and pathway analysis at DNA level (Eisen, 2007). Experimental workflow for metagenomic survey was conducted as given in Figure 3.4. Qulified DNA samples were sheared into smaller fragments by nebulization firstly. Then the overhangs resulting from fragmentation were converted into blunt ends by using T4 DNA polymerase, Klenow Fragment and T4 Polynucleotide Kinase. After adding an A (adenine) base to the 3' end of the blunt phosphorylated DNA fragments, adapters were ligated to the ends of the DNA fragments. Then short fragments were removed with Ampure beads. Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System were used to qualify and quantify the sample libraries. The qualified libraries were then sequenced via Illumina HiSeqTM platform.

Bioinformitics analysis pipeline for metagenomic survey was demonstrated in Figure 3.5. ABI StepOnePlus Real-Time PCR System was used to qualify and quantify the sample

libraries. The qualified libraries were then sequenced via Illumina HiSeqTM platform. Qualified sequencing reads that produced by Illumina platform were preprocessed and then assembled de novo with SOAPdenovo2 (Luo et al., 2012) and Rabbit (You et al., 2013) MetaGeneMark (Zhu et al., 2010) was then used to predict genes from assembled contigs, building a project specific gene catalog. Preprocessed reads were also mapped to IGC database and mapped genes were retieved and integrated into the gene catalog. CD-Hit (Li and Godzik, 2006) was used to remove redundancy. The gene catalog was blasted against public databases including nr, Swiss-Prot, COG, KEGG, GO, CAZy, eggNOG and ARDB to obtain functional and taxonomic annotation.

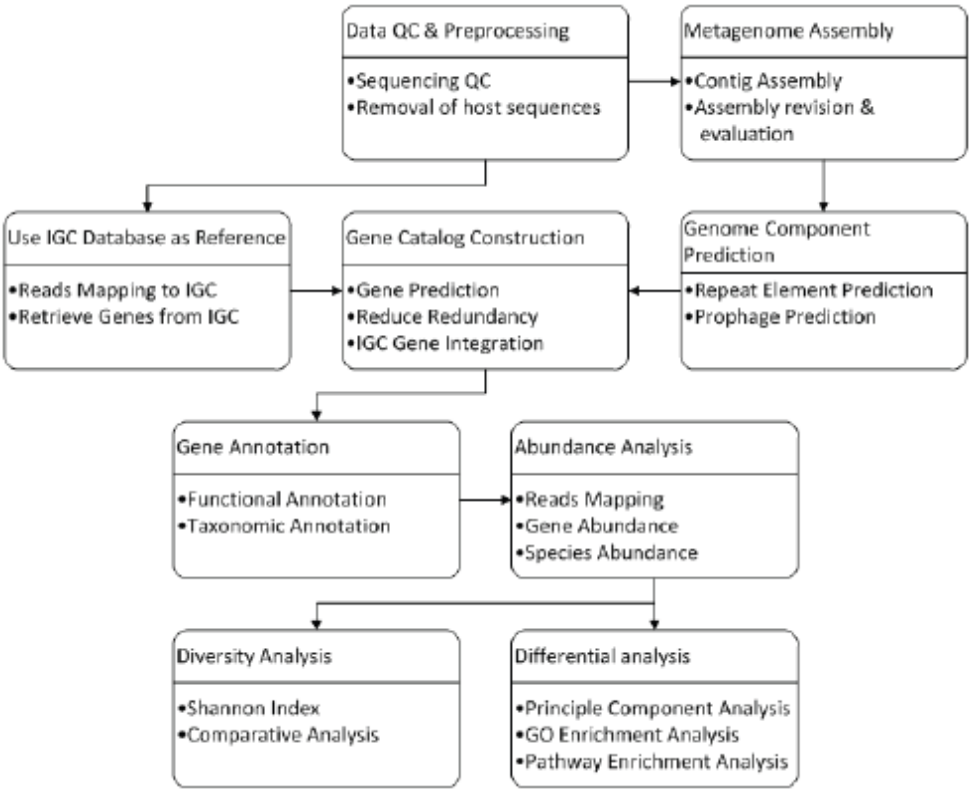

Figure 3.6. Bioinformatics analysis pipeline for metagenomic survey.

3.5.3. Strain Identification and Phylogenetic Analysis

Fungal DNA was sequenced with Strain Identification method in order to identify the species which have been isolated from ruminal fluid and cow manure. Strain identification and phylogenetic analysis of the isolated anaerobic fungi were carried out by the complete internal transcribed spacer (ITS; partial 18S, complete ITS 1, 5.8S, ITS 2, and partial 28S)

and D1/D2 domain at the 5’ end of the large-subunit (LSU) ribosomal DNA were amplified, using the primer pairs ITS1 (5’- TCC GTA GGT GAA CCT GCG G-3’)/ITS4 (5’- TCC TCC GCT TAT TGA TAT GC-3’) and NL1 5’-GCA TAT CAA TAA GCG GAG GAA AAG-3’)/NL4 (5’-GGT CCG TGT TTC AAG ACG G-3’), respectively (Fliegerova et al. 2006; Dagar et al. 2011). Care was taken to delimit the different regions of the rRNA locus in a consistent manner, as suggested by Hibbett et al. (1995), using the consensus sequences CATTA/CAACTTCAG (end of 18S/start of 5.8S) and GAGTGTCATTA/ TTGACCTCAAT (end of 5.8S/start of 28S). Phylogenetic reconstruction was conducted within the Geneious v6 bioinformatics package (Drummond et al. 2011), using MAFFT (v7.017 (Katoh et al. 2002)) for sequence alignment and Mr Bayes for phylogenetic analysis (Huelsenbeck & Ronquist 2001).

3.5.4. Illumina Miseq

In order to show molecular dynamics in all anaerobic digesters fed with manure, microalgae and macrolagae, Illumina NGS technology was used. Changes in microbial community structure were detected by lllumina MiSeq sequencing technology. Quantity and diversity of bacterial and archaeal communities which are responsible for biogas production during anaerobic digestion processes were analyzed by lllumina MiSeq. In order to find out the effect of bioaugmentation of anaerobic rumen fungi on microbial communities, DNA samples at steady state conditions for biogas production were selected and analyzed for all anaerobic digesters fed with different substrates; animal manure, microalgae and macroalgae. In addition, anaerobic seed sludge providing anaerobic bacteria and archaea for biogas production processes in anaerobic digesters was individually analyzed to show microbial community composition in the anaerobic sludge. Variations in the phylum distribution of the bacterial and archaeal communities in the anaerobic sludge were investigated by high-throughput Illumina Miseq.

The V4-V5 hypervariable region of the 16S rRNA gene was reproduced with region-specific primers which were designed to contain Illumina adaptor and barcode sequences 518F-926R for bacteria, 518F-958R for archaea) (Ma et al., 2015). A double round of PCR and dual indexing on PTC-200 DNA Engine Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., CA, USA) was used to carry out generation of sample amplicons.

Target regions were extracted with PCR rounds which were described according to Aydın (2016a). A picogreen assay and a Fluorometer (SpectraMax GeminiXPS 96-well plate reader) were utilized in order to adjust the concentrations of amplicons. After determination of concentration, they were put in the same amounts (~100 ng) to a single tube. The following procedure was used to move away short undesirable fragments and clean the amplicon pool. Firstly, the size of pool was determined using AMPure beads (Beckman Coulter), the product was conducted on a 1% gel, gel was cut and column was purified with Qiagen MinElute PCR purification kit and pool size was determined again with AMPure beads. PCR containing Illumina adaptor-specific primers was used to adjust the last quality of amplicon pool. After that, products of PCR were conducted on a DNA 1000 chip for the Agilent 2100 Bioanalyzer. The last amplicon pool was accepted on the condition that if long fragments were not identified after PCR. If there were short fragments, the procedure was repeated again. The KAPA 454 library quantification kit (KAPA Biosciences) and the Applied Biosystems Step One plus realtime PCR system were used to quantify the amplicon pool which is clean. Finally, Illumina MiSeq paired-end 300 bp protocols (Illumina, Inc., San Diego, CA, USA) was performed to procure sequences.

3.5.5. Real time quantitative PCR (qPCR)

qPCR was used in all of the anaerobic digesters to determine optimum fungi concentrations. qPCR assay was accomplished in triplicate using an ABI 7500 SDS system (Applied Biosystems, Foster City, California) during all digesters by qPCR protocols, which were amplified using previously specific primer sets for anaerobic fungi (Forward primer: 5'-GAGGAAGTAAAAGTCGTAACAAGGTTTC-3', Reverse primer: 5'-CAAATTCACAAAGGGTAGGATGATTT-3'). An optimum primer concentration of 350 nm and a final $MgCl_2$ concentration of 4mM were used for the qPCR under the following cycle conditions: denaturation at 94 ^{o}C for 4 min followed by 35 cycles of 96 ^{o}C for 45 s, 56 ^{o}C for 45 s, and 72 ^{o}C for 1 min followed by a final extension at 72 ^{o}C for 5 min. Detailed information on the qPCR analysis has been reported in the previous study (Denman and Mcsweeney, 2006).

18S rRNA gene and the internal spacer (ITS) region are generally used objective genes and they provide to quantify anaerobic rumen fungi by means of qPCR in addition to identification and community analyses of anaerobic fungi (Lockhart et al., 2006). While sequencing of the 18S rRNA gene is highly preserved in anaerobic rumen fungi species, the products of PCR cannot be directly sequence by ITS region and ITS region shows high diversity for anaerobic rumen fungi groups (Schoch et al., 2012). On the other hand, the sequence of 28S rRNA gene can be considered as the best favorable gene for phylogenetic identification of anaerobic rumen fungi. In addition, Tan and Cao (2014) demonstrated that the combination of DNA regions of 18S, 28S and ITS provides more certain information about fungal diversity and each DNA region causes a different consequence. Although fungal abundance can be comprehensively provided by quantification of anaerobic rumen fungi with qPCR, it is hard to associate with results of culture dependent enumeration (TFU) or the real biomass owing to various ratios of DNA/biomass content in species of anaerobic rumen fungi and based on specific growth stages of each culture (Dollhofer et al., 2015). The principal findings of this thesis are that qPCR method can be used to control the effect of bioaugmentation and it can be sufficient for determination of anaerobic rumen fungi.

3.6. Statistical analysis

R 3.1.1 analysis was used to conduct Statistical analyses (www.r-project.org). Histogram, q-q plots and the Shapiro-Wilk's test were performed to examine data normality. Variance homogeneity was also investigated by using The Levene's test. One-way analysis of variance (ANOVA) or independent-samples t-test was used to check against the variations in biogas production and microbial community dynamics between different inoculums ratios of anaerobic fungi and R0. In order to provide multiple comparing, The Tukey's test was used. Values of tests were pointed out as mean and standard deviation. The applicability of microbial community and inoculum ratios was determined by Pearson's test as a correlation test. Important difference were detected at the $p< 0.05$ level of importance.

4. RESULTS AND DISCUSSION

In the context of this thesis, serious digestion studies were conducted in order to comprehend the effect of rumen fungi for improvement of biogas production on anaerobic digesters. Initially, rumen fluid was analyzed with Metagenomic Analysis in order to identify anaerobic rumen fungi in ruminal fluid. After that, isolated and cultivated rumen fungi from rumen fluid and manure were analyzed by using Strain Identification and Phylogenetic Analysis techniques so as to identify species of anaerobic rumen fungi. Finally, anaerobic digesters fed with animal manure, microalgae and macroalgae were set up and performed semi-continuously in order to understand the effect of anaerobic rumen fungi on biogas production. Performance of anaerobic digesters was evaluated with biogas and biomethane production. Inhibition effect of the digesters was controlled with measurement of VFAs. Finally, microbial community dynamics during anaerobic digestion process were identified according to Illumina Miseq and qPCR analyses.

4.1. Results of Metagenomics Analaysis of Anaerobic Rumen Fungi

Anaerobic fungi are new group of organisms which inhabit in rumen ecosystem. These fungi possess a lifecycle varying between zoospore (a motile flagellated form) and thallus (a non-motile vegetative reproductive form). Anaerobic rumen fungi play important role in the physicochemical degradation of plant cell walls (Dollhofer et al., 2015). Metagenomic survey of rumen fluids was revealed that bacteria (50%), protozoa (30%), fungi (10%) and bacteriophages (10%) exist in the rumen. Diversity of anaerobic fungi in the rumen fluid was also investigated by metegenomic survey as shown in Fig.4.1. These organisms are classified in phylum *Neocallimastigomycota*. The results of metagenomic survey showed that six different genera of anaerobic fungi are found in the samples of rumen fluid. These genera were Cyllamyces (19%), Caecomyces (15%), Anaeromyces (10%), Neocallimastix (10%), Piromyces (9%) and Orpinomyces (7%) respectively.

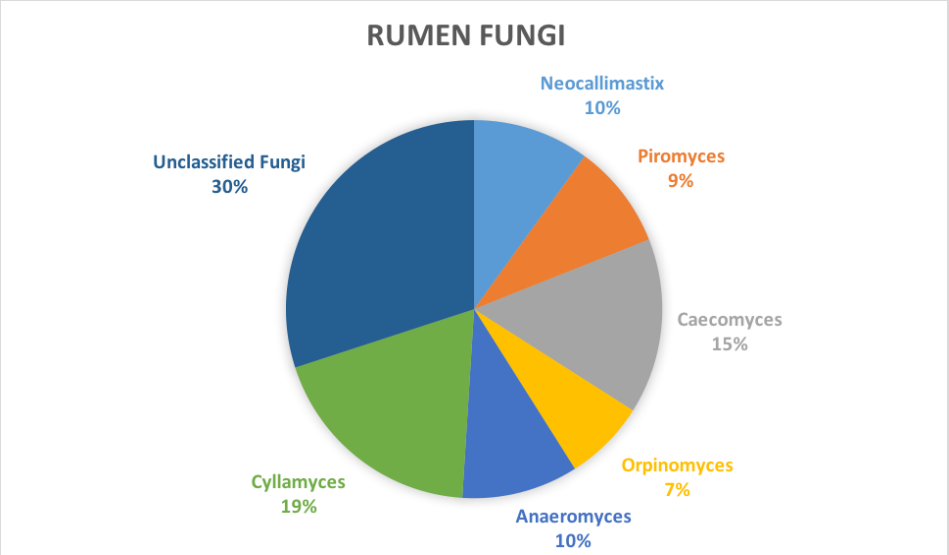

Figure 4.1. Diversity of anaerobic fungi in the rumen fluid.

Anaeromyces are principal commercial source of xylanases which degrade the lignocellulosic compounds. Xylanase is produced by *Anaeromyces* and it can be used as a key enzyme in biotechnology studies. *Piromyces* have an important role for the digestion of rigid plant cell wall. Neocallimastix play a pivotal role in the rumen because they can physically and enzymatically attack the lignocellulosic materials. *Orpinomyces* are also another conspicuous species because they can easily solubilize lignocellulose and they can produce all enzymes which is required to effectively hydrolyze cellulose and hemicelluloses compounds. It was assumed that one of the primary reasons why anaerobic rumen fungi evolved the resistant and influential activity of cellulolytic hemicellulolytic and lignocellulosic capability is horizontal gene transfer from bacteria. Horizontal gene transfer (HGT) has an important effect on specification and enzyme activities of anaerobic rumen fungi. (Fitzpatrick, 2012).

4.2. Results of Strain Identification and Phylogenetic Analysis of Isolated Rumen Fungi

According to results of Strain Identification and Phylogenetic Analysis, species were identified with based on the results of Next Generation analyzing. Four strains of anaerobic rumen fungi which were isolated from rumen fluid were shown in Table 4.1. *Piromyces sp.* (strain KSX1), *Neocallimastix frontalis* EB 188 (ATCC 76100), *Anaeromyces sp.* (AF-CTS-BTrA1) and *Orpinomyces sp.* (strain C1A) were isolated from cow rumen fluid.

Table 4.1. Isolated strains of anaerobic rumen fungi.

From cow rumen fluid	
Piromyces sp.	**strain KSX1**
Neocallimastix frontalis	**EB 188 (ATCC 76100)**
Anaeromyces sp.	**AF-CTS-BTrA1**
Orpinomyces sp.	**strain C1A**

4.3. Result of Microbial Community Composition in the Anaerobic Seed Sludge

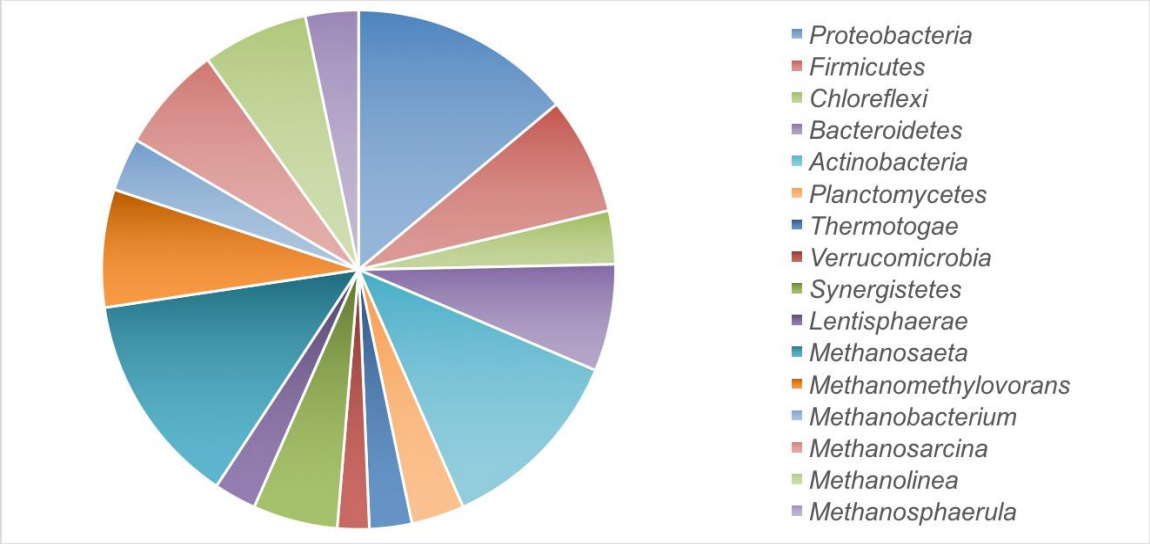

Figure 4.2. Community composition in the anaerobic seed sludge.

Variations in the phylum distribution of the bacterial and archaeal communities in the anaerobic sludge were investigated by high-throughput Illumina Miseq as seen in Fig.4.2. 10 communities of bacteria and 6 communities of archaea were found in the anaerobic sludge .It was found that *Preteobacteria* are the most dominant bacterial community and *Methanoseata* are the most dominant archaeal communities. *Actinobacteria, Firmicutes, Bacteroidetes* and *Synergistetes* were the other dominant bacterial communities respectively. Although *Chloroflexi, Planctomycetes, Thermotogoe, Verrucumicrobia* and *Lentisphaerare* were identified in the sludge, they were not dominant communities and they are found in small quantities. After *Methanoseata, Methanomethylovorans,*

Methanolinea and *Methanosarcina* were the other dominant archaeal communities respectively. *Methanobacterium* and *Methanosphaerula* also found in the sludge, but they were at the low percentage. Although bacterial communities were found high in number, percentage of bacterial communities and archaeal communities were almost equal to each other.

4.4. Results of Animal Manure Bioaugmented with Anaerobic Rumen Fungi

4.4.1. Biogas and Methane Production

Biogas production which is an important parameter to understand the performance of anaerobic digesters bioaugmented with anaerobic rumen fungi was recorded with milligas counters and it was shown in Figure 4.3.

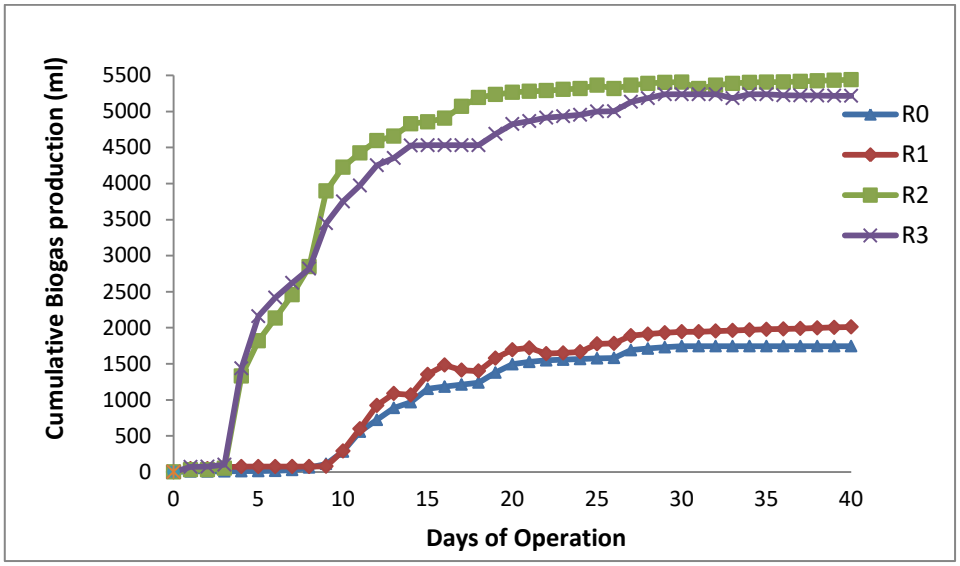

Figure 4.3. Biogas production in anaerobic digesters fed with animal manure.

The results of biogas production showed that there is an increase in the biogas production during 40 days. Increasing in biogas production continued between the start-up stage and the steady stage. End of a 40 days' period, it reached the steady stage in which biogas production is stable in anaerobic digesters. R_2 (15% of fungi) and R_3 (20% of fungi) digesters previously achieved the steady stage in comparison with the other digesters. Thus, it can be said that anaerobic rumen fungi in high concentration provided to achieve

steady stage easily. The highest biogas production was also observed in R_2 (15%) digester about 5500 ml. After R_2 digester, the maximum biogas production R_3 (20%), R_1 (5%), R_0 (control) digesters were 5000, 2000, 1500 ml, respectively. The lowest biogas production was observed in R_0 digester, because probably it wasn't bioaugmented with anaerobic rumen fungi. Although there was an important difference in biogas production among all digesters, there wasn't a conspicuous different between R_1 digester and control digester.

Because methane from biogas is an excellent alternative energy source in order to replace the use of non-renewable fossil fuels with renewable energy, methane production is considerably important parameter. In addition, it is also important for evaluation of the performance of anaerobic digester bioaugmented with anaerobic rumen fungi. Methane production was calculated according to the methane content of the biogas produced. Methane productions in digesters are given in Figure 4.4.

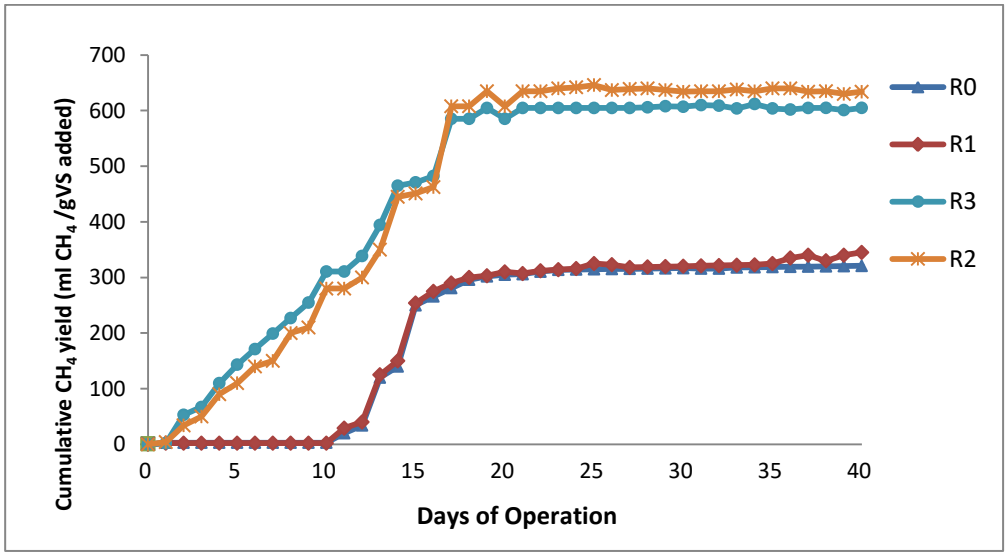

Figure 4.4. Methane production in anaerobic digesters fed with

As shown in Figure 4.4, in this study it was demonstrated that there is an increase, which continued between the start-up stage and the steady stage, in the methane production. End of a 40 days' period, it reached the steady stage in which methane production is stabile in all anaerobic digesters. Digesters of R_2 and R_3 previously achieved the steady stage in comparison with the digesters of R_0 and R_1. Thus, it can be said that anaerobic rumen fungi in high concentration provided to achieve steady stage easily. However, it was also showed that high concentration of anaerobic rumen fungi provided

high methane yield considering methane production. Methane yield in R_0, R_1, R_2 and R_3 was different from each other. The highest methane production was observed in R_2 digester as almost 60% of total biogas. After R_2 digester, the maximum methane production was observed in R_3, R_1 and R_0 digesters respectively. The lowest methane production was observed in R_0 digester because probably it wasn't bioaugmented with anaerobic rumen fungi. According to all methane production results, it was showed that the concentration of 15% anaerobic rumen fungi is the most efficient amount for improvement of biomethane production in anaerobic digesters fed with animal manure.

4.4.2. Volatile Fatty Acids Production

The major VFAs; acetic acid and propionic acid and the others; isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocopric acid, caproic acid and heptonic acid were measured according to results of GC. Temporal variations in VFA concentration are depicted in Fig.4.5 a, b, c, d for all digesters fed with animal manure. Except for acetic acid and propionic acid, the other concentrations of VFAs weren't detected. For all digesters, VFAs were consumed at the end of the day 40. The results showed that acetic acids and propionic acids reached the maximum concentration between day 5 and day 15 in digester R_0 and R_3, respectively. However, both acetic acids and propionic acids reached the maximum concentration between day 1 and day 10 in digester R_2. Although it was observed an increase the concentration of acetic acid in all digesters between day 1 and day 10, the highest concentration of acetic acid was observed in digester R_0 (653 mg/L). The highest accumulation of propionic acid was detected in digester R3 (741 mg/L) and R_1 (599 mg/L). Because these concentrations are not over 1,500 – 2,000 mg/L, it cannot be said that there is an inhibition for all digesters (Labatut and Gooch, 2012). In addition, because it has been suggested that propionic acid is the best indicator of process instability by Ahring et al. (1995), a decrease in accumulation of propionic acids after day 10 can show a stabile process for all digesters. Moreover, the lowest concentration of propionic acid was detected in digester R_2 where the highest biogas is and methane production. This means that digester R_2 has the most stable process and so the highest biogas and biomethane production were observed. Decrease in all VFAs accumulation after day 1 in digester the R_2 can be also evidence that the accumulation of VFAs may contribute to a reduced rate of biogas production.

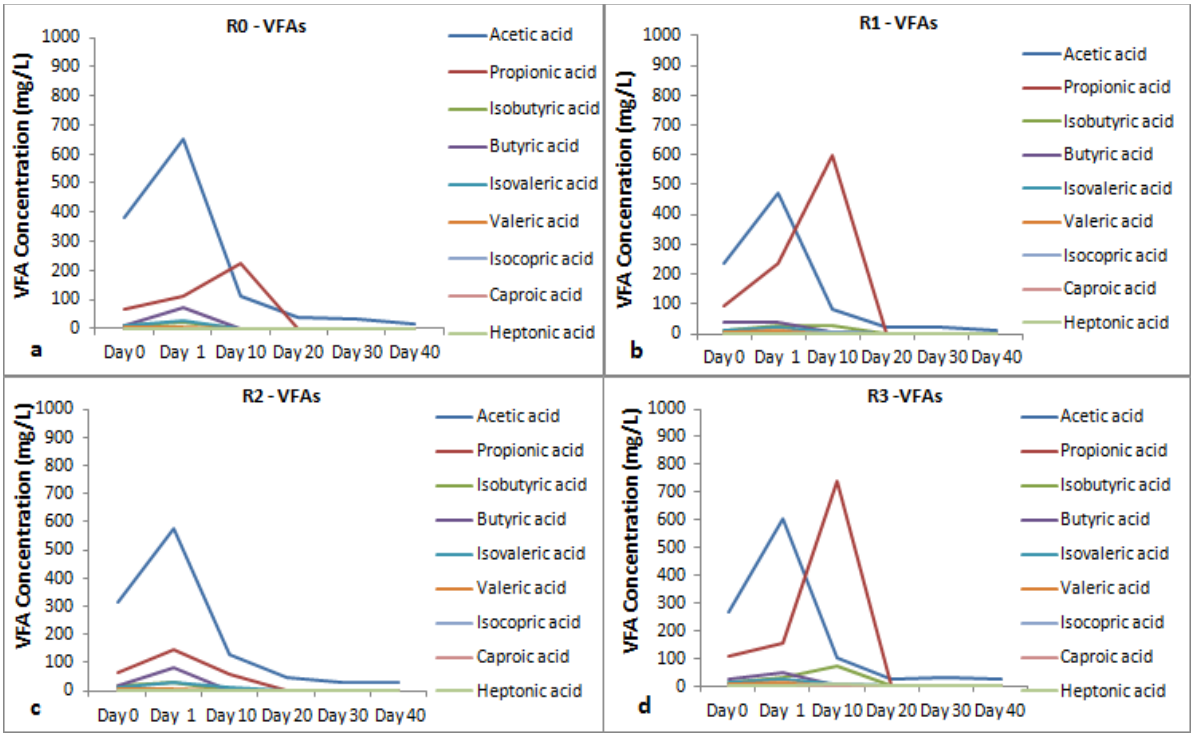

Figure 4.5. VFA profiles in digesters (a) R_0, (b) R_1, (c) R_2 and (d) R_3.

4.4.3. Microbial Community Dynamics in Anaerobic Digesters

Variations in the phylum distribution of the bacteria and archaeal communities in the samples at steady state conditions for biogas production of anaerobic digesters fed with animal manure and bioaugmented with rumen fungi were investigated by high-throughput Illumina Miseq as seen in Fig. 4.6., 4.7., and 4.8.

10 phyla and 15 genera of bacteria were found in the samples of anaerobic digesters as seen in Fig. 4 and 5. It was found that *Synergistetes* are the most dominant bacterial community in digester R_0, *Actinobacteria* are the most dominant bacterial community in digester R_1, *Lentisphaerae and Verrucomicrobia* are the most dominant bacterial community in digester R_2 and *Firmicutes* are the most dominant bacterial community in digester R_3.

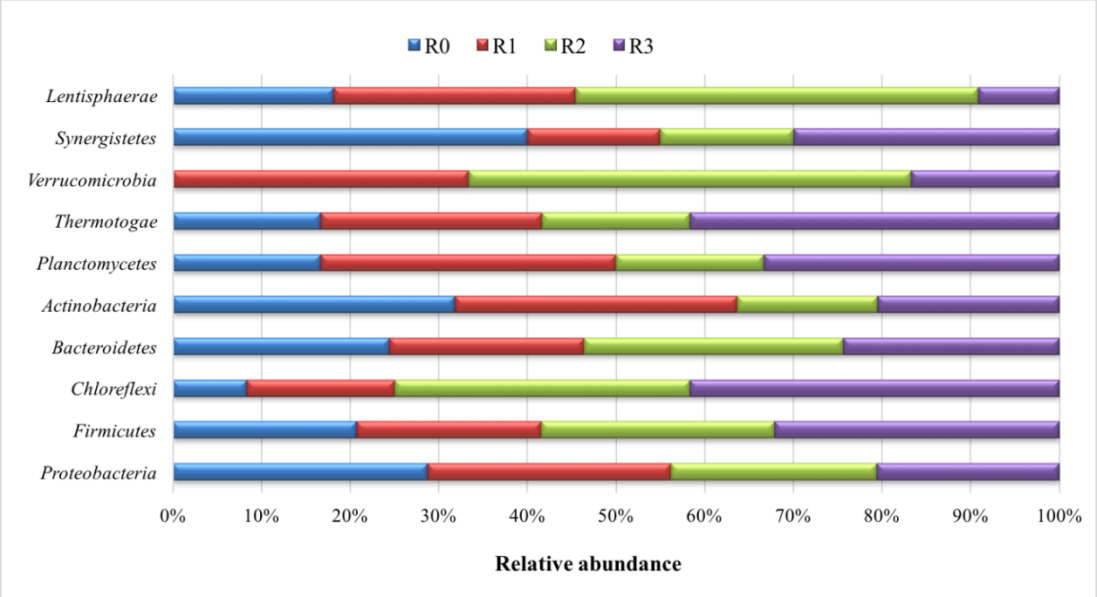

Figure 4.6. Bacterial community dynamics in digesters R_0, R_1, R_2 and R_3.

When investigating the bacterial genera, it was revealed that *Mycobacterium*, *Kosmotoga* and *Rhodopirellula*, *Clostridium* and *Longilinea* and *Clostridium* are the most dominant genera in digesters R_0, R_1, R_2 and R_3 respectively. *Clostridium* is quite important genus for production of butanol, butyric acid, acetone and iso-propanol. While *Chloroflexi* were found in small quantities in digester R_0, it was showed that digester R_3, which has the highest concentration of rumen fungi, contains the highest phylum of *Chloroflexi.* Thus, it was showed that *Chloreflexi* is positively affected by the addition of anaerobic rumen fungi. In addition, it was observed that higher concentration of rumen fungi negatively affects the phyla of *Lentisphaerae* and *Actinobacteria*. However, it was showed that *Lentisphaerae* and *Verrucomicrobia* are the most dominant phyla for biogas production according to results of digester R_2 which has the highest biogas production among anaerobic digesters fed with animal manure. It can be said that *Lentisphaerae* and *Verrucomicrobia* became dominant because anaerobic rumen fungi provide the increase of hydrolysis in anaerobic digestion. Although *Mycobacterium* is the least dominant genus of bacteria in digester R_2, the results implied that *Clostridium* and *Longilinea* acted as the main genera with efficient animal manure degradation capability in the R_2 digester, where the highest methane yields are.

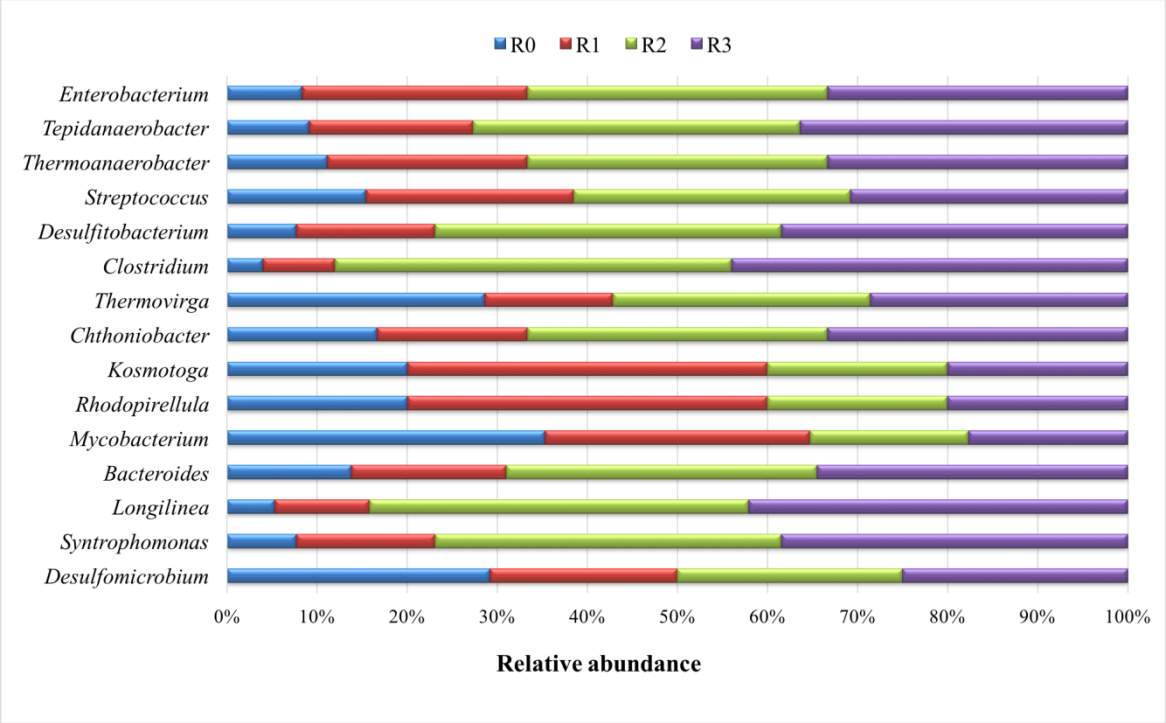

Figure 4.7. Relative abundance of dominant bacterial genus in digesters R_0, R_1, R_2 and R_3.

The identification of the archaea communities is illustrated in Fig. 4.8. It was found that 4 phyla and 8 genera of Achaea in the anaerobic digesters fed with animal manure and bioaugmented with anaerobic rumen fungi. The most dominant archaeal communities are *Methanosarcinales*, *Methanoasaeta*, *Methanobacteriales* and *Methanomicrobiales* in digesters of R_0, R_1, R_2 and R_3 respectively. While investigating the archaeal genera, it was found that *Methanobacterium kanagiense*, *Methanobacterium uliginosum*, *Methanolinea sp.* and *Methanolinea sp.* are the most dominant genera in digesters of R_0, R_1, R_2 and R_3 respectively. Although *Methanobacterium kanagiense* is the least dominant genus in digester R_2 which has the highest biogas production, *Methanolinea sp.* is the most effective genus for biogas production. Therefore, it can be said that anaerobic rumen fungi are more influential for *Methanolinea sp.* Moreover, it was also implied that the main pathway for methane production is provided by *Methanolinea sp.* in digester R_2.

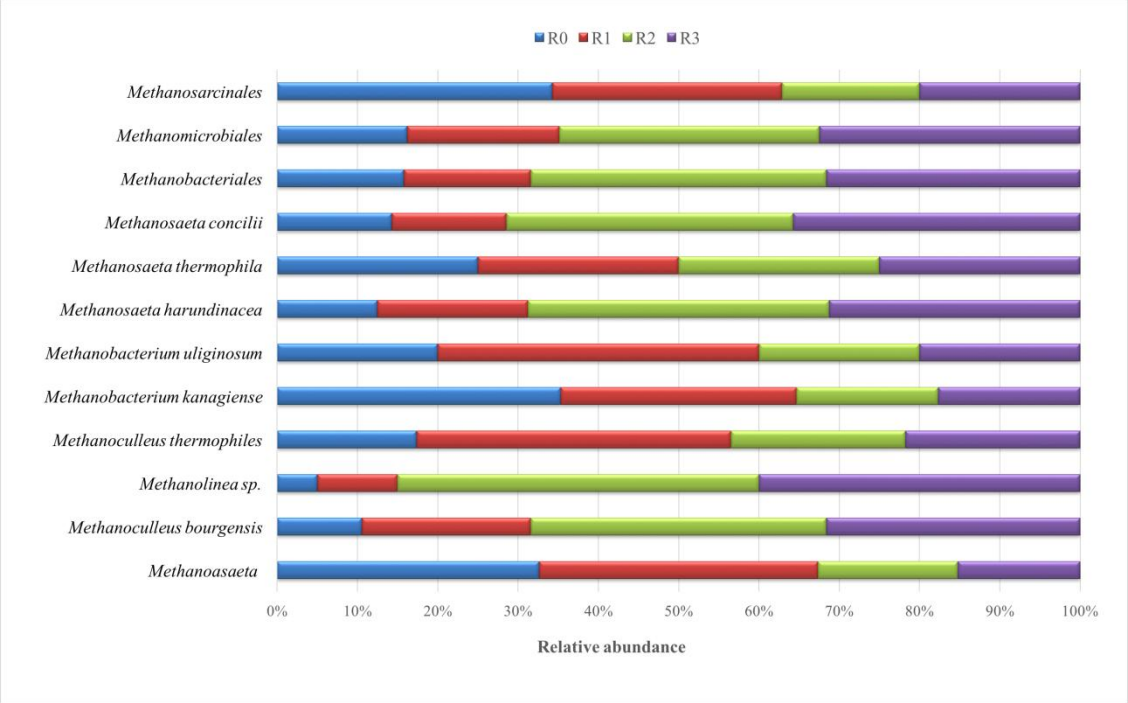

Figure 4.8. Archaeal community dynamics in digesters R_0, R_1, R_2 and R_3.

According to all molecular results of distribution of the bacteria and archaeal communities, it was proven that anaerobic rumen fungi are more effective on phylum of *Lentisphaerae*, genus of *Clostridium* and *Methanolinea sp.* in terms of the highest biogas production. Therefore, it can be more effective the follow-up of them for control instead of investigation of fungi variations.

qPCR method was used to quantify the fungal 18S rDNA copy number. 18S rDNA sequence specific primers were used to quantify total fungus present in the anaerobic digesters fed with animal manure for the real time PCR assays. Results of the qPCR assay can be shown in Fig.4.9. The results of the qPCR showed that while the number of total cells of fungi decreased in time in digester R_1 (5%) which contains low concentration of anaerobic rumen fungi, it increased in time in digester R_2 (15%) and R_3 (20%) which contain relatively high concentration of anaerobic rumen fungi. Thus, it can be said that anaerobic rumen fungi in low concentration cannot be sufficient to compete with anaerobic microbial communities in anaerobic digester and anaerobic rumen fungi cannot obtain in digester R_1. Because the production of biogas and biomethane is lowest in digester R_1, it can be said that low concentration of anaerobic rumen fungi is insufficient to degrade animal manure. However, anaerobic rumen fungi in relatively high concentrations can

increasingly obtain in digesters R_2 and R_3 during 40 days. On the other hand, it was revealed that the number of total cells of fungi was lower in digester R_2 (15%), where the highest biogas is, than the digester R_3 (20%). Therefore, it can be said that the highest number of total cells of anaerobic rumen fungi cannot provide the effective degradation of animal manure and biogas production for anaerobic microbial communities in anaerobic digester. It is probably because rumen fungi become dominant to anaerobic microbial community which is responsible for biogas production in anaerobic digestion process. Although anaerobic rumen fungi provide the degradation of inert lignocellulosic compounds in animal manure, it is required that anaerobic rumen fungi cannot be dominant to anaerobic microbial communities so as to continue anaerobic digestion process by anaerobic bacteria and archaea. As a consequence, qPCR method can be preferred because quantification of anaerobic rumen fungi with qPCR method shows similar results with next generation sequencing and qPCR is considerably economic method.

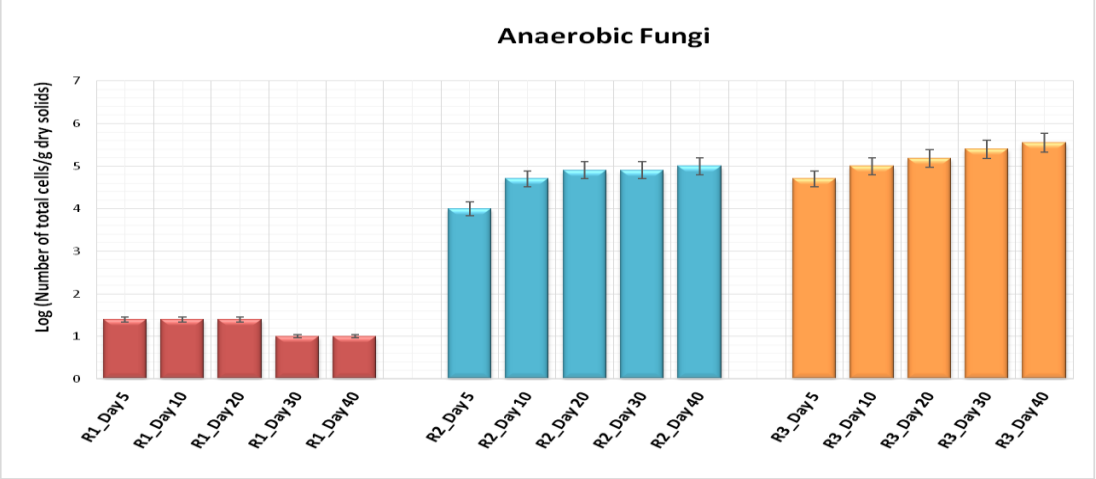

Figure 4.9. Quantities of anaerobic fungi in digesters R_1, R_2 and R_3.

As a consequence of the all results of anaerobic digesters fed with animal manure and bioaugmented with anaerobic rumen fungi, it can be said that degradation of animal manure and biogas performance were enhanced by bioaugmentation with anaerobic rumen fungi. It was observed that there is a 60% increase in methane production due to enhanced biodegradation of lignocellulosic compounds in animal manure. Although some VFA accumulation in digester was the result of process imbalance before 20 days, the accumulation didn't cause the inhibition. According to result of next generation of R_2 digester, which has the highest biogas production, anaerobic rumen fungi are more

effective on phylum of *Lentisphaerae*, genus of *Clostridium* and *Methanolinea sp.*. Main pathway for effective methane production from animal manure is provided by *Methanolinea sp*. Results of qPCR revealed that anaerobic rumen fungi cannot be dominant to anaerobic microbial communities for proceeding of anaerobic digestion process by anaerobic bacteria and archaea even if anaerobic rumen fungi provide the degradation of inert lignocellulosic compounds in animal manure.

4.5. Results of Microalgae Bioaugmented with Anaerobic Rumen Fungi

4.5.1. Biogas and Methane Production

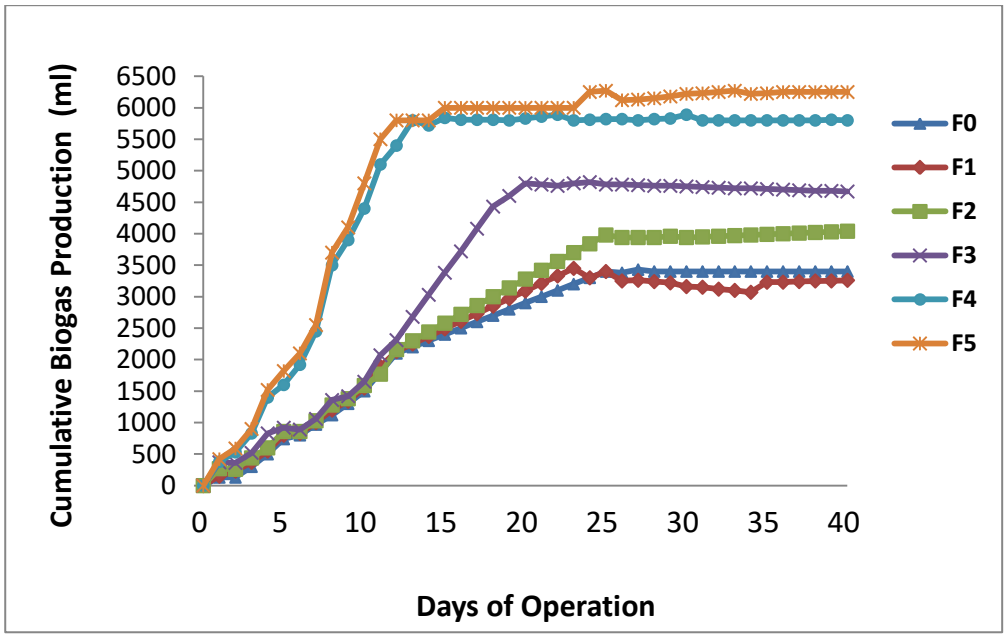

Figure 4.10. Biogas production in anaerobic digesters fed with microalgae.

The results of biogas production showed that there is an increase in the biogas production during 40 days. Increasing in biogas production continued between the startup stage and the steady stage. Biogas production approximately reached to the steady stage, in which biogas production is stable, between day 15 and 20 in all digesters. While the highest biogas production was measured in digester F_5 (6250 ml), F_4 (5800 ml), F_3 (4670 ml) and F_2 (4040 ml), the lowest biogas production was measured in F_1 (3260 ml) and F_0 (3400 ml) respectively. However, it was revealed that digester F_5 and F_4, which were bioaugmented with high concentration of anaerobic rumen fungi, previously achieved the steady stage in comparison with the other digesters which have lower biogas production.

Therefore, it can be said that anaerobic rumen fungi in high concentration provided to achieve steady stage easily. Because digester F_0, which wasn't bioaugmented with anaerobic rumen fungi, and F_1 (1%) achieved lowest biogas production and F_5 which was bioaugmented with highest concentration of anaerobic rumen fungi (%20) achieved the highest biogas production, it can be said that anaerobic rumen fungi are positively effective for biogas production on anaerobic digesters fed with microalgae *Haematococcus pluvialis*. In addition, biogas production increases as long as concentration of anaerobic rumen fungi increases and the most effective concentration of anaerobic rumen fungi is 20 % for biogas production from microalgae *Haematococcus pluvialis.*

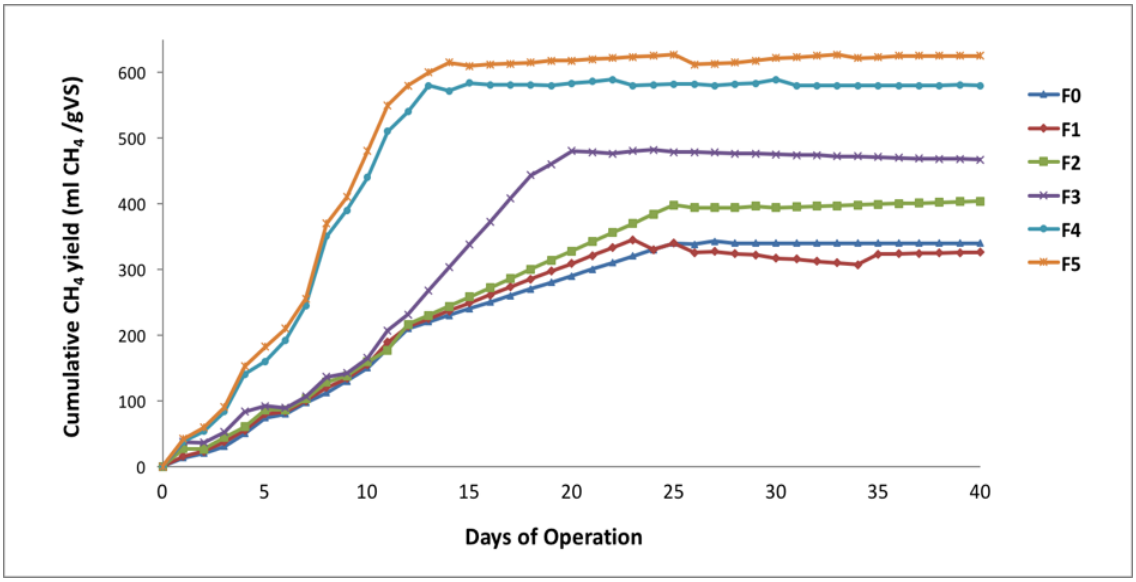

Figure 4.11. Methane production in anaerobic digesters fed with microalgae.

Methane production was also calculated according to the methane content of the biogas produced. Methane productions in digesters are given in Figure 4.11. According to the results of methane production, it was showed that there is an increase in the methane production. Increasing in methane production continued between the start-up stage and the steady stage. End of a 40 days' period, it reached the steady stage in which methane production is stabile in anaerobic digesters. Digesters F_4 and F_5 previously achieved the steady stage in comparison with the other digesters. Thus, it can be said that anaerobic rumen fungi in high concentration provided to achieve steady stage easily. However, it was also showed that high concentration of anaerobic rumen fungi provided high methane yield considering methane production. Methane yield in F_0, F_1 (1%), F_2 (5%), F_3 (10%), F_4

(15%) and F_5 (20%) was different from each other. The highest methane production was observed in digester F_5 (57% of total biogas). After F_5 digester, the maximum methane production was observed in F_4, F_3, F_2, F_0 digesters respectively. The lowest methane production was observed in F_1 digester. Although there was an important difference in methane yield among all digesters, there was not a conspicuous different between the digester F_1 and control digester.

4.5.2. Volatile Fatty Acids Production

The concentrations of VFAs were evaluated according to results of GC so as to evaluate and control the anaerobic digestion process. Temporal variations in VFA concentration are depicted in Fig.4.12. a, b, c, d, e, f for all digesters fed with microalgae *Haematococcus pluvialis.* Although all VFAs such as acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocopric acid, caproic acid and heptonic acid were measured through anaerobic digestion processes, accumulation of acetic acid and propionic acid, which are considerably significant indicator for inhibition effect on anaerobic digestion processes, was observed at some periods. However, VFAs were consumed at the end of the day 40 for all digesters. The results showed that acetic acid (600-700 mg/L) increased between day 1 and day 10 in all digesters. However, the accumulation of acetic acid decreased after day 10 and consumed at the end of the day 40 in all digesters. It was also observed that concentration of acetic acid in digester F_4 and F_5 was lower than the other digesters. When comparing the concentration of propionic acid, it was showed that increase in propionic acid was different in the all digesters. The highest concentration of acetic acid was observed in digesters F_0, F_1 and F_2. Concentration of propionic acid was considerably low in digester F_3, F_4 and F_5. Moreover, the lowest accumulation of propionic acid (220 mg/L) was observed in digester F_5 where the highest biogas production is. It was also investigated that as inoculums ratio of anaerobic rumen fungi increase, the accumulation of propionic acid decreases. Because propionic acid is the best indicator of process instability, it can be said that the digester F_5 has the most stable process and the most efficient biogas and biomethane production (Ahring et al. 1995). In addition, it is hard to mention about the inhibition because concentration of acetic acid and propionic acid are below 1,500 – 2,000 mg/L in the all digester (Labatut and Gooch, 2012).

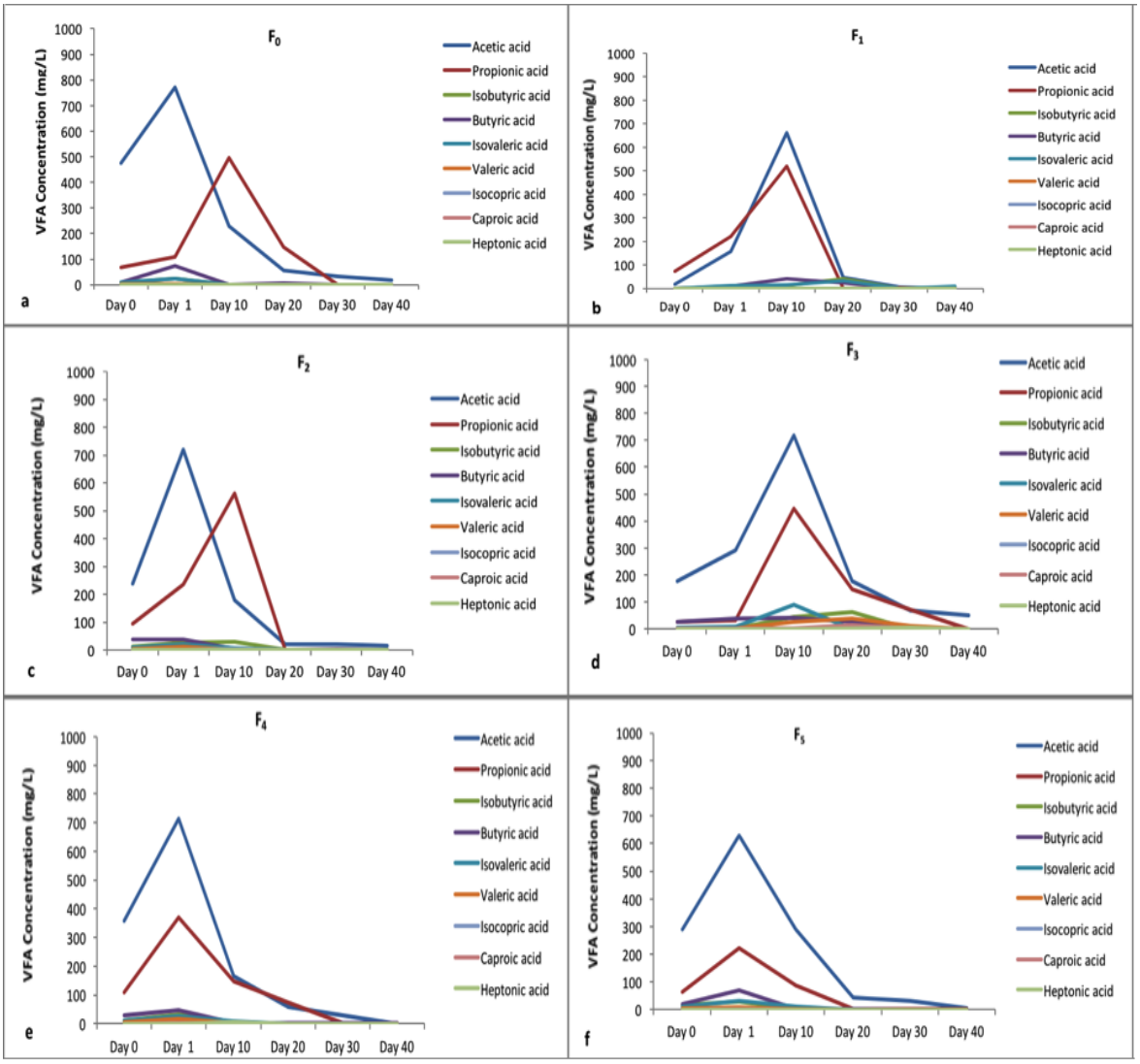

Figure 4.12. VFA profiles in digesters (a) F_0, (b) F_1, (c) F_2, (d) F_3, (e) F_4, (f) F_5.

4.5.3. Microbial Community Dynamics in Anaerobic Digesters

Microbial community analysis by using high-throughput Illumina MiSeq is a promising culture-independent method in order to investigate the variety of microalgae in anaerobic digestion. Thus, this approach can be beneficial to comprehend the microbial relationship between acetogens and methanogens. It can be also comprehended how these hinder the enhancement of biogas production during anaerobic digestion. Illumina Miseq analysis was used to investigate anaerobic digesters at a high inoculum-to-substrate ratio. Variations in the phylum distribution of the bacteria communities were determined according to the different inoculum percentages of anaerobic rumen fungi as seen in Fig. 4.13.

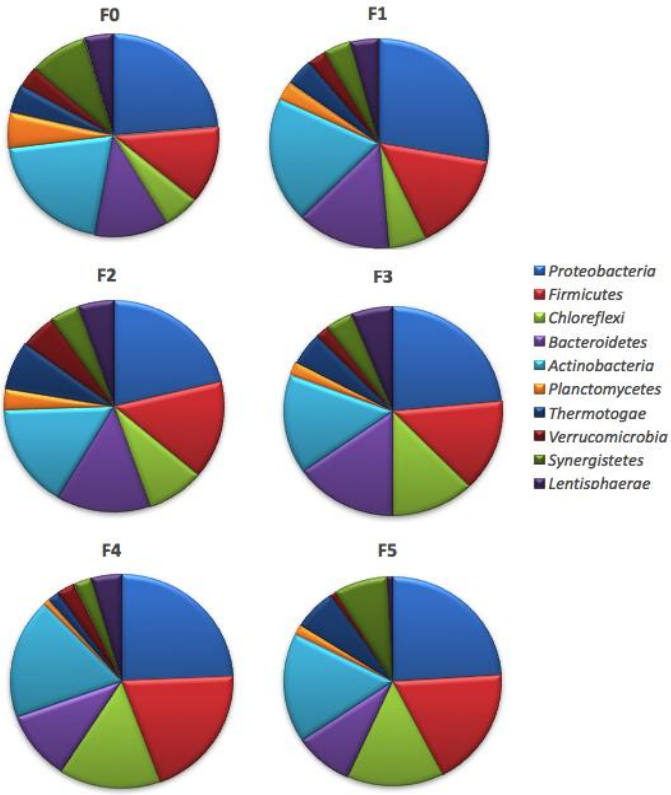

Figure 4.13. Bacteria communities of digesters F_0, F_1, F_2, F_3, F_4 and F_5.

It was showed that bacterial communities in anaerobic digesters are affected by different concentration of anaerobic rumen fungi. *Proteobacteria* was the most dominant community in all digesters. *Actinobacteria*, *Firmicutes* and *Bacteroidetes* were the other dominant communities respectively in all digesters. All digesters contained *Synergistetes*, *Planctomycetes*, *Chloreflexi*, *Lentisohaerae*, *Thermotogae* and *Verrucomicrobia* in small quantities. Digester F_0 was dominated by *Proteobacteria* and *Actinobacteria*. In addition to *Proteobacteria* and *Actinobacteria*, the digesters F_1 and F_2 were dominated by *Firmicutes* and *Bacteroidetes*. *Chlorofexi* which was one of the most dominant communities in F_3 and F_4 digesters, dominancy of *Chloroxi* relatively increased in F_5. F_4 and F_5 digesters, in which have higher methane productions, were dominated by *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Chloroflex* respectively. Thus, it can be said that combination of these communities is highly influential for methane yield. Despite the fact that *Proteobacteria* was dominant in digesters F_0, F_1, F_2, F_3, F_4 and F_5, bacterial community dynamics could change as a result of the varied inoculum ratios of anaerobic rumen fungi in the anaerobic digesters. It was observed that community of *Proteobacteria*, *Chloroflexi*, *Firmicutes* became dominant when concentration of anaerobic rumen fungi increased. According to

the bacterial community results, it was implied that the *Firmicutes* and *Chloroflexi* are the main phylum influenced the effective degradation capability of microalgae cell in digesters F_4 and F_5 in which the highest methane production was observed.

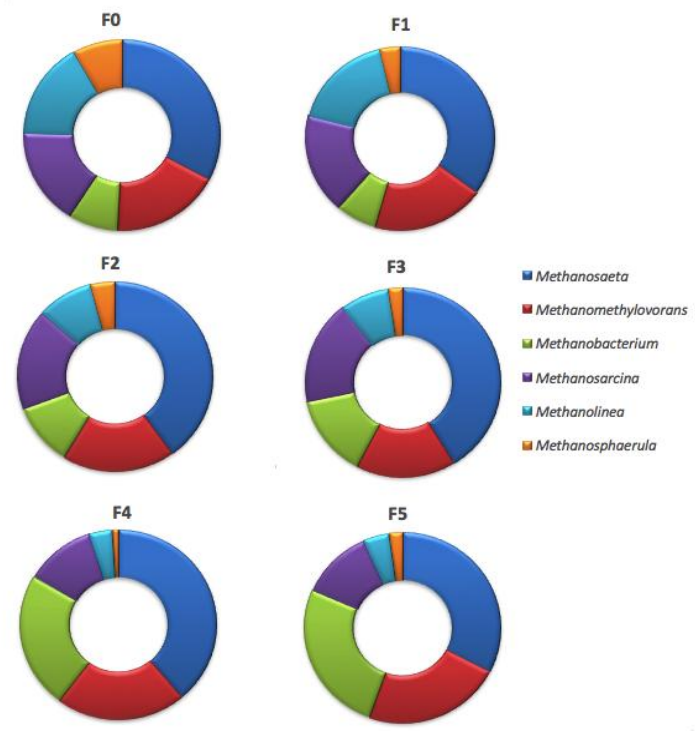

Figure 4.14. Archaea communities of digesters F_0, F_1, F_2, F_3, F_4 and F_5.

Variations in the phylum distribution of the archaea communities were determined according to the different inoculum percentages of anaerobic rumen fungi as seen in Fig. 4.14. It was showed that archaeal communities in anaerobic digesters are influenced by different concentration of anaerobic rumen fungi. *Methanosaeta* was the generally most abundant community in digesters F_0, F_1, F_2, F_3, F_4 and F_5. In addition to *Methanosaeta*, *Methanomthylovarans*, *Methanobacterium*, *Methanosarcina*, *Methanolinea* and *Methanosphairula* were present in the all digesters. *Methanosphairula* was the lowest community in all digesters. Digesters F_0 and F_1 were dominated by *Methanosaeta*, *Methanomthylovarans*, *Methanosarcina* and *Methanolinea*. Subsequent to *Methanosaeta*, *Methanomthylovarans* and *Methanosarcina* were the other dominant phylum in digesters F_2 and F_3. While community of *Methanosphairula* considerably decreased, community of *Methanobacterium* became dominant in F_4 and F_5 digesters, in which are observed higher methane yield. Therefore, it was demonstrated that abundance of *Methanosaeta*,

Methanobacterium and *Methanomethylovorans* communities increase as concentration of anaerobic rumen fungi in digester increases. As a consequence of these results, it was found that *Methanosaeta*, *Methanobacterium* and *Methanomethylovorans* improved the main pathway for methane production in digester F_5. The diversity of archaeal methanogenic communities conspicuously changed due to bioaugmentation with anaerobic rumen fungi at a high inoculum ratio (20%) in the F_5 digester.

qPCR method was used to quantify the fungal 18S rDNA copy number. 18S rDNA sequence specific primers were used to quantify total fungus present in the anaerobic digesters fed with *Haematococcus pluvialis* for the real time PCR assays. Results of the qPCR assay can be shown in Fig.4.15. The results of the qPCR showed that the number of total cells of fungi decreased in time in the digesters containing low concentration of anaerobic rumen fungi (F_1, F_2 and F_3) despite the fact that it is required to increase in time. Thus, it can be said that anaerobic rumen fungi in low concentrations cannot be sufficient to degrade algal biomass and to compete with anaerobic microbial communities in anaerobic digester. Thus, anaerobic rumen fungi cannot obtain in digesters F_1, F_2 and F_3. However, it was showed that the number of total cells of fungi increased in time in digesters containing high concentration of anaerobic rumen fungi (F_4 and F_5). It shows that anaerobic rumen fungi in high concentrations can provide the effective degradation of algal biomass for anaerobic microbial communities in anaerobic digester and hence anaerobic rumen fungi can obtain in digesters F_4 and F_5 through 40 days. Because they increasingly exist during 40 days in the digester, they can degrade the inert lignocellulosic compounds in microalgae and they can provide to enhance biogas production from microalgae *Haematococcus pluvialis*. Although quantification of anaerobic rumen fungi with qPCR method shows similar results with next generation sequencing, qPCR is a considerably economic method.

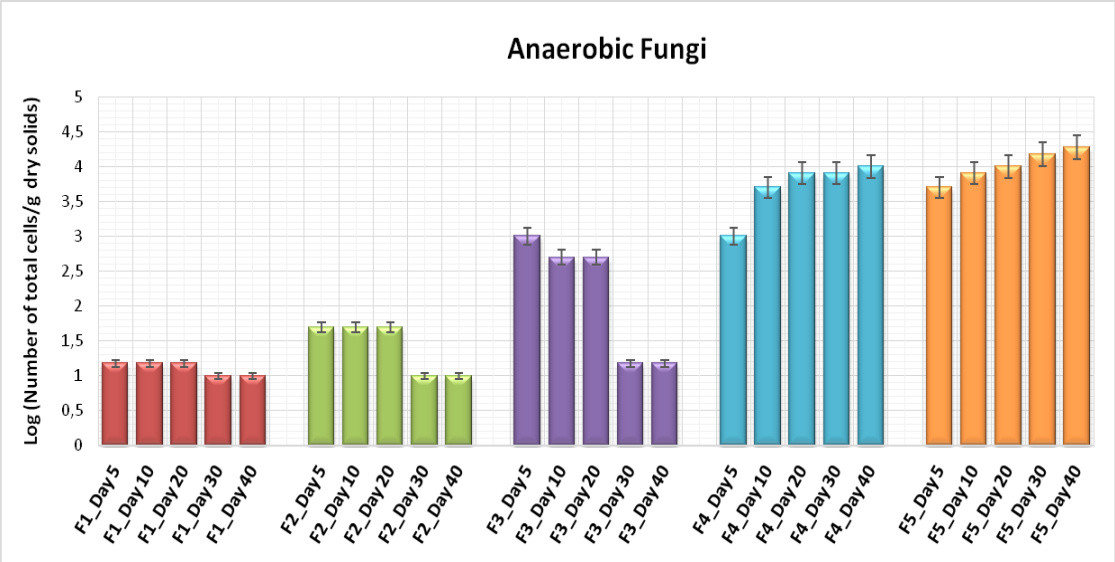

Figure 4.15. Quantities of anaerobic fungi in digesters F_1, F_2, F_3, F_4 and F_5.

As a consequence of the all results of anaerobic digesters fed with microalgae and bioaugmented with anaerobic rumen fungi, bioaugmentation with anaerobic fungi (20%) was determined to represent the most energy-efficiency method of producing methane from microalgae *H. Pluvialis*. 6250 ml biogas production and 57% methane yield were provided thanks to anaerobic rumen fungi in the anaerobic digesters fed with microlagae. Inhibition effect was not observed in the anaerobic digesters according to the measurements of VFAs. It was showed that syntrophic acetate-oxidizing bacteria and acetoclastic methanogen play a crucial role in methane production from microalgae cell degradation. qPCR results demonstrated that anaerobic rumen fungi in high concentration (20%) obtain during 40 days, degrade the inert lignocellulosic compounds in microalgae and they can provide to improve biogas production from microalgae. Biomethane production from microalgae was significantly achieved by bioaugmentation with anaerobic rumen fungi. Quantity and diversity of bacterial and archaeal communities were improved and fermentation performance was increased thanks to addition of rumen anaerobic fungi. qPCR results demonstrated that anaerobic rumen fungi in high concentration (20%) obtain during 40 days, degrade the inert lignocellulosic compounds in microalgae and they can provide to improve biogas production from microalgae. Because the diversity of archaeal methanogenic communities conspicuously changed due to bioaugmentation with anaerobic rumen fungi at a high inoculum ratio (20%) in digester F_5, it was suggested that

Methanosaeta, *Methanobacterium* and *Methanomethylovorans* are required for the degradation of microalgae biomass.

4.6. Results of Macroalgae Bioaugmetated with Anaerobic Rumen Fungi

4.6.1. Biogas and Methane Production

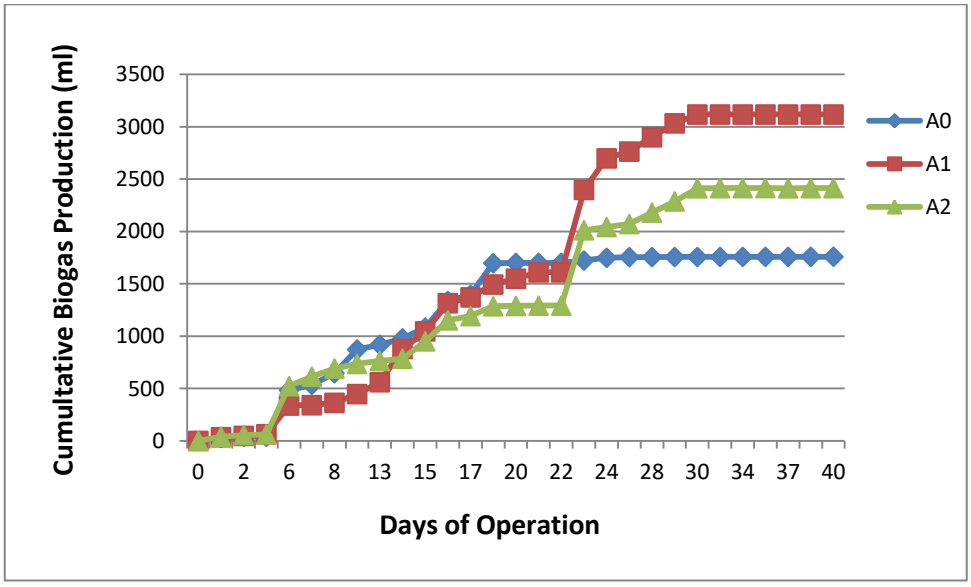

Figure 4.16. Biogas production in anaerobic digesters fed with macroalgae.

Biogas production, which is a significant parameter to understand the performance of anaerobic digesters, were recorded with milligas counters. Anaerobic digesters fed with macroalgae *Ulva lactuca* and biougmentated with anaerobic fungi are given in Figure 4.14. It was showed that there is an increase in the biogas production during 40 days in all digesters. Increasing in biogas production continued between the startup stage and the steady stage. End of a 40 days' period, it reached the steady stage in which biogas production is stable in anaerobic digesters. Digester A_0 which wasn't bioaugmented with anaerobic rumen fungi previously achieved the steady stage in comparison with the other digesters. In addition, the lowest biogas production was also observed in digester A_0. Digester A_1 containing rumen fungi in concentration of 15% achieved a higher biogas production (about 3500 ml) than the other digesters. Biogas production in digester A_2 and A_0 were almost 2500 and 1800 ml respectively. As a consequence of these biogas production results, it can be said that anaerobic rumen fungi are positively effective for

biogas production on anaerobic digesters fed with macroalgae *Ulva lactuca* and the most effective concentration of anaerobic rumen fungi is 15%. Above concentration of 15% anaerobic rumen fungi causes to decrease biogas production on anaerobic digesters fed with macroalgae *Ulva lactuca*.

Methane production was calculated according to the methane content of the biogas produced in order to evaluate the performance of anaerobic digester bioaugmented with anaerobic rumen fungi. Since methane from biogas is an excellent alternative promising energy source and this study was conducted to improve methane production, results of methane production are considerably important parameter.

As shown in Figure 4.15., in this study, it was showed that there is an increase in the methane production for all digesters. The increase in methane production continued between the startup stage and the steady stage. Although control digester (A_0) previously reached the steady stage between 15-20 days, the increase in methane production proceeded until the end of the day 35 in digesters A_1 and A_2. Thus, it can be said that anaerobic rumen fungi in anaerobic digesters fed with macroalgae *Ulva lactuca* provide to produce methane for a longer time when compared to control digester. However, it was also showed that the concentration of anaerobic rumen fungi is important parameter to improve methane production. According to results, the highest methane production (54,7% of total biogas) was observed in digester A_1 which contains 15% concentration of anaerobic rumen fungi. On the other hand, methane production in digester A_2 containing 20% concentration of anaerobic rumen fungi has lower methane production than digester A_1. As a consequence of these results, it can be concluded that bioaugmentation of anaerobic rumen fungi is considerably effective treatment method to improve methane production on anaerobic digesters fed with macroalgae *Ulva lactuca.* And also, the most effective concentration of anaerobic rumen fungi is 15% for effectively methane production.

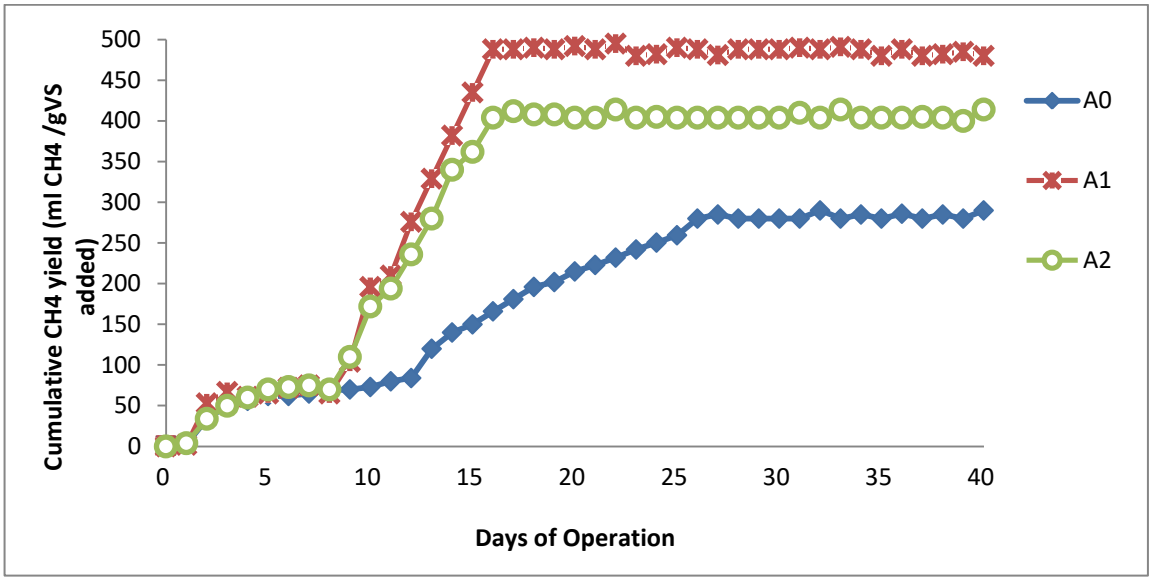

Figure 4.17. Methane production in anaerobic digesters fed with macroalgae.

4.6.2. Volatile Fatty Acid Production

Changes in volatile fatty acid (VFA) level were investigated according to results of GC in order to indicate the process instability of anaerobic digesters fed with macroalgae because VFAs are reliable method for the evaluation and control of the anaerobic digestion process. Temporal variations in acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocopric acid, caproic acid and heptonic acid are shown in Fig 4.16. a,b,c,d. Although VFAs were consumed at the end of the 40 days in all digesters, some accumulations of VFAs were detected during anaerobic digestion processes. Especially accumulation of acetic acid and propionic acid is important indicator for inhibition effect on anaerobic digestion processes. The results demonstrated that the accumulation of acetic acid and propionic acid decreases after day 10 even if there is an increase until day 10 in digester A_1 and A_2 which were bioaugmented with anaerobic rumen fungi. It was observed that there is an increase in digester A_0 at day 20. Although temporal changes in concentration of VFAs are regarded as stability until day 10, propionic acid (230 mg/L) increased at day 20 in digester A_0. However, it cannot be said that there is an inhibition effect on the digester because the concentration of total VFA is typically below 500 mg/L is accepted as normal (Labatut and Gooch, 2012). Accumulation of acetic acid was approximately measured below 500 mg/L in all digester, but, the lowest concentration of propionic acid (273 mg/L) was detected in digester A_1 where the highest

biogas production is. Because propionic acid is the best indicator of process instability, it can be said that digester A_1 has the most stable process and the most efficient biogas and biomethane production (Ahring et al. 1995).

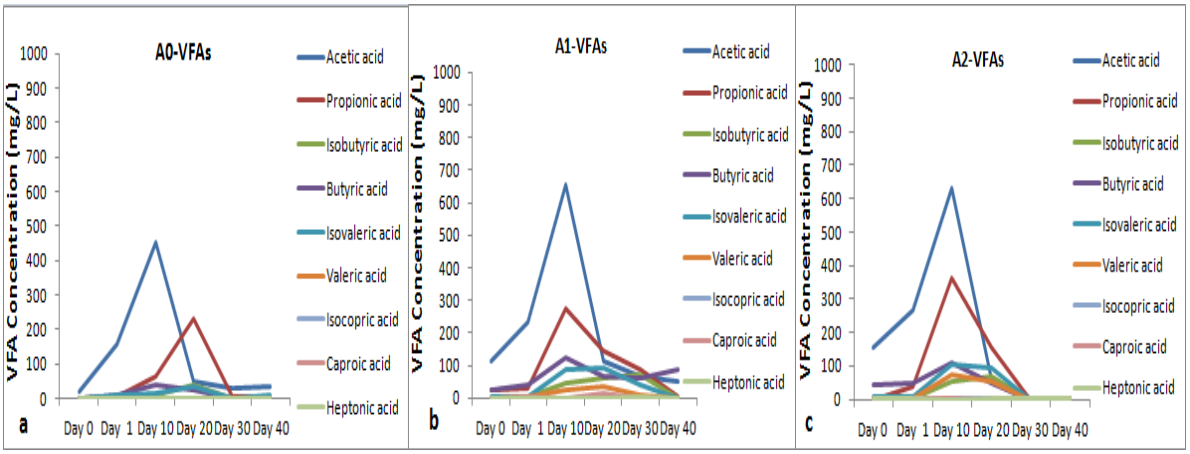

Figure 4.18. VFA profiles in digesters (a) A_0, (b) A_1 and (c) A_2.

4.6.3. Microbial Community Dynamics in Anaerobic Digesters

Variations in the phylum distribution of the bacteria and archaeal communities in the samples of anaerobic digesters fed with macroalgae *Ulva lactuca* and bioaugmented with rumen fungi were investigated by high-throughput Illumina Miseq. 15 genera of bacteria were found in the samples of anaerobic digesters as seen in Fig.4.19. It was revealed that *Mycobacterium* are the most dominant bacterial genus in digester A_0, *Syntrophpmonas* are the most dominant bacterial genus in digester A_1 and Thermoanaerobacter are the most dominant bacterial genera in digester A_2. Although genera of *Clostridium, Desulfobacaterium, Enterobacterium, Tepidanaerobacter, Longilinea, Bacteriodes* and *Sytrophomonas* were found at the lowest quantities in digester A_0, it was observed that they started to be dominant as concentration of rumen fungi increases in digester A_1 and A_2. Thus, it can be said that anaerobic rumen fungi are in a mutualistic relationship with these bacterial genera during anaerobic digestion processes. On the other hand, it was showed that genera of *Mycobacteirum* and *Thermovirga* decreased as concentration of rumen fungi increases. It means that genera of *Mycobacterium* and *Termovirga* were negatively affected by anaerobic rumen fungi. *Syntrophomonas, Desulfomicrobium* and *Clostridium* were the most dominant genera respectively in digester A_1 where the highest

biogas and biomethane production observed. Therefore, it can be said that these genera are more effective bacterial genera for biogas production from macroalgae *Ulva lactuca.*

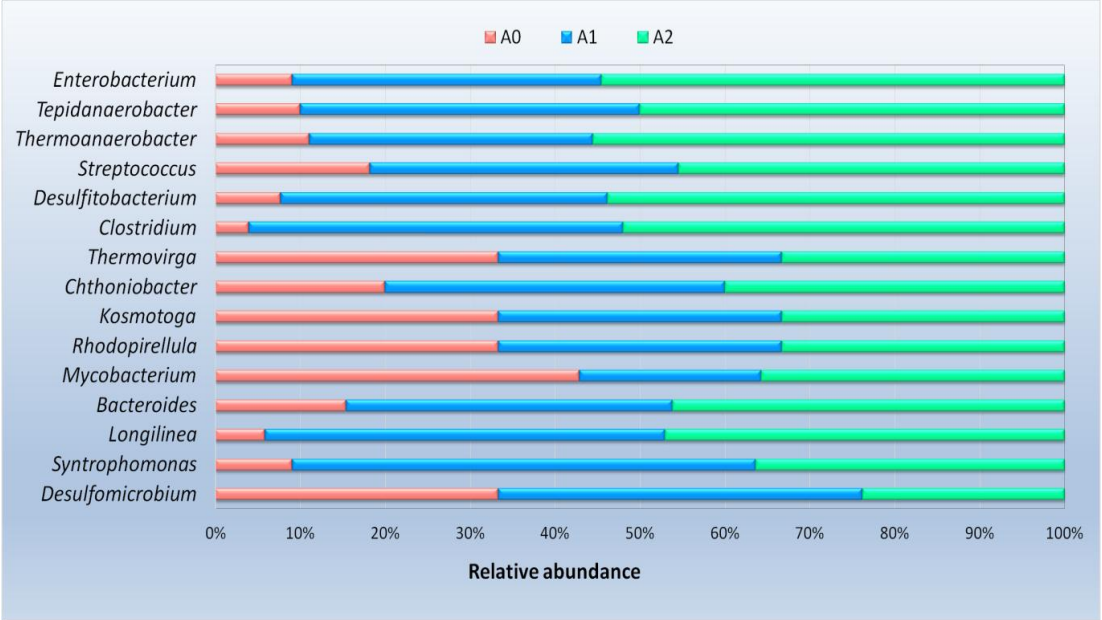

Figure 4.19. Bacteria communities of digester A_0, A_1 and A_2.

The identification of the archaea communities is illustrated in Fig.4.20. It was found that 12 genera of Acrhaea in the anaerobic digesters fed with macroalgae *Ulva lactuca* and bioaugmented with anaerobic rumen fungi. The most dominant archaeal communities are *Methanobacterium* in digester A_0, *Methanolinea* in digester A_1 *and Methanoculleus* in digester A_2. The least dominant genera are Methanolinea, *Methanosarcinales, Methanocelleus* and *Methanoasaeta* in digesters A_0, A_1 and A_2 respectively and it was observed that these genera increased as concentration of rumen fungi increases. Therefore, it can be said that *Methanolinea sp, Methanosarcinales, Methanocelleus* and *Methanoasaeta* were positively affected by anaerobic rumen fungi. On the other hand, *Methanobacterium, Methanoasaeta* and *Methanosarcilanes* decreased as concentration of rumen fungi increases. Thus, it can be said that *Methanobacterium, Methanoasaeta* and *Methanosarcilanes* are negatively affected by anaerobic rumen fungi. While investigating the result of digester A_1, where the highest biogas and biomethane production are, it was found that *Methanolinea* and *Methanobacteriales* are the most dominant genera. Thus, it can be said that *Methanolinea* and *Methanobacteriales* are more effective archaeal genera

for biogas production from macroalgae. It was investigated that main pathway for methane production is provided by *Methanolinea* and *Methanobacteriales* in digester A_1.

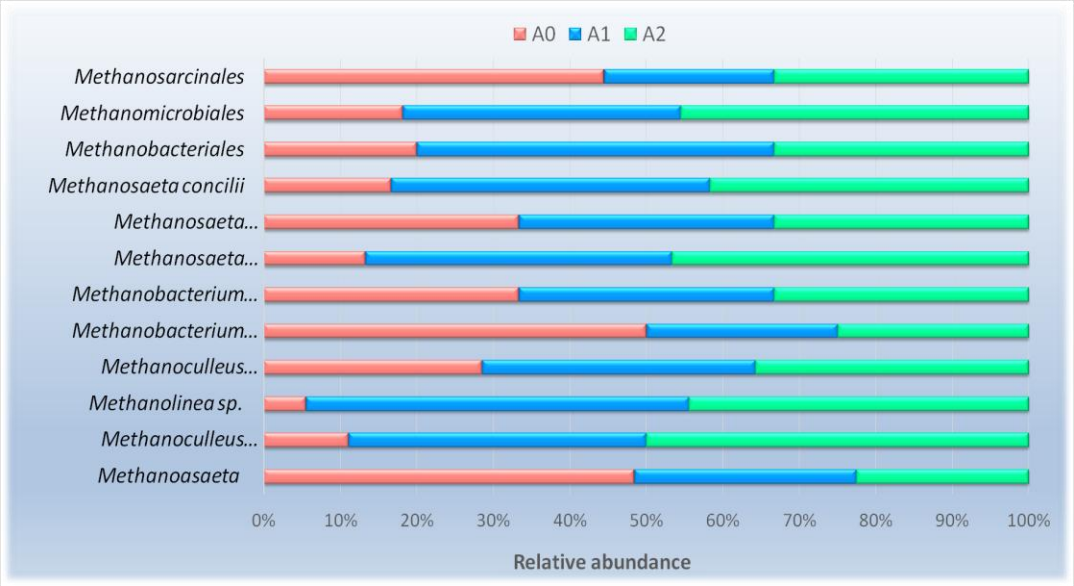

Figure 4.20. Archaea communities of digesters A_0, A_1 and A_2.

Changes in anaerobic rumen fungi were identified using qPCR method. 18S rDNA sequence specific primers were used to quantify total fungus present in the anaerobic digesters fed with macroalgae *Ulva lactuca* for the real time PCR assays. Results of the qPCR assay can be shown in Fig.4.21. The results of the qPCR showed that the number of total cells of fungi nearly remained unchanged in digester A_1. However, the number of total cells of fungi increased in time in digester A_2 containing high concentration of anaerobic rumen fungi. Because the production of biogas and biomethane is highest in digester A_1, it can be said that high number of total cells of fungi cannot sufficient to improve biogas production. It is probably because rumen fungi become dominant to anaerobic microbial community which is responsible for biogas production in anaerobic digestion process. Despite the fact that anaerobic rumen fungi provide the degradation of lignocellulosic compounds in macroalgae, it is required that anaerobic rumen fungi cannot be dominant to anaerobic microbial communities in order to continue anaerobic digestion process by anaerobic bacteria and archaea. In digester A_1, the number of total cells of fungi are considerably adequate for biodegredation of compounds and after production of biomethane by anaerobic bacteria and methnanogens. It can be said that as the number of total cells of fungi increases, anaerobic microorganism which are responsible for biogas

production in anaerobic digester cannot effectively perform. Thus, concentration of anaerobic rumen fungi and anaerobic microorganisms should be in balance to provide sufficient biomethane yield.

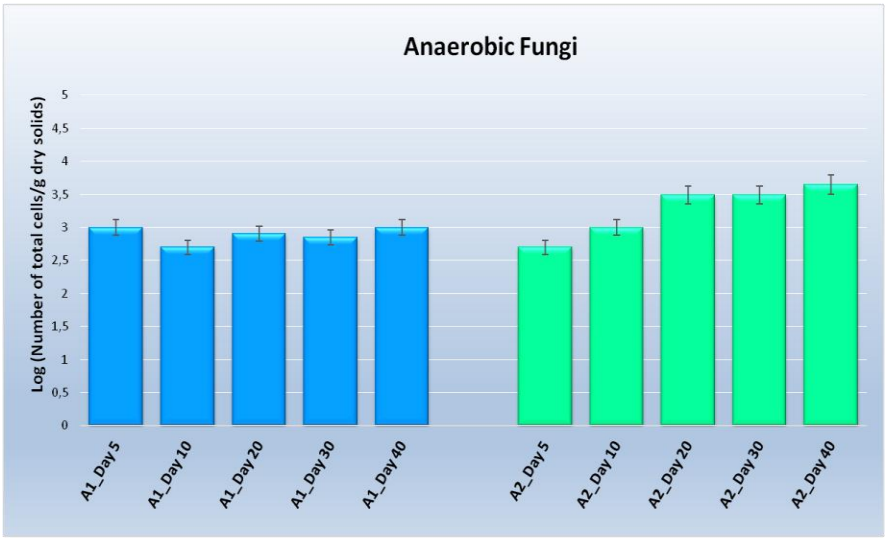

Figure 4.21. Quantities of anaerobic fungi in digesters A_1 and A_2.

According to all molecular results of distribution of the bacteria and archaeal communities, it was proven that anaerobic rumen fungi are more effective on bacterial genera of *Syntrophomonas*, *Desulfomicrobium*, *Clostridium* and archaeal genera of *Methanolinea* and *Methanobacteriales* in terms of the highest biogas production. Moreover, it was proven that highest number of total cells of anaerobic rumen fungi cannot provide the effective degradation of macroalgae and biogas production for anaerobic microbial communities in anaerobic digester. Illumina Miseq sequencing can be more effective the follow-up of them for control instead of investigation of fungi variations. On the other hand, qPCR method can be preferred because quantification of anaerobic rumen fungi can be analyzed with qPCR method and qPCR is considerably economic method.

As a consequence of the all results of anaerobic digesters fed with macroalgae and bioaugmented with anaerobic rumen fungi, bioaugmentation with anaerobic fungi was determined to represent the most energy-efficiency method of producing methane from macroalgae *Ulva lactuca.* Biomethane production from macroalgae was significantly achieved by bioaugmentation of anaerobic rumen fungi. It was demonstrated that the most effective concentration of anaerobic rumen fungi is 15% and above concentration of 15%

anaerobic rumen fungi causes to decrease biogas production on anaerobic digesters fed with macroalgae *Ulva lactuca.* 3500 ml biogas production and 54,7% methane yield were provided thanks to anaerobic rumen fungi in the anaerobic digesters fed with macrolagae. The result of VFAs showed that there is no inhibition effect in the anaerobic digesters. It was showed that anaerobic rumen fungi are more effective on bacterial genera of Syntrophomonas, Desulfomicrobium, Clostridium and archaeal genera of *Methanolinea* and *Methanobacteriales* for improvement of biogas production. qPCR results demonstrated that quantification of anaerobic rumen fungi.

5. CONCLUSION

The anaerobic rumen fungi can be considered as a novel and promising microorganism in order to improve performance of anaerobic digesters and biogas efficiency for lignocellulosic biomass. The main aim of this study was to determine the effects of anaerobic rumen fungi on anaerobic digesters fed with different lignocellulosic compounds. In this concept, anaerobic rumen fungi were systematically examined for enhancement of biogas production from animal manure, microalgae *Haematococcus pluvialis* and macroalgae *Ulva lactuca.*

Bioaugmentation with anaerobic rumen fungi at concentration of 15% was determined to represent the most energy-efficiency method of producing methane from animal manure in anaerobic digester systems. 5500 ml biogas and 60% methane production were achieved by bioaugmentation of anaerobic rumen fungi. Phylum of *Bacteroidetes*, genera of *Clostridium* and *Longilinea*, phylum of *Methanobacteriales* and genus *Methanolinea sp.* are the most dominant microorganism for effective degradation of animal manure and higher biogas production. In addition, main pathway for effective methane production from animal manure is provided by *Methanolinea sp.*

It was found that 6250 ml biogas production and 57% methane yield were provided thanks to anaerobic rumen fungi in the anaerobic digesters fed with macrolagae. The increases in methane yield observed remained in the same order of magnitude, regardless of the inoculum ratios of anaerobic fungi. Bioaugmentation with anaerobic fungi (20%) was determined to represent low-cost method of increasing methane production (6250 ml). *Firmicutes* and *Chloroflexi* were the main phylum influenced the effective degradation capability of microalgae cell. *Methanosaeta*, *Methanobacterium* and *Methanomethylovorans* improved the principle pathway for methane production from microalgae *H. pluvialis.*

Anaerobic rumen fungi in concentration of 15% achieved a higher biogas production (3500 ml) in the anaerobic digesters fed with macroalgae. 54,7% methane production were provided thanks to bioaugmentation of anaerobic rumen fungi. Anaerobic rumen fungi are

more effective on bacterial genera of *Syntrophomonas, Desulfomicrobium, Clostridium* and archeal genera of *Methanolinea* and *Methanobacteriales* in terms of the highest biogas production.

It was demonstrated that anaerobic rumen fungi can be a promising alternative to enhance biogas production from different types of lignocellulosic compounds thanks to their non-specific extracellular ligninolytic enzymatic system. Although next generation sequencing can provide comprehensive information about the microbial community dynamics in anaerobic digesters, qPCR which is a considerably economic method can be used to control the effect of bioaugmentation and it can be sufficient for determination of anaerobic rumen fungi.

In addition to manure, microalgae and macroalgae, bioagmentation with rumen fungi can be also used for different types of micro-macroalgae and wide range of substrates having high lignin content in order to degrade biomass and enhance the biomethane production through anaerobic digestion process. Because anaerobic rumen fungi have a non-specific extracellular ligninolytic enzymatic system, they can be effectively used for biodegradation of different substrates. Thus, biougmentation of anaerobic rumen fungi can be considered as an advantageous and promising method for biotechnology studies in different fields.

Although different concentrations of anaerobic rumen fungi of 4 species (*Piromyces sp*, *Neocallimastix frontalis*, *Anaeromyces sp.* and *Orpinomyces sp.*) were used in this study, effects of various combination of different species on biogas production can be investigated in the future studies. For example, combination of 2 species or 3 species can be researched and their performance on biodegradation and biogas production during anaerobic digestion processes can be compared. In addition to different combinations of anaerobic rumen fungi, it can be also studied which species are more effective for enhancement of biogas production. As a consequence of these studies, anaerobic rumen fungi can be used in the real treatment plants in large scale.

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