BIOMETHANE POTENTIAL OF PRE-TREATED MACROALGAE AND CORN STOVER BY *Trametes versicolor* ENTRAPPED IN CA-ALGINATE BEADS

by

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Dedicated to Dr. Öykü Irigül Sönmez and Büşra Öztürk

For the love of science…

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ABSTRACT

BIOMETHANE POTENTIAL OF PRE-TREATED MACROALGAE AND CORN STOVER BY *Trametes versicolor* ENTRAPPED IN CA-ALGINATE BEADS

Microorganisms especially fungi are utilized in biological pre-treatment of lignocellulosic biomass. In this particular study, *Trametes versicolor* were entrapped in Ca-alginate beads in order to use them in the pre-treatment of macroalgae (*Ulva* sp.) and corn stover prior to anaerobic digestion process. After pre-treatment, anaerobic co-digestion of corn stover and macroalgae was carried out. According to results, laccase activities of samples were high enough to process pre-treatment and activity of enzyme in sample containing corn stover only showed dramatic increase in the 2nd day of pre-treatment. The concentration of reducing sugars was increased after 24 hours for pre-treatment samples, and then tended to decrease following 6 days of pre-treatment. Methane yields of samples were increased by 26%, 24%, and 15% for macroalgae, corn, and co-digestion of both substrates, respectively. Soluble chemical oxygen demand and volatile fatty acid concentrations of digesters were obtained as strengthening the methane yield results. Next-generation sequencing analysis was conducted by using ION PGM^{TM} in order to determine microbial communities in samples taken from the 20th day of anaerobic digestion process. Significant differences in microbial communities including bacteria and archaea were determined between non-treated and pre-treated digesters and pre-treatment of substrates increased microbial diversities inside of anaerobic digesters according to bioinformatics analysis. Overall, the results indicated that *T. versicolor* entrapped in Ca-alginate beads can be utilized in the pre-treatment of lignocellulosic biomass and macroalgae biomass in order to decrease the cost and time of the operation.

ÖZET

CA-ALGİNAT KÜRECİKLERİNE HAPSEDİLMİŞ *Trametes versicolor* İLE ÖNARITIM YAPILMIŞ OLAN MAKROYOSUN VE MISIR ARTIKLARININ BİYOMETAN POTENSİYELİ

Mikroorganizmalar özellikle mantar olanlar lignoselülozik biyokütlenin önarıtımı için kullanılmaktadır. Bu çalışmada, Ca-alginat küreciklerine hapsedilmiş *Trametes versicolor*, anaerobik çürütme prosesi öncesinde makroyosun (*Ulva* sp.) ve mısır atıklarının ön arıtımı için kullanıldı. Ön arıtımdan sonra, mısır atıkları ve makroyosunun birlikte anaerobik çürümesi yürütüldü. Sonuçlara göre örneklerin lakkaz enzim aktivitesi ön arıtım işlemi için yeterince yüksekti ve ön arıtımın ikinci gününde yalnızca mısır atıkları içeren örneğin lakkaz enzim aktivitesi hızlı bir yükseliş gösterdi. Ön arıtım örneklerinde indirgeyici şeker miktarları 24 saat sonra arttı ve ön arıtımın kalan 6 günü düşüşe meyil gösterdi. Örneklerin metan verimlilikleri makroyosun, mısır atıkları ve iki substratın birlikte çürütülmesi sonucu sırasıyla %26, %24 ve %15 artış göstermiştir. Çürütücülerin çözünmüş kimyasal oksijen ihtiyacı ve uçucu yağ asitleri konsantrasyonlarından elde edilen sonuçlar, metan verimliliği sonuçlarıyla örtüşmüştür. ION PGM^{TM} kullanarak anaerobik çürütücü prosesinin 20.gününde alınan örneklerin mikrobiyal komünitelerini belirlemek için yeni nesil gen dizileme analizi yapıldı. Ön arıtım yapılmış ve yapılmamış çürütücülerin arke ve bakteri bazlı mikrobiyal komünitelerinde önemli farklar tespit edildi ve biyoinformatik analizlerine göre substratların ön arıtımı çürütücülerin içindeki mikrobiyal çeşitliliği artırdığı görülmüştür. Genel olarak sonuçlar *T. versicolor* hücreleri hapsedilmiş Ca-alginat küreciklerini, işlem süresini ve maliyetini düşürmek için lignoselülozik ve makroyosun biyokütlelerinin ön arıtımı için kullanılabileceğine işaret etmiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Unit
Ag_2SO_4	Silver Sulphate	
CH_4	Methane	mL
CO_2	Carbon dioxide	
H_2	Hydrogen	
H_2O	Water	
H_2O_2	Hydrogen Peroxide	
H_2S	Hydrogen Sulfide	
H_2SO_4	Sulphuric Acid	
H_3PO_4	Phosphoric Acid	
HCl	Hydrochloric Acid	
$HgSO_4$	Mercuric Sulphate	
HNO_3^-	Nitric Acid	
$K_2Cr_2O_7$	Potassium Dichromate	
NaOH	Sodium Hydroxide	
NO_3^-	Nitrate	
S	Elemental Sulphur	
SO_4^{-2}	Sulphate	
µl	Microliter	

Abbreviation	Explanation	Unit
ABTS	2-2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)	
AD	Anaerobic Digestion	
C:N	Carbon:Nitrogen	
COD	Chemical Oxygen Demand	mg/L
CON-MA	Control Macroalgae	
CON-CS	Control Corn Stover	
CON-Co	Control Both Substrates	
dH_2O	Distilled Water	
DGGE	Denaturing Gradient Gel Electrophoresis	

DNA	Deoxyribonucleic Acid	
DNS	Dinitrosalicylic Acid	
dNTP	Deoxyribonucleotide Triphosphate	
ENS	Effective Number of Species	
I:S	Inoculum:Substrate	
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectrometry	
FISH	Fluorescence in situ Hybridization	
GC	Gas Chromatography	
GC-MS	Gas Chromatography-Mass Spectrometry	
NGS	Next-Generation Sequencing	
OLR	Organic Loading Rate	g TVS/L-day
OTUs	Operational Taxonomic Units	
PCR	Polymerase Chain Reaction	
PDA	Potato Dextrose Agar	
QIIME	Quantitative Insights Into Microbial Ecology	
PRE-MA	Pre-treatment of Macroalgae	
PRE-CS	Pre-treatment of Corn Stover	
PRE-Co	Pre-treatment of Both Substrates	
Q-PCR	Quantitative PCR (Real-Time PCR)	
RNA	Ribonucleic Acid	
sCOD	Soluble Chemical Oxygen Demand	mg/L
SSYE	Soluble Starch Yeast Extract	
S:S	Substrate:Substrate	
TKN	Total Kjeldahl Nitrogen	
TS	Total Solids	mg/L
TVS	Total Volatile Solids	mg/L
VFA	Volatile Fatty Acid	mg/L

1. INTRODUCTION

Energy demand increases day by day, along with rising life standards, developing technology, and increasing population in Turkey and the world. Although energy from fossil fuels continues to supply a major part of this requirement today, fossil fuels are not seen as sustainable energy solutions due to being finite and the source of damaging the nature. Renewable energy researches that can be solutions to the environmental problems such as climate change, waste disposal, and energy crises, continues to gain importance. Within the scope of energy politics, Turkey intends to produce at least 30% of its energy requirement until the year of 2023 by renewable energy sources (ETKB, 2014). While satisfying the energy need, one of the renewable energy sources, biomass energy, has the quality of being solution to the problem of energy supply without being a cause of environmental pollution. Biogas is produced by anaerobic digestion process to the use of heat, electricity, and transportation. Biogas is obtained by renewable energy sources and has the potential of replacement the energy that is produced by fossil fuels.

In terms of biomass that is based on agriculture and animal husbandry, Turkey is quite rich country, and its potential of annual biomass average is known approximately 109.4 million tones. Agricultural waste, animal waste, and industrial waste are highly significant source of biomass and annually estimated about 50-65 million tones in Turkey. After agricultural production, the majority of remained in fields are burned as chaff or leaved alone in the field. If agricultural and animal wastes, which are not stabilized, are used for fields, pollutants in these wastes can cause environmental pollution and a decline in production (Sümer et al., 2016). In recent years, one of the significant problems in many countries, in parallel with the inescapable increase in population and the consumption of natural resources, has been how these wastes can be safely and sustainably disposed.

Anaerobic digestion of organic wastes can be an alternative way by providing disposal possibility in a safe and sustainable way because wastes obtained from digesters can be used as fertilizer thanks to their rich nutrient content; moreover, biogas that is a renewable energy source is produced also by anaerobic digestion process. Renewable energy sources are divided into 5 main headings: Hydraulic, wind, solar, geothermal, and biomass energy. Biomass energy usage needs to be increased in future years in order to decrease the amount of un-treated waste disposal and be an alternative energy source instead of fossil fuels.

In this study, it was aimed to increase biomethane potential of macroalgae and corn stover by a novel pre-treatment technique that was applying Ca-alginate beads entrapped with white-rot fungi, which was *Trametes versicolor*. Efficiency of pre-treatment was measured by comparing biogas and methane yields obtained from anaerobic batch digestion tests. Later, microbial community and diversity was determined by bioinformatics analyses.

2. LITERATURE REVIEW

2.1. Background of Anaerobic Digestion

Anaerobic digestion is biological process that organic waste is broken down gradually to obtain energetically valuable gas form by diverse microbial communities. (Esposito et al., 2012) Anaerobic digestion process produces biogas that is a renewable energy source as indicated in Figure 2.1. This process also occurs in nature such as mammary intestine, sediments, white-ant digestion system and waterlogged soil (Ward et al., 2008). Waste water, farm, industrial, domestic and agricultural waste, plant biomass, crop residues, and energy crops (especially cultivate to produce meta-rich biogas) are used to provide carbon neutral local renewable energy (Lehtomaki et al., 2007).

Animal manure and organic waste are processed together in an anaerobic digester to produce biogas. This biogas is used for basic human needs required energy such as electricity, transportation and heating. The stabilized sludge from the anaerobic digestion is used as a fertilizer to the plantation, thus providing sustainability.

Methane production from anaerobic digestion technology obtained various biological wastes is ideal in many respects. It becomes more and more used worldwide due to economic and environmental benefits. In addition, using biogas compared to the natural gas has more advantages because i) they are produced from renewable sources ii) they do not release greenhouse gases into the atmosphere iii) they are local, so it is independent iv) they provide disposal of most organic wastes which are break down hardly v) they help to solve waste management problems (Chandra et al., 2012).

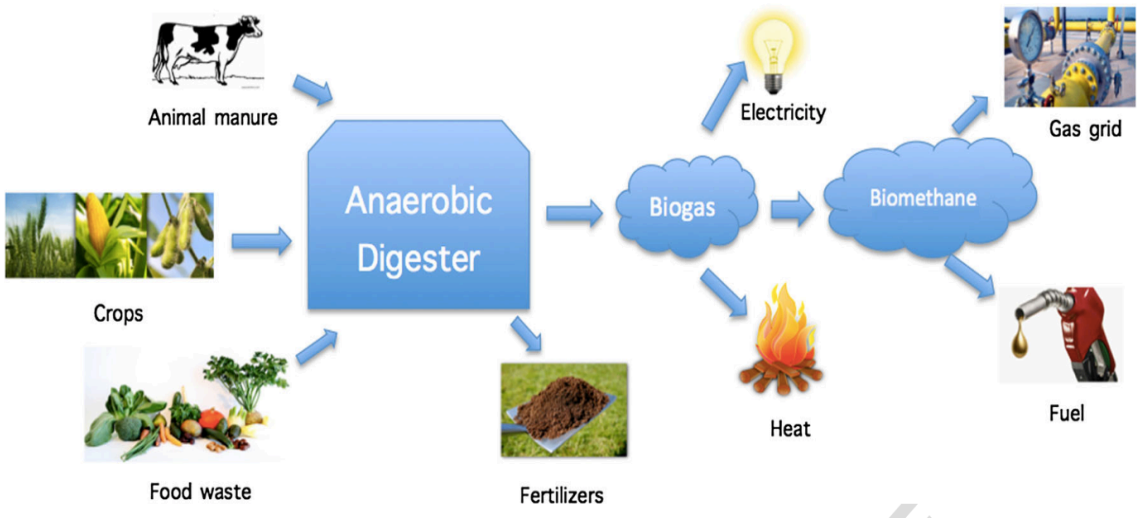

Figure 2.1. The valuable products obtained in AD process and substrates for anaerobic digesters (Bozan et al., 2017).

Remaining crop residues after harvesting process have rich amounts of lignocellulose and they can be a potential for biogas production as renewable energy source (Chandra et al., 2012). Energy crops are types of biomasses. Residues remained after crop harvesting are used to decrease energy demand by the application of anaerobic digestion process (Weiland, 2010).

Biogas is a type of gas produced under anaerobic conditions while degradation of biomass occurs by microbial activities. Wastes used to produce biogas can extend to various types: industrial wastewater, food industry, and agricultural wastes (Sawatdeenarunat et al., 2015). Biogas consists of mainly methane (55-75%), carbon dioxide (25-45%), and rare amounts of nitrogen, hydrogen, hydrogen sulphur, and oxygen. It can also contain ammonia and aromatic hydrocarbons depending on the type of substrate (Tsavkelova and Netrusov, 2012).

2.1.1. Process Biochemistry

Biogas production by anaerobic digestion is a very complex biological process possessing four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Each step is carried out by different groups of microorganisms (Gerardi, 2003). Steps of AD process are shown in Figure 2.2. Decomposition of substrates cause COD consumption and this COD conversion ratio in each step is shown in Figure 2.3.

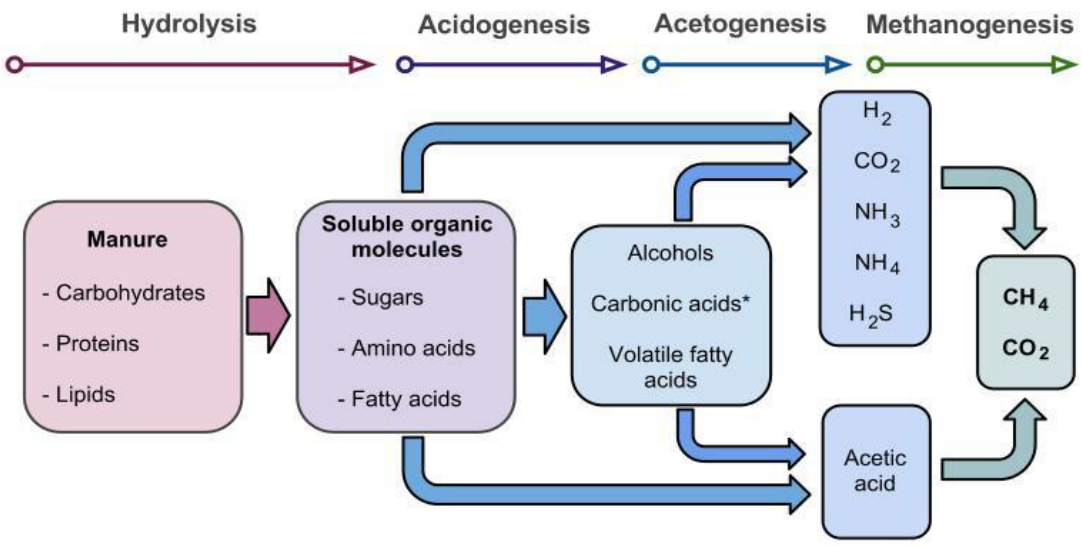

Figure 2.2. Four steps in AD process (Rea, 2014).

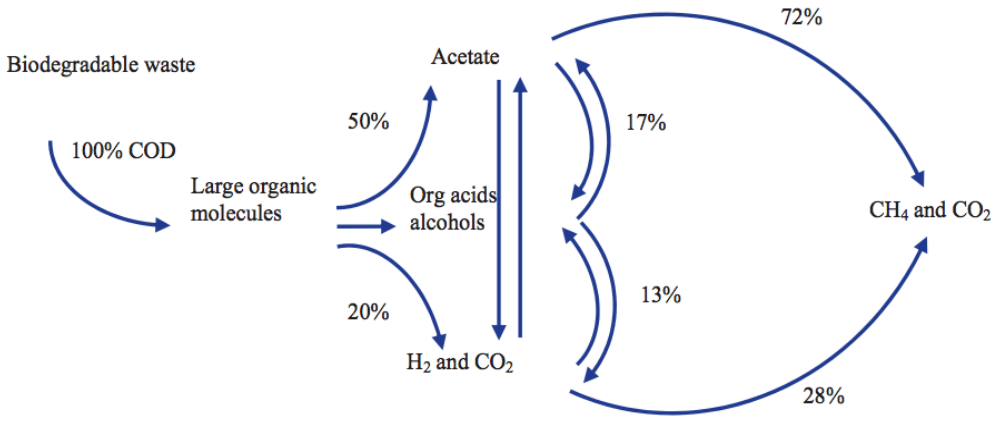

Figure 2.3. COD conversion ratios during AD steps (Chandra et al., 2012).

Hydrolysis is a step used by bacteria in order to degrade complex biological compounds, such as cellulose and proteins, for making them available nutrients to cells. Degraded compounds are absorbed by bacteria, and then they are converted to VFAs, ethanol, and propionate by serial breakdown reactions. Later, acetate and hydrogen are formed by microorganisms utilizing VFAs as substrate. Degradation of acetate and reduction of CO_2 and H_2 cause methane production in the end of AD process (Gerardi, 2003).

2.1.1.1. Hydrolysis. The first step of AD process is hydrolysis. In this step, complex organic compounds are converted into smaller soluble compounds (Haandel and Lettinga, 1994). Anaerobic microorganisms promote the conversion of cellulose, carbohydrate, and lipids into soluble monomers by the help of their extracellular enzymes. Covalent bonds existing in complex organic compounds were broken down aided by water molecules. Carbohydrates, lipids, and proteins were

converted into basic sugar molecules, fatty acids, and aminoacids, respectively. Degradation of carbohydrates can take shorter time than protein and lipid degradations that can take several days. However, degradation of lignocellulose and lignin is much slower and most of the time not completed after digestion process (Deublein and Steinhauser, 2008).

2.1.1.2. Acidogenesis. Products coming from hydrolysis step were go further conversion process in acidogenesis step. In this step, volatile fatty acids (VFA) and CO_2 were produced by using soluble hydrolysis products as substrate by the help of acidogenic bacteria. Mostly acetic acids were produced in this step; however, propionic, butyric, isobutyric, valeric, and isovaleric can be synthesized unless conditions for AD process were stable (Liu et al., 2008).

2.1.1.3. Acetogenesis. Products synthesized by acidogenic bacteria were processed to produce acetate, hydrogen, and carbon dioxide that were the precursors for methane production. There is a symbiotic relationship between methanogens and acetogenic microorganisms (Chandra et al., 2012).

2.1.1.4. Methanogenesis. The last step of AD process is methanogenesis. Methane as main energy carrier is produced as last step of anaerobic digestion proceeded by methanogenesis. It can be produced in natural environments having anaerobic conditions such as swamps, sediments, and rumen and this natural production of methane is a huge part of methane emissions (Sun, 2015). This step is limited by methanogenic activity because methanogens can be fragile to environmental alterations. Therefore, this step is rate-limiting step of AD process.

2.1.2. Process Microbiology

Particular microorganisms involved in AD process and their metabolic activities depend on operational conditions of digesters, environmental conditions, and chemical composition of feedstock or waste used in the process (Manyi-Loh et al., 2013). Efficiency and stability of AD process rely on harmonic and syntrophic relationships of microorganisms with each other (Vanwonterghem et al., 2014). Serial biochemical reactions are carried out by microorganisms in order to provide methane and CO_2. Microorganisms involved in this process are hydrolytic fermentative microorganisms, acidogenic microorganisms, hydrogen-producing acetogenic microorganisms, hydrogen-utilizing acetogenic microorganisms, carbon dioxide reducing methanogens, and acetoclastic methanogens as shown detailed way in Table 2.1 (Chernicharo, 2007).

Table 2.1. Microorganisms involved in AD process (Korres et al., 2013).

Stage of AD	*Major taxonomic entities identified*
Hydrolysis and acidogensis	• Fungi *Trichoderma (e.g. T. reesei), Thermomonospora, Ralstonia* and *Shewanella, Penicillium, Aspergillus* and *Humicola* • Bacteria e.g. *Bacteroides, Butyrivibrio, Clostridium, Cellulomonas, Fusobacterium, Selenomonas, Streptococcus, Peptococcus* and *Campylobacter. Actinomycetes* such as *Streptomyces* • *Pseudomonas mendocina, Bacillus halodurans, Clostridium hastiforme, Gracilibacter thermotolerans*, and *Thermomonas haemolytica. Synergistete.*
Acetogenesis	• Most acetogens are in the phylum *Firmicutes* e.g. *Moorella thermoacetica.* • *Spirochaetes.* • δ-*proteobacteria* e.g. *Desulfotignum phosphitoxidans.* • *Acidobacteria* e.g. *Holophaga foetida* • Exclusively acetogenic bacteria e.g. *Acetobacterium* and *Sporomusa* • Genera with acetogenic and non-acetogenic species e.g. *Clostridium, Ruminococcus, Eubacterium, Thermoanaerobacter, Treponema.*
Methanogenesis	Exclusively anaerobic, methane-producing *Archaea* from the phylum *Euryarchaeota,* with • 6 orders: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, Methanopyrales, Methanocellales*, and • 31 genera e.g. *Methanosarcina, Methanobrevibacter/ Methanobacterium Methanosaeta*

Hydrolytic step is generally performed by extracellular enzymes produced in fermentative bacteria in AD process, which has anaerobic conditions. Reaction rate of this step relies on several parameters such as pH and temperature (Gerardi, 2003). Heterogenic microorganisms are mostly taking a role in hydrolysis step of AD process. For instance, *Clostridium* spp. can have an impact on the decomposition of cellulose; moreover, *Bacillus* spp. can be a part of protein and fats degradation (Noike et al., 1985; Lema et al., 1991). Classification of extensive microorganisms conducting hydrolysis step was listed as aminolytic, lipolytic, proteolytic, and cellulolytic bacteria (Payton and Haddock, 1986).

Acidogenic step consists of conversion of soluble monomers, which are produced in hydrolysis step, into CO_2, alcohols, hydrogen, and short-chain organic acids. This process is carried out by acidogenic anaerobic bacteria. Amounts of hydrogen ions produced in this step regulate the variety of final products (Ren et al., 2007). In the end, acetate and hydrogen can be produced by the metabolic activity of fermentative microorganisms, which can digest amino acids and sugars. For instance, *Clostridium* sp. can conduct the production of butyric acid, butanol, isopropanol, and acetone; moreover, butyrate can be synthesized by *Butyribacterium* (Macy et al., 1978). There are different pathways and diverse reactions performed in this step. Beta-oxidation reactions are the type of reaction responsible for degradation of Fatty acids and Butyric acid. If acrylic and succinate pathways are used in the decomposition of carbohydrates, then propionic acid formation occurs (Chernicharo, 2007).

In acetogenic step, products formed during acidogenesis are precursors in acetogenesis step. Acetogenic bacteria, which can be hydrogen-producing or hydrogen-consuming, perform acetate, hydrogen, and CO_2 production by utilizing products coming from acidogenesis as substrates. Two types of mechanisms constitute acetogenic step: acetogenic hydrogenetation and dehydrogenation. Fermentation of hexoses or utilizing carbon dioxide and hydrogen to produce acetate is the step called acetogenic hydrogenation; furthermore, the step called acetogenic dehydrogenation refers to oxidizing long-chain and short-chain VFAs by the metabolic function of obligate hydrogen-producing or obligate proton-reducing bacteria (Gavala et al., 2003). Syntrophobacter wolinii, Syntrophus buswellii, Methaobacterium bryantii, and Desulfovibrio can be given as example of widespread acetic acid forming bacteria (Gujer et al., 1983).

Methanogenesis step is performed by group of key microorganisms called methanogens that can be found in fresh waters and sea, wastewater, and digestion system of herbivores in nature. Methanogens are members of archaea domain and they can be sensitive to pH variations, VFAs concentrations, free ammonia and ammonium ion concentrations. There are 6 classes of methanoges: *Methanopyrales*, *Methanobacteriales*, *Methanosarcinales*, *Methanococcales*, *Methanocellales*, and *Methanocellales*. Acetate is a precursor for almost over 70% methane production of anaerobic digestion process and it is utilized by *Methanosarcinales* (Manyi-Loh et al., 2013).

2.1.3. Anaerobic Digestion Parameters

There are particular parameters needed to be adjusted and continuously in order to have efficient biogas production in anaerobic digestion process. Microbial growth conditions should be determined and followed to succeed AD process. Control parameters needed to be followed in AD systems can differ depending on added substrate and inoculated seed. Substrate composition and temperature are the main parameters determining the performance and the stability of AD process (Moset et al., 2015). Unstable anaerobic digesters can be detected by following variations in alkalinity, pH, VFAs consumption, biogas and methane production, and CO_2 formation (Gerardi, 2003).

2.1.3.1. Alkalinity, pH, and volatile fatty acids. Most hydrolytic bacteria function in pH ranging between 5-7.2. Methanogens have smaller range compared to hydrolytic bacteria for pH that is between 6.5-7.5 (Azman, 2016). VFAs are intermediate compounds produced after degradation of complex organic molecules. VFAs variation in digester is one of the important indicators showing

the stability of digestion process. VFAs accumulation indicates overload of substrate into digester. pH under 6.2 can have toxic affect on methanogens. However, acidogenic bacteria can adapt the environment having pH around 4.5-5.0. VFAs accumulation can decrease pH and inhibit the methanogenic activity, and then pH decrease can be observed dramatically. Alkalinity should be followed because of its buffering capacity of pH alterations (Sun, 2015).

2.1.3.2. Temperature. Psychrophilic (0-20°C), mesophilic (20-40°C), and thermophilic (40-60°C) temperatures can be applied in AD systems. Temperature is a crucial parameter that can have impact on kinetic and thermodynamic structure of AD. Thermophilic digesters show more decrease in pathogen population and the sludge obtained from these digesters is more stabile (Gavala et al., 2003). Also, microorganisms grow faster and biochemical reactions rates increase under thermophilic conditions. Methanogens are sensitive to temperature alterations; thus, change in temperature should be adjusted to minimal levels (Chen and Neibling, 2014). However, mesophilic digesters are more preferred because they have high process stabilization and less energy consumption and also methanogen diversity is higher under mesophilic conditions compared to thermophilic conditions.

2.1.3.3. The organic loading rate. The amount of daily added organic material per m^3 of digester processing volume is called the organic loading rate (OLR). It is usually measured according to Total Volatile Solids (TVS), Total Solids (TS), and Chemical Oxygen Demand (COD). It is a significant parameter that should be reported for specifically semi-continuous and continuous reactors. AD process can be affected by the organic loading rate because stability and productivity of the system is fragile. High OLR, which can enrich the growth of different microorganisms, decrease the requirements for capacity of digester and energy supply for heating; nevertheless, high OLR can have impact on accumulation of VFAs and ethanol, unequal distribution of mixing and heat in digesters (Liu et al., 2017).

2.1.3.4. The solids retention time. It is a parameter to observe digester performance and TVS consumption. TVS is used as indicator representing organic content of digester. TVS consumption relies on temperature and solids retention time. Stability of AD system also depends on microbial activities and solids retention time can determine the dominant microorganisms in digesters. Solids retention time refers the residence time of dissolved organic compounds in the digesters (De la Rubia, 2006). It can be regulated according to loading rate. Methanogens have less growth rate and hydrolysing ratio under low temperatures; thus, solids retention time should be kept longer

compared to higher temperatures (De Mes et al., 2003). Degradation of proteins, carbohydrates, and lipids are accomplished in 10 days at 35°C in AD process (Abdelgadir, 2014).

2.1.3.5. Toxicity. There are specific toxic elements that should be avoided in AD. Free oxygen elements should not exist in the anaerobic digesters. There are also compounds containing oxygens that can have negative impact on digestion process such as NO_3^-, H_2O_2, and SO_4^{-2}. Substrate consisting of excessive amount of sulphate can induce growth of sulphate-reducing bacteria and that can cause the production of H_2S, which is a chemical responsible from odor problem also. High concentrations of H_2S can have inhibition effect on AD process. Ammonium is another compound being toxic in high concentrations for AD systems and it has been crucial problem needed to be solved for especially substrates containing high amount of ammonia such as manure.

2.2. Biogas Production by Anaerobic Digestion Systems

Biomass arises an alternative resource for energy production as renewable energy in recent years (McKendry, 2002). Anaerobic digestion process produces biogas counting methane and carbon dioxide by diverse microorganisms under anaerobic conditions (Frigon and Guiot, 2010).

There are several types of biomass that can be applied in AD process in order to produce biogas. Substrates utilized in anaerobic digesters include crop residue, macroalgae, microalgae, manure, and so on. There are numerous applications of biogas generated by AD process: electricity, cooling process, heating, etc. After generating biogas, digestate, which is very qualified fertilizer, can be incorporated with soil (Weiland, 2010). The main reason searching for alternative feedstock is to reach high methane yield (Prochnow et al., 2009).

2.2.1. Feedstocks

Most of the biomass utilized in biogas production is categorized as agriculture, waste, and forests. They can also be classified as non-woody and woody biomass. Classification of biomass is done based on their organic content such as carbohydrate, protein, lignin, cellulose, and lipids (Braun, 2007). On the other hand, there is also another classification as terrestrial and aquatic biomass in order to distinguish them in broad range.

There are several substrates utilized in AD systems; for instance, animal waste (manure), crop residue and waste, municipal solid waste, forestry wastes, industrial wastes, sewage, and so on. Co-

digestion of some of these substrates is another approach to increase biogas yield of anaerobic digesters (Weiland, 2010).

One of the substrates used in AD systems is maize (*Zea mays*) that grows extensively in global range. Maize was farmed on a field of 162 million hectare in 2010 (FAOSTAT, 2012). Maize has high amount of starch that is an important precursor for glucose. In AD systems, corn stover, maize grains, corn-cob mix, and grains silage are used because they have less fibre and high starch amount that cause easily accessible compounds for biodegradation process but they have also high-cost; therefore, it is generally used as animal nourishment and left-overs are mixed with other substrates for AD systems.

Another substrate utilized in AD process is macroalgae. Algae have been recommended as fuel supply for a long time even during world wars (McHugh, 2003). It can be non-food biomass that will not affect the food supply if it is used in AD system compared to other feedstocks used as food supply also. Production of greenhouse gases by algae is fewer amounts; moreover it is more sustainable than other substrates. Species called *Ulva* is generally chosen as biomass to be used also in food industry thanks to its crucial amount of trace elements, vitamins, and fibers (Nielsen et al., 2012). It is highlighted that 20 times more biomass can be produced by macroalgae compared to cereal crops (Bruhn et al., 2011).

2.2.2. Process Technologies

There are lots of configurations and digester types in AD systems. These configurations are determined according to total solids in biomass, number of steps and phases in digestion process, process temperature, substrate loading, organic loading rate, and retention time (Korres et al., 2013). Digester types can be generally categorized as: (1) Dry and wet digestion process; (2) batch, semi-continuous, and continuous digestion process; (3) single-phase and multi-phase digestion process. Anaerobic digesters can be divided into two sub-categories; first one is called as wet digestion that has TS value less than 15%; and the second one is called as dry digestion that has TS value higher than 15%.

AD process can be conducted as single-phase, two-phases, or three-phases. Single-phase process contain only one digester tank processing digestion. In two-phases process, sludge obtained as output of first digestion tank is used as substrate for second digestion tank. Two-phases process

is more desirable compared to single-phase process. Multi-phases digesters provide different physical conditions for the growth of specific microbial communities (Korres et al., 2013).

In batch digestion systems, digesters are fed with substrate in the beginning and later process is left to complete digestion steps. There are significant differences between first phase and second phase in batch digesters. In first phase, acidogenesis rate is faster than methanogenesis step; furthermore, organic acids are converted to biogas in second phase (Nasir et al., 2014).

Carbon to nitrogen ratio (C:N) is an important parameter having impact on AD process. There are numerous studies conducted to find best C:N ratio for AD process. Some studies showed that C:N ratio should be kept between 13:1-20:1 (Zhu, 2007; Kumar et al., 2010). Co-digestion of particular substrates can manage to keep C:N ratio in working conditions for AD. On the other hand, co-digestion can be an efficient method also to prevent the inhibition caused by high sulphur and ammonia concentration and create a stable tampon capacity for digestion process (Zhang et al., 2013).

Biogas and methane yield might be increased by correct selection of substrates for co-digestion process. Substrates for co-digestion and their mixing ratio must be determined carefully because there are some parameters affecting co-digestion process that are pH, substrate ratio, TVS, seed and temperature (Abdelhay et al., 2016). For instance, digesters containing only manure can make process unstable because of its very low C:N ratio, which is around 5:1-8:1 (Zhang et al., 2013). If they are mixed with crop residues, digestion stability can increase thanks to its increased C:N ratio (Møller et al., 2004).

2.3. Enhancement of Biogas Production in Anaerobic Digestion

2.3.1. Pre-treatment Techniques

Lignocellulosic compounds can be barrier for the anaerobic digestion process. This structure is well-designed structure in order to protect the biomass against the degradation enzymes. Lignin is composed by tightly bonded of cellulose and hemicellulose. Therefore, it becomes harder for microorganisms, which exist in AD process, to reach the cellulose and hemicellulose for a successful degradation. Thus, some pre-treatment techniques have been developing in recent years in order to degrade lignin and reach to cellulose. Lignocellulosic biomass can be found in energy crops, manure, forest residues, municipal solid waste, and also algal biomass. There are other

compounds also inhibiting the AD process and those compounds should have been pre-treated before digestion process (Taherzadeh and Karimi 2008; Sims, 2003).

Macroalgae have small quantity of lignin and cellulose compared to other substrates containing large amount of lignin such as energy crops. Yet, it should be still pre-treated in order to increase biomethane potential of macroalgae (Nikolaisen et al., 2011). New desirable techniques should be developed to decrease the limitations of anaerobic digestion of macroalgae (Saqib et al., 2013).

2.3.1.1. Physical pre-treatments. Mechanical treatment of substrates is generally conducted in order to increase the surface area by decreasing the size of the substrates. This process can be given as an example for physical pre-treatment process. It has been shown that certain particle sizes can increase the efficiency of biogas production in AD systems (Sun and Cheng, 2002). There is also another technique for physical pre-treatment, which is extrusion. By applying high temperatures, biomass can be extruded with shear forces and mixing (Chinnadurai et al., 2008).

2.3.1.2. Chemical pre-treatments. pH varying between acidic, neutral, and basic conditions is useful for the application of chemical pre-treatments. In acidic conditions, lignin is hydrolysed to xylose, through hemicellulose. Xylose is one of the monomer of hemicellulose. HNO_3, H_3PO_4, H_2SO_4, HCl, and organic acids such as acetic acid are used to supply acidic conditions in chemical pre-treatment processes (Mosier et al., 2004). Also, Ionic liquid (IL) is generally applied to provide neutral environment (Li et al., 2009; Cruz et al., 2013). Furthermore, solubilizing lignin can be achieved by alkaline treatment, which can also reduce the amount of crystallinity and detach lignin carbohydrate complexes and enlarge inner surface area of the cellulose (Kumar and Wyman, 2009).

2.3.1.3. Physico-chemical pre-treatments. There are two methods extensively applied: CO_2 explosion and steam explosion. It has been shown that two steps steam explosion can improve the hydrolysis of sugars around 13% and fermentation process. Both methodology needs high pressures and that can increase the cost, which is not desirable (Balan et al., 2014).

2.3.1.4. Biological pre-treatments. Lignin can be degraded by extracellular enzymes produced by some specific microorganisms especially fungi. White-rot, brown-rot, and soft-rot fungi can exclusively used in the biological pre-treatment of AD systems containing lignocellulosic substrates. Lignin decaying enzymes such as laccase and peroxidase can be efficiently applied in pre-treatment. Compared to physical and chemical treatments, biological pre-treatment can be more cost-efficient and sustainable. Nevertheless, it is slower process compared to other pre-treatment

techniques. Also, hydrolysing efficiency of fungi is lower than chemical pre-treatment process (Balan et al., 2014).

Fungi that can degrade lignin can be categorized as soft-rot, white-rot, and brown-rot fungi. Lignocellulose is a well-built structure and extracellular enzymes are needed to degrade it. Also, degradation method should be oxidative more than hydrolytic degradation because of its C-C and ether bonds. Irregularity of lignin structure desire less precise enzymes rather than other degradative enzymes (Kirk and Cullen, 1998). Lignin peroxidase, manganese peroxidase, H_2O_2-forming enzymes, versatile peroxidase, and laccase are the enzymes responsible for the decomposition of lignin (Wong, 2009). Several enzymes degrading lignin can be released by white-rot fungi and also there are some other enzymes such as cellulases, hemicellulases, and xylanases released by white-rot fungi. Most of the white-rot fungi can produce manganese peroxidase and laccase but lignin peroxidase can only be produced by certain white-rot fungi (Isroi et al., 2011).

White-rot fungi have being used in pre-treatment of lignocellulosic biomass and there are different effects obtained from studies. Yu and his colleagues (2010) observed that decomposition of lignocellulosic biomass increased over 54.6% for corn straw after 30 days of incubation by the pre-treatment with *T. versicolor*. Zhang and his colleagues (2007) stated that there can be over 20% decrease in lignin content in bamboo culm if the pre-treatment process is applied four weeks by using *T. versicolor* and *Echinodontium taxodii*. Oxidation of lignin by white-rot fungi is not precise; therefore, lignin can be degraded completely by pre-treatmen process (Isroi et al., 2011). Another study conducted by Hom-Diaz and his colleagues (2016) is that methane yield of microalgal biomass increased by 74% and 20% after applying pre-treatment process with *T. versicolor* broth and commercial laccase enzyme, respectively. This study indicates that there might be other released factors, not only laccase enzyme, by *T. versicolor* affecting the increase in biodegradability of microalgal biomass.

2.3.2. Bioaugmentation

Supplementing of specific or mixed microbial community into a desired system is called Bioaugmentation process (Venkiteshwaran et al., 2015). It has being used for long time ago and it seems a promising technique for biomethanization processes (Zhang et al., 2015). There are particular studies showing the effect of Bioaugmentation on degradation of phenol, benzene, 3-chlorobenzoat, and pentachlorophenol in digesters (Venkiteshwaran et al., 2015). This technique is

applied to increase biomethane potential of animal manure, chicken wastes, and oil products (Peng et al., 2013).

2.4. Molecular Methods Applied For Microbial Diversity Analyses in Anaerobic Digesters

Anaerobic digesters contain diverse amounts of microbial communities and these communities have a balanced relationship with each other. They are responsible from the beginning to the end of AD periods. Therefore, it is important to get information about how this balanced relationship works and which microbial communities exist in the anaerobic digesters. There are some specific methods in order to determine microbial communities and find out about microbial ecology of digesters: Real-time polymerase chain reaction (Q-PCR), denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH), metagenomics, and next-generation sequencing (NGS). The applications of molecular methods are summarized in Figure 2.4

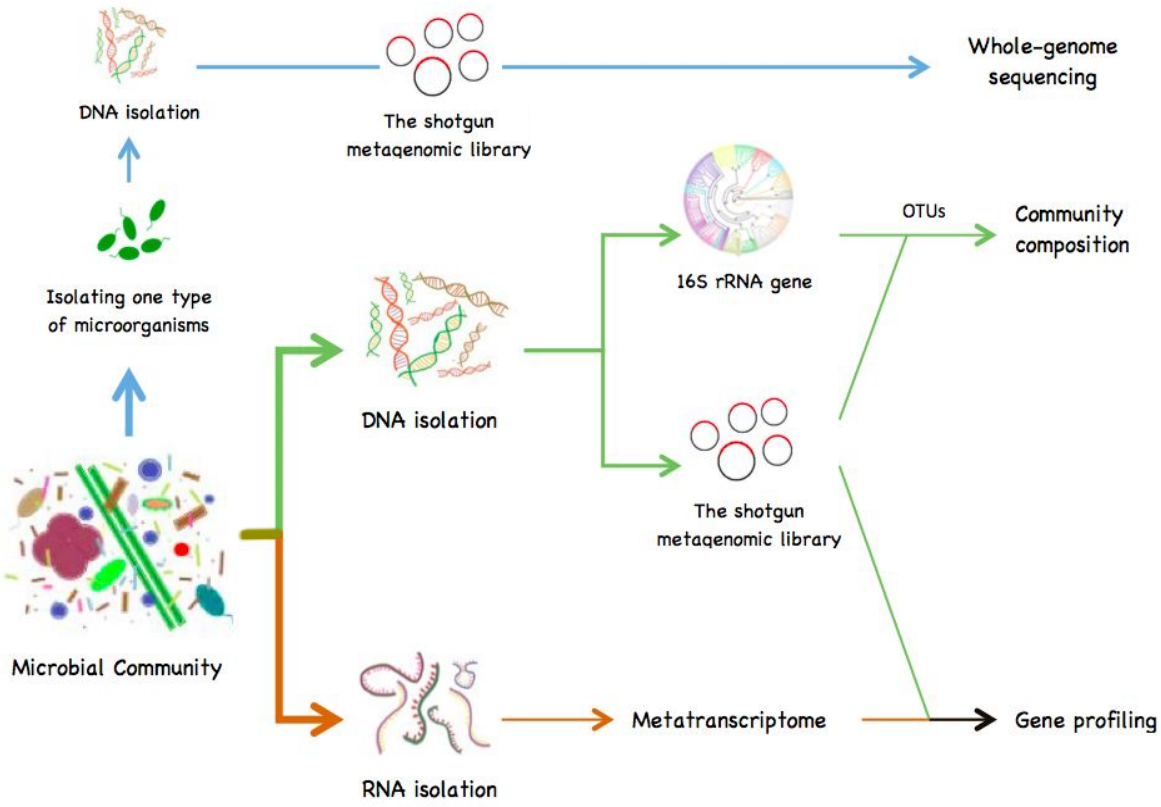

Figure 2.4. The applications of molecular methods (Bozan et al., 2017).

2.4.1. Real-Time Polymerase Chain Reaction (Q-PCR)

Specific parts of DNA can be amplified by using polymerase chain reactions (PCR), which was developed after the purification of thermostable DNA polymerase that can process in very high temperatures without unfolding. PCR technology is applied to multiply a gene or a DNA fragment to millions of copies by using a DNA template, designed primers (designed small fragment of DNA or RNA), DNA polymerase (resistant to high temperatures), dNTP (DNA nucleotide bases), and buffer solutions. There are three steps of PCR: Denaturing, annealing, and extending. In denaturing step, DNA and primers are heated up to very high temperatures to break down the hydrogen bonds and denature the DNA. In annealing step, attachment of primers to DNA template occurs. In extending step, primers start to add dNTP to elaborate the target section of DNA. These three steps go over several cycles to obtain lots of copies of target DNA fragment. Real-time PCR is a promoted technique of PCR that can show the amplifying step in real-time by fluorescence dying. It is also called quantitative PCR (Q-PCR) because it gives an idea for gene expressions. Q-PCR is used to amplify 16S RNA gene or some functional genes to count the microorganisms related metabolically anaerobic digestion process digesters (Malinen et al., 2003)

2.4.2. Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is generally applied popular technique to get some information about microbial communities existing in anaerobic digesters. The methodology is based on observing the differences in short or medium length DNA sequences according their melting points. It can be applied to verify phylogenetic characteristic of microbial populations; furthermore, it is used in microbial ecology, food industry, and clinical diagnostics (Madigan et al., 2008).

2.4.3. Fluorescence in situ Hybridization (FISH)

Fluorescence in situ hybridization (FISH) is a technique applied to illustrate the microbial communities related to anaerobic digestion process. Fluorescent staining is applied to show microorganisms under fluorescent microscopy. Specific probes targeting bacterial and archaeal ribosomal RNA are operated for staining. However, every bacteria and archaea might not be observed under fluorescence microscopy. Several kinds of microorganisms can be visualized same time by binding them with different probes targeting each microbial group (Speicher et al., 1996).

2.4.4. Metagenomics

Metagenomics is based on analysis of recovered genomes from samples, which is taken directly from environment. This methodology is also called with different names such as microbial ecogenomics or environmental genomics. It can be applied directly to analyse samples containing environmental genomes without need of cultivation of microorganisms. It is very beneficial method thanks to its analysis capacity of complete genome compared to analysis obtained by PCR, which gives only information about multiplied specific DNA fragments (Schloss and Handelsman, 2013).

2.4.5. Next-Generation Sequencing (NGS)

Next-generation sequencing (NGS) has been a popular method recently to analyse transcription factors, genetic variations, whole-genome, and microbial communities. It is very applicable methodology to determine exact microbial communities in lab-scale or big-scale anaerobic digesters (Azman, 2016). There are researches aiming to obtain data about microbial communities, whole-genome of species, and enzyme diversities in samples. It is developed year by year in order to decrease the cost and increase the efficiency and speed of sequencing. There are different approaches for sequencing in next-generation sequencing, which are Illumina sequencing, pyrosequencing, single molecule real time, and Ion Torrent (Bozan et al., 2017). The method gives reliable quantitative information about species existing in digesters. In Table 2.2, NGS instruments are compared based on not only their strategy for library construction and sequencing principle but also their quality of run and their run time.

Table 2.2. Comparison of NGS instruments (Metzker, 2010).

Instrument	Foundation year	Library construction	Sequencing principle	Bases per read	Run time	Quality
454 Pyrosequencing	2005	emPCR on microbeads	Pyrosequencing	800 bp	23 h	Q20-Q30
Illumina Miseq	2007	Bridge-PCR on flow cell surface	Reversible terminator sequencing by synthesis	2x150 bp	27 h	>Q30
Illumina Hiseq	2007	Bridge-PCR on flow cell surface	Reversible terminator sequencing by synthesis	2x150 bp	11 days	>Q30
Ion torrent	2010	emPCR on microbeads	Semiconductor-based sequencing by synthesis	200 bp	4.5 h	>Q20
Pasific Biosciences	2011	None	Single-molecule, real-time DNA sequencing by synthesis	800 bp	2 h	>Q50 consensus >Q10 single

There are several applications of NGS in anaerobic digestion systems. For instance, Akyol and his colleagues (2016) conducted a research to determine microbial communities inside of seed sludge. Moreover, Öner and her colleagues (2018) studied the differences in microbial communities between control digesters and bioaugmented digesters. Some studies applied NGS are shown in Table 2.3.

Table 2.3. NGS techniques applied for microbial community diversity in anaerobic digesters (Bozan et al., 2017).

Lignocellulosic substrate	Temperature	NGS Technique	Dominant Bacteria	Dominant Archaea
Cow manure and food waste	35 °C	Pyrosequencing	Unclassified *Bacteroidetes*	*Methanosarcina*
Cow manure and food waste	50 °C	Pyrosequencing	*Clostridia*	*Methanosarcina*
Manure	37 °C	Pryrosequencing	*Bacilli*	*Methanosarcina*
maize silage (63%), green rye (35%) and low amounts of chicken manure (appr. 2%)	41 °C	Pyrosequencing	*Clostridium thermocellum ATCC 27405*	*Methanoculleus marisnigri* JR1
Seed sludge	37 °C	Pyrosequencing	*Clostridia*	-
Maize silage and pig manure	37 °C	Solid NGS	*Clostridium thermocellum*	*Methanoculleus marisnigri*
Scenedesmus obliquus and maize silage	37 °C	Ion Torrent	*Clostridia*	*Methanosarcina*
Wastewater treatment plant sludge fed with microcrystalline cellulose and glucose	55 °C	Illumina	*Clostridium*	*Methanothermobacter*

2.5. Ca-alginate Beads and Their Applications

Ca-alginate structure is an extensively used material in order to immobilize cells. Alginates contain combined 1,4-glycosidic bonds of D-mannuronic and α-L-guluronic acid. It is widely used biopolymer for the entrapment of cells. Structure of alginate is illustrated in Figure 2.5. It can form solid polymer by the addition of polyvalent metal cations. When the alginate solution is simply dropped into $CaCl_2$ solution, bead formation occurs by crosslinking of Ca with carboxyl groups and this formed bead does not dissolve by itself if it is not treated with special solutions. (Klein et al., 1983).

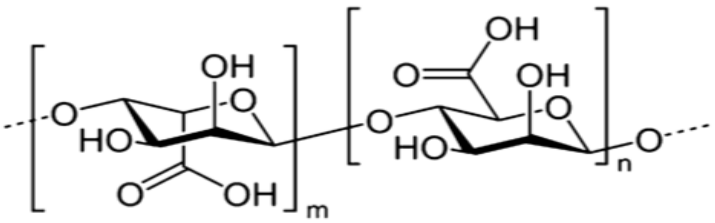

Figure 2.5. Structure unit of alginate biopolymer (Klein et al. 1983).

Cell can be immobilized by using Ca-alginate bead formation. After immobilization, secretion of some compounds and absorption of nutrients, which are enough parameters to keep cells alive, can be still carried out by cell. Thus, there are lots of applications of cells entrapped in Ca-alginate beads. It was determined that immobilized cells in matrix can grow denser quantities compared to cells growing as free cells in media. There are two approaches for entrapment of cells; one of them is to keep cells alive inside the beads for the process, and the other one is to entrap the non-alive cells (Nussinovitch, 2010).

Different types of cells, which can be fungi, animal cells, bacteria, yeast, and so on, can successfully be entrapped into Ca-alginate beads without loosing their biocatalytic activities (Nussinovitch, 2010). There are several studies carried out by using cell-entrapped Ca-alginate beads as biocatalyst.

This technique has being used as decolorizing material in effluents having colour problems. *Trametes versicolor*, which is a white-rot fungi releasing several types of oxidizing enzymes, is generally entrapped into Ca-alginate beads to overcome colour problem. For instance, Pallerla and Chambers (1996) studied removal of colour and adsorbable organic halides in lab-scale bioreactor by applying *T. versicolor* entrapped in Ca-alginate beads. Moreover, Dominguez and his colleagues (2007) removed also chemical called anthracene, which is a kind of polycyclic aromatic hydrocarbons (PAH), by again entrapped *T. versicolor* in Ca-alginate beads. Furthermore, there is also a study conducted based on co-immobilization of *Phanerochaete chrysosporium* with enzyme mixed obtained from *T. versicolor* into Ca-alginate beads in order to increase the degradation capacity of beads for dye removal (Li et al., 2015). There are also studies conducted with entrapped beads, containing alive and dead fungi cells, for the removal of metal contaminants such cadmium (Arica et al., 2001).

3. MATERIALS AND METHODS

3.1. Sampling and Characterization

Anaerobic seed sludge containing microbial communities such as methanogens and bacteria was obtained from big-scale anaerobic digesters operating at mesophilic temperature (41°C) at Sütaş located in Bursa, Turkey. *Ulva* sp., which is a type of macroalgae species, used in this study was collected from Marmara Sea, a region close to Sultankoy, Tekirdag, Turkey. Corn stover was harvested from a local farmland at Tekirdag, Turkey. Corn stover was dried under sun for 5 days before usage. Collected substrates were shredded into small pieces and they were stored at +4°C. After gathering all samples, they were characterized based on TS, TVS, TKN, pH, alkalinity, COD and C:N ratio according to Standard Methods (APHA, 2005).

Trametes versicolor ATTC 42530, which is a type of white-rot fungi species, was purchased from American Type Culture Collection (ATTC) and it was stored at -80°C.

3.2. Cultivation of *Trametes versicolor*

Trametes versicolor ATTC 42530 was grown and sub-cultured in potato dextrose agar (PDA) (Bioshop, Canada Inc.) every 10 days at 25°C during this study. For the further growth of fungi, media containing specifically soluble starch and $CuSO_4$ was prepared in order to induce laccase enzyme activity (Kocyigit et al., 2012; Revankar and Lele, 2006). Ingredients for soluble starch yeast extract (SSYE) medium are shown in Table 3.1. First, soluble starch was dissolved in distilled water by microwave by mixing every 2 minutes until it dissolved completely. Then, other ingredients shown in Table 3.1 were added and pH was adjusted to 5.0. In the end, mixture was filled with distilled water up to 1 L. Sterilization of media was managed by autoclaving at 121°C for 15 min.

Table 3.1. SSYE medium recipe.

Chemical	Concentration (g/L)
Soluble starch	20
Yeast extract	2.5
$CuSO_4.5H_2O$	0.004
Na_2HPO_4	0.05
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.50
$CaCl_2$	0.01
$ZnSO_4.7H_2O$	0.01
$FeSO_4.7H_2O$	0.01

Three agar discs sized 1 cm in diameter were taken from colonies grown in PDA in order to inoculate the 200 ml SSYE medium. Inoculated media was left for incubation for 7 days at 25°C at 150 rpm. Inoculation of cells was conducted under sterile conditions by using Bunsen burner. Inoculation of cells to SSYE medium is illustrated in Figure 3.1. Laccase enzyme activity was followed during the incubation.

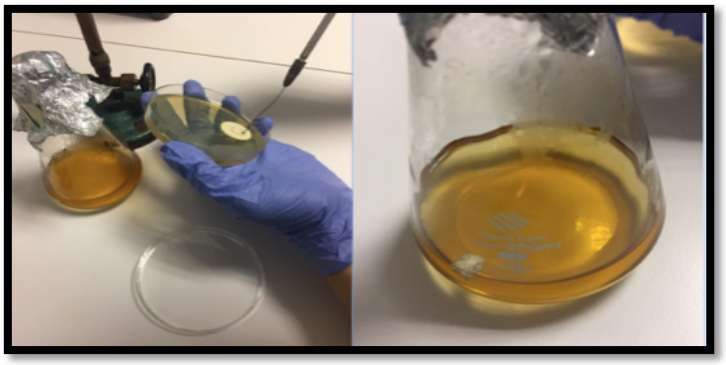

Figure 3.1. Inoculation of cell to SSYE medium.

3.3. Ca-Alginate Bead preparation and Entrapment of Cells

T. versicolor grown in soluble starch yeast extract medium was centrifuged at 10,000 rpm for 15 min. Biomass obtained after centrifugation was washed by distilled water and centrifuged again at similar conditions. Washing process was applied two times. After biomass was homogenized by utilizing a commercial blender for 5-10 sec under sterile conditions, they were sterilized by autoclaving at 121°C for 15 min.

20 ml homogenized-cells were mixed with autoclaved Na-alginate solution that was prepared by adding 4 g Na-alginate into 80 ml dH_2O. 4% Na-alginate was aimed to obtain in the end. Mixture of Na-alginate and cells was later dropped into 2% $CaCl_2$ solution by Pasteur pipette in order to obtain bead formation. Prepared beads were left in 2% $CaCl_2$ for 1 hour. Cells entrapped in Ca-alginate beads, which were later used for pre-treatment, were obtained by this methodology. Similar method was applied to control samples containing Ca-alginate beads, but without cells. In the preparation of empty Ca-alginate beads, distilled water was used instead of cells. Methodology for the Ca-alginate bead preparation was conducted under sterile conditions by using laminar flow hood. Bead formation is illustrated by drawing in Figure 3.2.

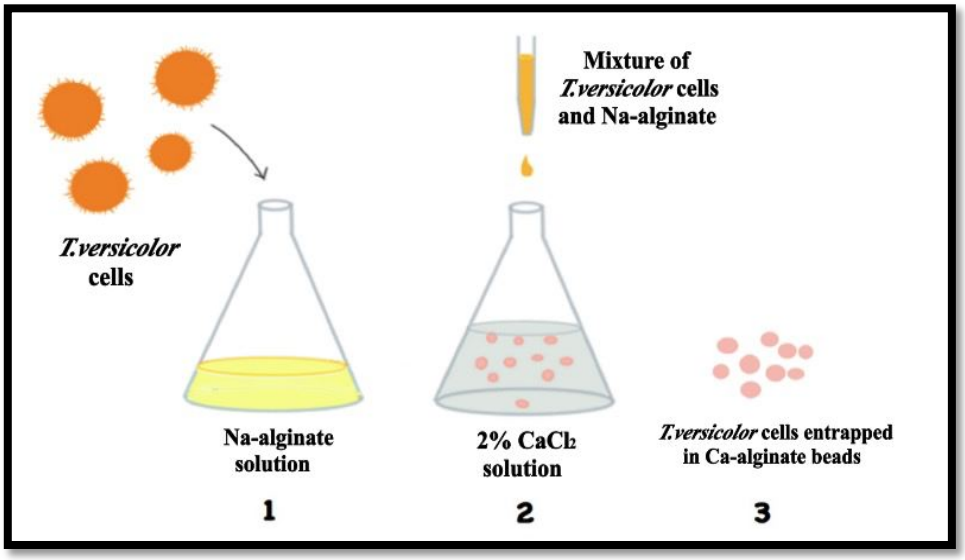

Figure 3.2. The bead formation.

3.4. Aerobic Pre-treatment of Substrates

Prior to pre-treatment, cells entrapped in Ca-alginate beads were grown in SSYE media at 25°C at 110 rpm for 2 days.

Amounts of *Ulva* sp. and corn stover needed for pre-treatment were calculated according to their amount in anaerobic batch reactors. Amounts required for each reactor in batch digestion are indicated in Table 3.2. Amount of substrates required in the pre-treatment process were measured according to required amount for replicates (6 replica in batch digestion) and required amount for analyses (80 ml). Calculated amounts for pre-treatment are shown in Table 3.3.

Table 3.2. Substrates and seed sludge amount required for each batch reactor types.

Status	Digester	*Ulva* sp. (g)	Corn stover (g)	Anerobic sludge (ml)
Without pre-treatment	Macroalgae digester	15,2	-	24
	Corn stover digester	-	1,82	24
	Co-digestion	7,6	0,91	24
With pre-treatment	Macroalgae digester	15,2	-	24
	Corn stover digester	-	1,82	24
	Co-digestion	7,6	0,91	24
Control	Seed sludge digester	-	-	24

Table 3.3. The total amounts of each substrate required for the aerobic pre-treatment.

Sample	*Ulva* sp (g)	Corn stover (g)	Tap water (ml)
Pre-MA	121,2	-	up to 320
Pre-CS	-	14,56	up to 320
Pre-Co	60,6	7,28	up to 320
Con-MA	121,2	-	up to 320
Con-CS	-	14,56	up to 320
Con-Co	60,6	7,28	up to 320

1 L Erlenmeyer flask was utilized for the pre-treatment process. 6 flasks labeled as Pre-MA (Pre-treatment of Macroalgae), Pre-CS (Pre-treatment of Corn Stover), Pre-Co (Pre-treatment of Both Substrates), Con-MA (Control for Macroalgae), Con-CS (Control for Corn Stover) and Con-Co (Control for Both Substrates) were filled with chopped substrates according to Table 3.3. Flasks filled with substrates were sterilized by autoclaving at 121°C for 15 min. Later, 20 ml of cells entrapped in Ca-alginate beads were added into Pre-MA, Pre-CS and Pre-Co. Similarly, 20 ml of empty Ca-alginate beads were added into Con-MA, Con-CS and Con-Co. All Erlenmeyer flasks were left for incubation at 25°C at 110 rpm for 7 days. Laccase enzyme activity was followed in Pre- samples during pre-treatment.

3.5. Set-up of Anaerobic Reactors

Based on study conducted by Akyol et al. (2016), inoculum to substrate ratio (I:S) and substrate to substrate ratio (S:S) were selected as 1:1 (g TVS). 120 ml serum bottles were utilized as batch reactors and active volume was determined as 80 ml. 6 replicates were applied for each kind of anaerobic digester. Each digester was containing 1.5 g TVS seed sludge and 1.5 g TVS substrate.

Each digester type was constructed according to Table 3.2 Based on this knowledge, Erlenmeyer flasks containing substrates shown in Table 3.3 were filled with right amount of seed sludge in the end of pre-treatment. Pre-MA and Con-MA were applied for macroalgae digestions only; Pre-CS and Con-CS were used for corn stover digestions only; Pre-Co and Con-Co were used for co-digestions. Pre- suffix was used for pre-treated substrates and Con- suffix was used to indicate control group.

80 ml sample was taken from each flask for analyses before conducting anaerobic digestion. Later, flasks containing mixture of substrates and seed sludge were divided into 6 serum bottles by adjusting 80 ml volume for each digester. pH of each reactor was adjusted to 7.0 ± 0.2. Each serum bottle was sealed tightly with metal crimps and rubber stoppers, then fluxed with N_2 for 1 min to provide anaerobic condition for microorganisms. They were left for incubation at 37± 1.0°C for 50 days and mixed manually by shaking three times a day to provide substrate accessibility for microorganisms. Anaerobic digester types and digester appearance are highlighted in Figure 3.3. Each serum bottle was opened for further analyses by 10 days intervals.

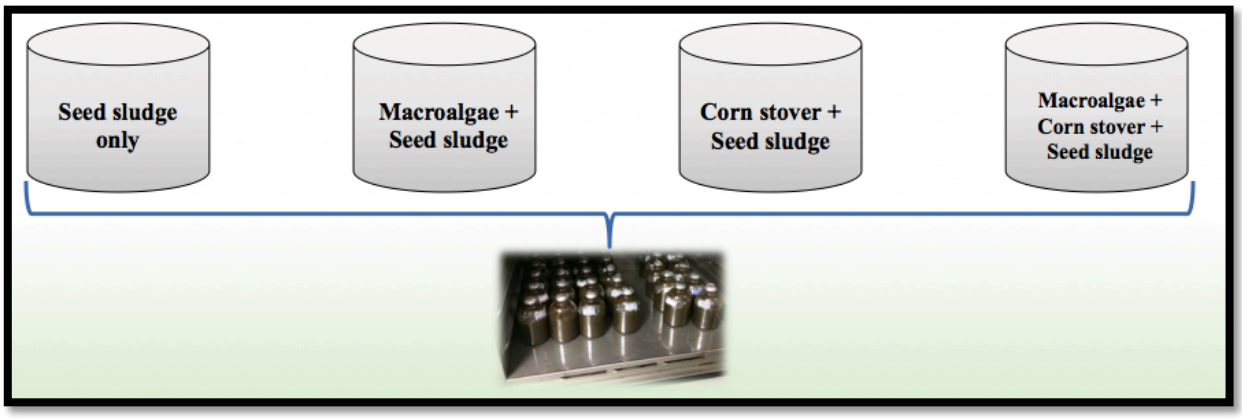

Figure 3.3. The types of batch digesters and illustration of digesters.

3.6. Analytical Measurements

Total solids (TS), total volatile solids (TVS), Total Kjeldahl Nitrogen (TKN), alkalinity, metal determination and soluble chemical oxygen demand (sCOD) analyses were conducted based on methods indicated in Standard Methods (APHA, 2005).

pH of the samples were determined by using pH-meter (pH 7110, Inolab) and alkalinity was measured by titration method with 0.1 N H_2SO_4. Amount of consumed acid was used for alkalinity calculation.

TKN is a method to analyse total ammonia nitrogen and organic nitrogen. Three steps are needed for conducting TKN experiment: digestion, distillation and titration. In this experiment, samples were dried and weighed approximately 0,1 g for digestion step. TKN analyses were conducted based on Standard Methods (APHA, 2005) and they were measured by spectrophotometer (HACH, DR/2010) at 460 nm by program coded as 399 (Hach et al., 1987).

Samples taken from digesters were centrifuged at 14,000 rpm at 4°C for 30 min. 0.45 µm pore size filters were used to filtrate the supernatant obtained after centrifugation. Filtrate was used to determine sCOD of samples. sCOD of substrates were conducted by utilizing prepared eluent solution based on TS calculation. The same filtration procedure was applied also to substrates. For sCOD analyses, digestion solution was prepared by using ingredients as shown in Table 3.4. The mixture was filled with deionized water up to 1 L. Sulphuric acid reagent was prepared by adding 10.12 g Ag_2SO_4 into 750 ml concentrated sulphuric acid in volumetric flask. Solution was left in the dark 1-2 days until it dissolved. It was completed up to 1000 ml with concentrated sulphuric acid after Ag_2SO_4 dissolved completely.

Table 3.4. Digestion solution preparation.

Chemicals	Concentration
$K_2Cr_2O_7$ (dried at 150°C, 2 h)	10.216 g/L
Concentrated H_2SO_4	167 ml/L
$HgSO_4$	33.3 g/L

2.5 ml diluted samples were taken and digested by 1,5 ml digestion solution and 3,5 ml concentrated sulphuric acid solution at thermoreactor (HACH, COD Reactor) at 150°C for 2 hours. After digested samples cooled down, absorbance of digested samples were measured by spectrophotometer (HACH, DR/2010) at 600 nm. Potassium hydrogen phthalate (KHP) was used to draw standard curve for the determination of sCOD.

The filtrate obtained for sCOD analyses was refiltered through 0.22 µm pore size filters for volatile fatty acid (VFA) analyses. 10 N phosphoric acid was added to final filtrates in the amount of 10% of total volume of final filtrates. By applying gas chromatography-mass spectrometry (GC-MS) (Perichrom, France and Agilent Technologies 6890N, USA), diversity and amounts of VFAs were determined.

Gas production was measured manually by a manometer (PM-9107 7000 mbar, Lutron Electronic Enterprise Co., LTD, Taiwan) every day and produced gas was released after

measurement. Gas composition was analysed by the utilization of gas chromatography (GC) (HP Agilent 6850) with a thermal conductivity detector (HP Plot Q column 30 m × 0.53 mm) for determining methane production. Helium was the carrier gas (2 ml/min) connected to instrument and 0.5 ml gas sample was injected by the help of syringe (2.5 ml).

C:N ratios of substrates and seed sludge were determined by elemental analysis instrument (ECS 4010 CHNSO Analyzer, COSTECH Analytical Technologies, INC., USA).

Macroalgae, corn stover and seed sludge were dried at 105°C for 24 hours and, later they were kept in desiccator until the metal analysis. Microwave Assisted Digestion Method was applied. Dried samples were grounded and digestion of samples was conducted by adding 8 ml HNO_3 and 2 ml H_2SO_4. Digestion was proceeded by utilizing the instrument called MARS 6 Microwave Accelerated Reaction System (CEM Corporation, USA). ICP-OES was applied for the determination of metals in samples.

Samples taken from pre-treatment process were centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was taken to measure laccase activity by spectrophotometric method (Kocyigit et al., 2012). 200 µl 5 mmol ABTS and 600 µl glycine-HCl (pH: 3.0) were added to cuvette, respectively. Cuvette was placed into spectrophotometer (UV160U UV/Vis, Shimadzu) and 400 µl supernatant of sample was added to cuvette. After addition of sample, immediate measurement was taken and alteration of absorption was followed during 5 min at 420 nm. The idea behind of this methodology is measuring oxidized ABTS amount at 420 nm (ε_{420} = 36 $mmol^{-1}$ cm^{-1}). Units/Liter (U/L) was used as unit to express the enzyme activity, which indicates the amount of enzyme that oxidized 1 µmol of ABTS per one minute under reaction conditions. Formula shown in Equation (3.1) was used to calculate the activity of enzyme.

$$Enzyme\ activity = \frac{\Delta E \times V_t}{\varepsilon \times d \times V_s} \times 10^3 \times Dilution\ factor \quad (3.1)$$

Equation (3.1) gives laccase enzyme activity. ΔE indicates the mean value of absorbance values measured in different minutes, ε shows extinction coefficient of ABTS, V_t shows volume of reaction, d indicates the distance of light passing through the sample, and V_s points to volume of sample containing enzyme. Multiplying with 10^3 was carried out to obtain unit as U/L.

DNS Assay is a method to show the reducing sugar concentration of a sample. Reducing sugar concentration of pre-treatment samples was measured with dinitrosalicylic colorimetric method (Miller, 1959). Concentration of reducing sugar determines the variation amount of reagent colour. DNS reagent was prepared according to Table 3.5.

Table 3.5. DNS reagent preparation.

Chemicals	Concentration
3,5-dinitrosalicylic acid [DNS]	10 g/L
Sodium potassium tartarate tetrahydrate ($KNaC_4H_4O_6 \cdot 4H_2O$)	300 g/L
2 N NaOH	200 ml/L

Standard glucose solution (1mg/ml) was utilized to draw standard curve. 0.2, 0.4, 0.6, 0.8 and 1 ml of standard glucose sample. 0.1 ml unknown sample were pipetted into test tube and dH_2O was pipetted to fill the all solutions to obtain 1 ml total volume. One tube was filled with only dH_2O. Two ml DNS reagent was added to mixtures and solutions were completed to 10 ml by dH_2O, and then incubated in boiling water for 5 min. After tubes cooled down, reducing sugar concentration of samples was measured by spectrophotometer (HACH, DR/2010) at 540 nm.

3.7. Molecular Techniques

3.7.1. DNA extraction

Genomic DNA from Soil Extraction Kit (Machenery-Nagel, Germany) was applied to isolate DNA of 20th day of digestion samples by considering manufacturer's procedure. 500 ml sample was taken from digesters and centrifuged at 12,000 rpm for 10 min. Supernatant was removed and 700 µl Lysis Buffer SL1 was used to homogenize pellet. Homogenized solution was pipetted into NucleoSpin® Bead Tube Type A containing ceramic beads. Later, 150 µl Enhancer SX was pipetted into solution and ribolyzer (Fast-prep FP120 BIO101/Savant, Thermo Electron Corporation) was utilized to destroy the cells for 30 sec at 5 m/s. Samples were centrifuged at 12,000 rpm for 2 min in order to reduce foam. After centrifugation, 150 µl Lysis Buffer SL3 was added to complete lysis steps. Samples were left incubation for 5 min at 0-4ºC, and then centrifuged for 1 min at 12,000 rpm. 700 µl supernatant was transferred into NucleoSpin® Inhibitor Removal Column, which was inserted in collection tube. Rest of the procedure was applied exactly as manufacturer's protocol. After completing DNA extraction, quality of isolated DNA was

determined by NanoPhotometer® by checking absorbance values of samples at 260 nm, 280 nm, and 230 nm.

3.7.2. Next Generation Sequencing

For next-generation sequencing analysis, universal 16S bacterial primers, which were 515F and 806R, were used for bacterial community analysis; moreover, archea349F and archaea806R primers were utilized to determine archaeal community analysis. PCR HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was applied as manufacturer's protocol for each sample (94°C-3 min / 28 x 94°C-30 s / 53°C-40 s / 72°C-1min). Primers used in this experiment were highlighted in Table 3.6.

Table 3.6. Primers used in NGS analysis.

Primers	Sequences
515F	GTGCCAGCMGCCGCGGTAA
806R	GGACTACVSGGGTATCTAAT
archea349F	GYGCASCAGKCGMGAAW
archaea806R	GGACTACVSGGGTATCTAAT

After PCR, all samples were diluted to obtain the same amplicon concentration and Agencourt Ampure Beads Kit (Agencourt Bioscience Corporation, MA, USA) was used for purification of amplicons.

Samples were sequenced in Ion PGM™ platform by using manufacturer's chemicals and following manufacturer's protocol. During analysing of results, short sequencing data (< 200bp) and sequencing data that does not contain barcodes were removed from the results. The obtained sequencing data was compared with the existing operational taxonomic units (OTUs) and samples containing over 97% similarities were listed. OTU groups were classified taxonomically based on M5RN database by using BLASTn (DeSantis et al., 2006). QIIME (Quantitative Insights Into Microbial Ecology) was used to determine meaningful taxonomic differences.

Also, bioinformatics analysis of obtained results was applied to find alfa-diversity indexes (Shannon index, Simpson index, Invsimpson index, Chao index, Pielou's evenness) and beta diversities comparing digesters to each other.

3.8. Statistical Analysis

R 3.1.1 analyses were utilized to carry out statistical analyses. Data normality was determined by applying q-q plots, histograms and Shapiro-Wilk's test. The Levene's test was used to find variance homogeneity. Values obtained from experiments were indicated with their mean values and standard deviations. The Tukey's test was examined to give possibility for multiple comparing. Pearson's test was applied to see applicability of microbial diversity and inoculum ratios. Level importance was selected as $p<0.05$.

4. RESULTS AND DISCUSSION

In this study, a new approach for pre-treatment process was applied by exposing aerobic fungi, which was *T. versicolor*, entrapped beads to lignocellulosic biomass in order to increase biomethane potential of anaerobic digesters. After entrapment of fungi into Ca-alginate beads, beads were exposed to macroalgae and corn stover substrates in order to increase the accessibility of cellulose by degrading lignin with laccase enzyme produced by fungi. Later, anaerobic batch reactors were constructed with pre-treated substrates to see the effect of pre-treatment process. Biogas and methane production were followed with sCOD and VFA consumption to evaluate process efficiency. Microbial community dynamics were determined and compared with each other by using NGS method. In the end, bioinformatics analyses were carried out to realize and compare the microbial diversity within digesters and between digesters.

4.1. Characterization Results

All the samples used in this study, they were characterized based on the methodology explained in Standard Methods (APHA, 2005). Results obtained from characterization are highlighted in Table 4.1. TS and TVS ratios were used to calculate the amount of substrates needed in anaerobic digesters. There were no obstacles gathered from the characterization results to construct anaerobic batch digesters. Elemental S analysis showed 3% S content in *Ulva* sp.

Table 4.1. The characterization results for *Ulva* sp., corn stover and seed sludge.

Samples	TS (% w/w)	TVS (% w/w)	TVS/TS (% w/w)	C:N	TKN (ppm)	pH	Alkalinity (mg $CaCO_3$/L)
Ulva sp.	15.5	9.9	64	16:1	2 937 ± 159	6.28 ± 0.2	800 ± 42
Corn stover	92.3	82.4	89	55:1	1 858 ± 201	5.38 ± 0.2	400 ± 30
Seed Sludge	9.5	6.2	65	11:1	2 811 ± 208	8.35 ± 0.2	18 600± 130

Metal analyses indicating 12 metals were conducted also according to Standard Methods (APHA, 2005) by using ICP-OES. Results gathered from metal analyses are indicated in Table 4.2. Metal levels of samples were not high enough to inhibit the AD process.

Table 4.2. Metal analysis of corn stover and *Ulva* sp. by ICP-OES (LD: less than detection limit).

Metal	Corn stover (mg/kg)	*Ulva* sp. (mg/kg)
Cr	2	2
Ni	1	4
Zn	268	151
Pb	1	LD
Mn	14	17
Fe	47	312
Al	28	376
Co	LD	LD
Si	148	186
Mo	LD	LD
Cu	3	6
Cd	0,3	0,3

4.2. Fungal Growth and Bead Formation

In the beginning, *T. versicolor* was inoculated in PDA media and incubated for 7 days at 25°C. After 7 days of incubation, grown *T. versicolor* cells had white colour covered the surface of PDA media. The grown *T. versicolor* cells were illustrated in Figure 4.1.

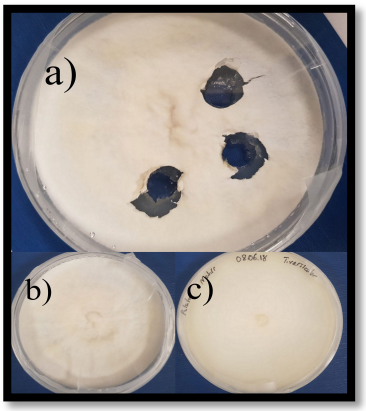

Figure 4.1. Grown *T. versicolor* in potato dextrose agar (PDA). a) Appearance of plate after agar discs extraction, b) bottom face of plate after growth, c) top face of plate after growth.

Grown *T. versicolor* cells on PDA media was extracted by agar discs method in order to inoculate SSYE media. The appearance of PDA media after extraction is shown in Figure 4.1. After incubation at 120 rpm at 25°C in SSYE media, cells formed white spherical colonies that can be seen by bare eyes as shown in Figure 4.2.

Figure 4.2. Grown *T. versicolor* in SSYE medium.

Grown cells in SSYE media were used in bead formation. Their spherical formation was an obstacle for the bead formation procedure. Therefore, a commercial blender was applied to blend them for 5-10 sec under sterile conditions. 20 ml cells were taken from blender for the entrapment of cells into Ca-alginate beads. After dropping mixture of Na-alginate and cell suspension into 2% $CaCl_2$ solution, bead formation was observed. Ca-alginate beads entrapped with *T. versicolor* cells had a white spherical appearance sized around 2-3 mm as shown in Figure 4.3. Distilled water was utilized instead of cell suspension to generate empty beads.

Figure 4.3. Ca-alginate beads entrapped with *T. versicolor*.

Formed beads were fragile and they were not suitable for a pre-treatment process conducted over 7 days. Therefore, maximum pre-treatment process was applied to observe also bead deformation. Ca-alginate beads should be strengthened by creating composite materials with the other biopolymers in order to use them repeatedly for longer times.

4.3. Pre-treatment Process

Before pre-treatment process, cells entrapped in Ca-alginate beads left for growing in SSYE medium for 2 days and they had 1318 U/L enzyme activity right before pre-treatment. The reason behind using commercial blender to chop biomass is that physical pre-treatment of lignocellulosic substrate like this process can increase the hydrolysis rate up to 23-59% (Zheng et al., 2014).

After 7 days of incubation, beads were collected manually and substrates obtained from this process were used for building anaerobic batch digesters. A methodology for collection of beads should be modified for the future studies such as putting beads inside small mesh bags.

4.3.1. Laccase Enzyme Activity

During pre-treatment, secretion of laccase enzyme from cells entrapped beads was followed by enzyme activity. Enzyme activity was measured each day via formula given in Figure 3.4. Three replicates were used to decrease inaccuracy of obtained values. Enzyme activity graph was drawn with the calculated activity values as highlighted in Equation (3.1). As it can be seen clearly from Figure 4.4, dramatic increase in laccase activity was observed in the pre-treatment of corn stover in the 2nd day of pre-treatment. Activities of laccase enzyme in Erlenmeyer flasks containing macroalgae only and both substrates had little increase in total and they were almost stable during pre-treatment. Erlenmeyer flask containing both substrates showed little increase in the end of pre-treatment process.

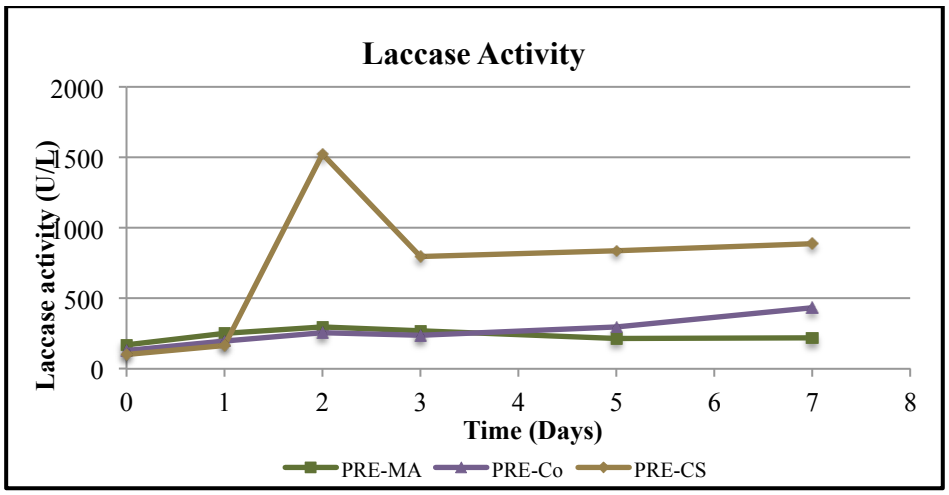

Figure 4.4. Laccase enzyme activity during 7 days in pre-treatment process (PRE-MA: Pre-treatment of macroalgae only, PRE-CS: Pre-treatment of Corn stover only, PRE-Co: Pre-treatment of both substrates).

The reason for the increase of the laccase activity in the corn stover pre-treatment process might be a result of starch content of corn stover because it has shown that starch can induce laccase activity dramatically (Kocyigit et al, 2012). Therefore, we added starch into SSYE media. Macroalgae content did not dramatically increase the laccase activity but it did not inhibit the activity also. Enzyme activity was almost stable during pre-treatment of macroalgae substrates; thus, cells entrapped with beads can still be used for pre-treatment of macroalgae substrates.

4.3.2. DNS Assay

As shown in Figure 4.5, standard curve was drawn with the data from a standard solution that was 1 mg/ml glucose solution. The equation for the fitted line was found as y=0.6061x-0.0206 and this equation was applied to find the reducing sugar concentrations in samples. R^2 is verified as 0.996 that is close to 1.0.

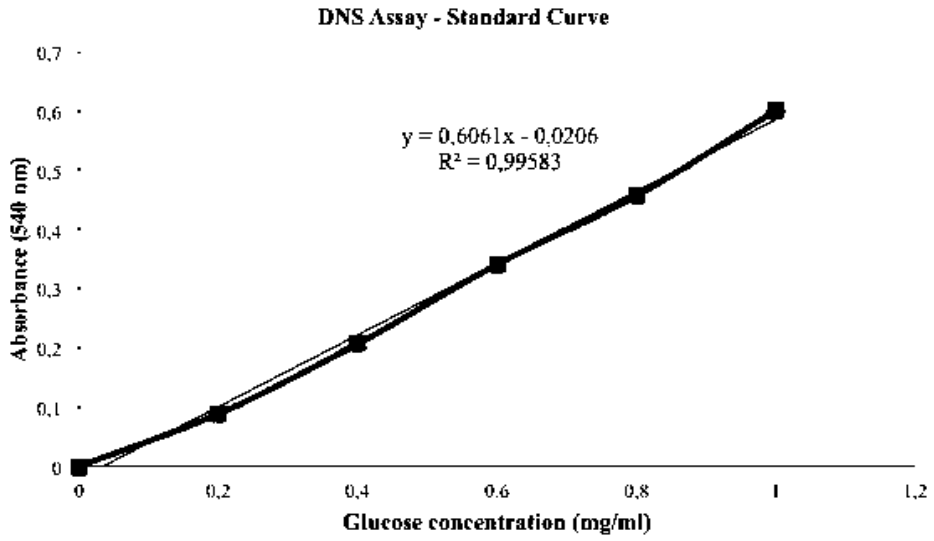

Figure 4.5. Standard curve for DNS assay (Equation and R^2 are given on graph).

As it can be stated from Figure 4.6, reducing sugar concentration of sample consisting of corn stover only and both substrates showed dramatic increase after 1 day of incubation and dramatic decrease until 4th day; however, sample consisting of only macroalgae had considerable increase after 1 day and later all samples showed fluctuation in concentration after 4th day until pre-treatment ended. After pre-treatment, it was predicted to detect certain increase in reducing sugar concentrations because of laccase enzyme.

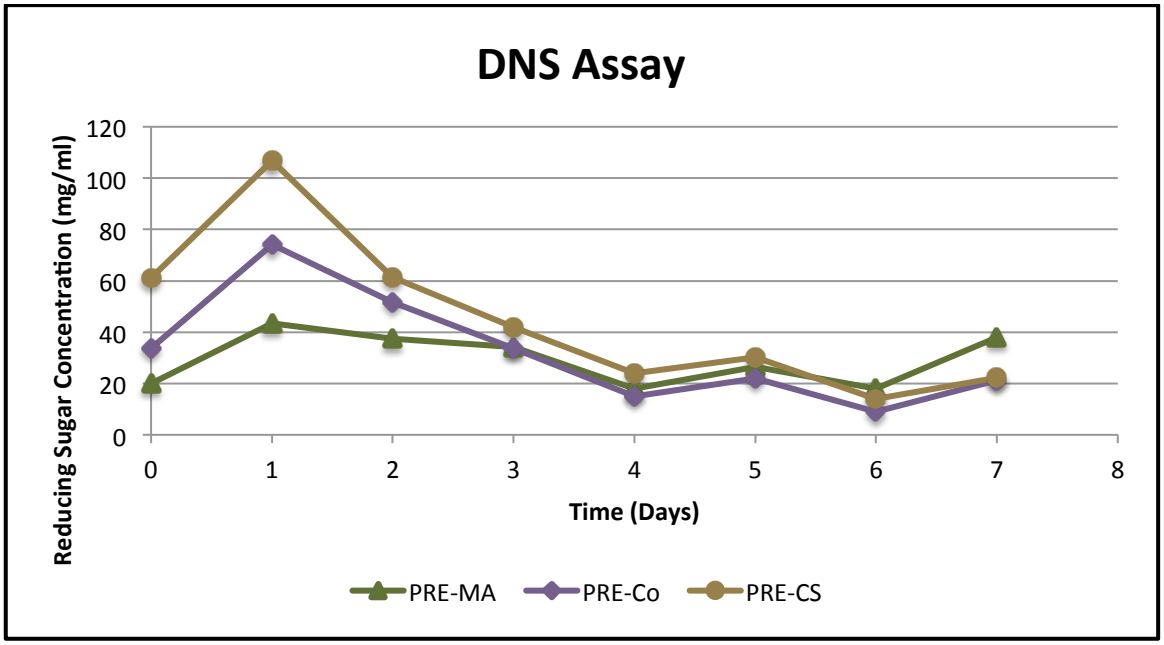

Figure 4.6. DNS assay showing reducing sugar concentration alteration during 7 days in pre-treatment process.

Laccase enzyme caused an increase in reducing sugar concentration in pre-treatment process because activity of enzyme was high enough to degrade during pre-treatment. It can be stated that the dramatic increase in the concentration of reducing sugar in pre-treatment of corn stover can probably induce the laccase activity in 2nd day of pre-treatment (Figure 4.4). Reducing sugar concentration started to be consumed after high production of sugar because *T. versicolor* needed carbon source for growing and reproduction as expected.

4.4. Anaerobic Batch Digestion

After constructing anaerobic batch digesters, several serial analyses were conducted in order to follow the digestion process. By 10 days intervals, volatile fatty acid (VFA) concentrations and chemical oxygen demand (COD) were used as an indicator to show the efficiency of digestion process. Biogas yield and methane yield were calculated with added TVS to digesters to show the efficiency of applied pre-treatment method for lignocellulosic biomass used in this study.

4.4.1. sCOD consumption

After drawing a standard curve by using KHP as standard, y=0.0004x (x: concentration of KHP, y: absorbance value at 600 nm) was found as equation of drawn curve. R^2 was found as 0.965, which was also close to 1.0.

By using the equation found from standard curve, sCOD values were measured during digestion period by 10 days intervals as indicated in Table 4.3. Also, graphic illustration of sCOD values of samples was drawn as it can be seen in Figure 4.7. All digesters containing pre-treated substrates showed higher values compared to digesters having non-treated substrates in the initial day of digestion. All samples showed dramatic increase after 10 days of digestion, later they start to decrease except the control digester of macroalgae only. Control macroalgae digester showed little increase until 30rd of digestion, then it started to decrease also. Seed sludge was measured as control of all digesters and it showed the lowest consumption as expected. Highest sCOD consumption was observed in digesters containing corn stover only and lowest sCOD consumption was seen in digester containing non-treated macroalgae only. Consumption in digester having pre-treated macroalgae only showed higher value (63%) compared to digester having non-treated macroalgae only (49%). Digesters containing corn stover only had similar but different consumption ratio; non-treated corn stover digester showed higher value. Co-digesters showed similar sCOD consumption ratio.

Table 4.3. The sCOD values of each digester during digestion period and total consumption of sCOD.

	sCOD (mg/L)						
Days	0	10	20	30	40	50	Consumption (%)
CON-MA	8375	12938	13375	10000	8938	6875	49
CON-CS	9000	15625	6125	4750	3125	1688	89
CON-Co	9313	12938	6563	6313	4563	4500	65
PRE-MA	10813	16063	13563	7120	6350	5900	63
PRE-CS	10875	13438	4813	4188	2688	2500	81
PRE-Co	13750	15500	12313	7625	6875	5438	65
Seed Sludge	2938	5750	3625	4063	3813		34

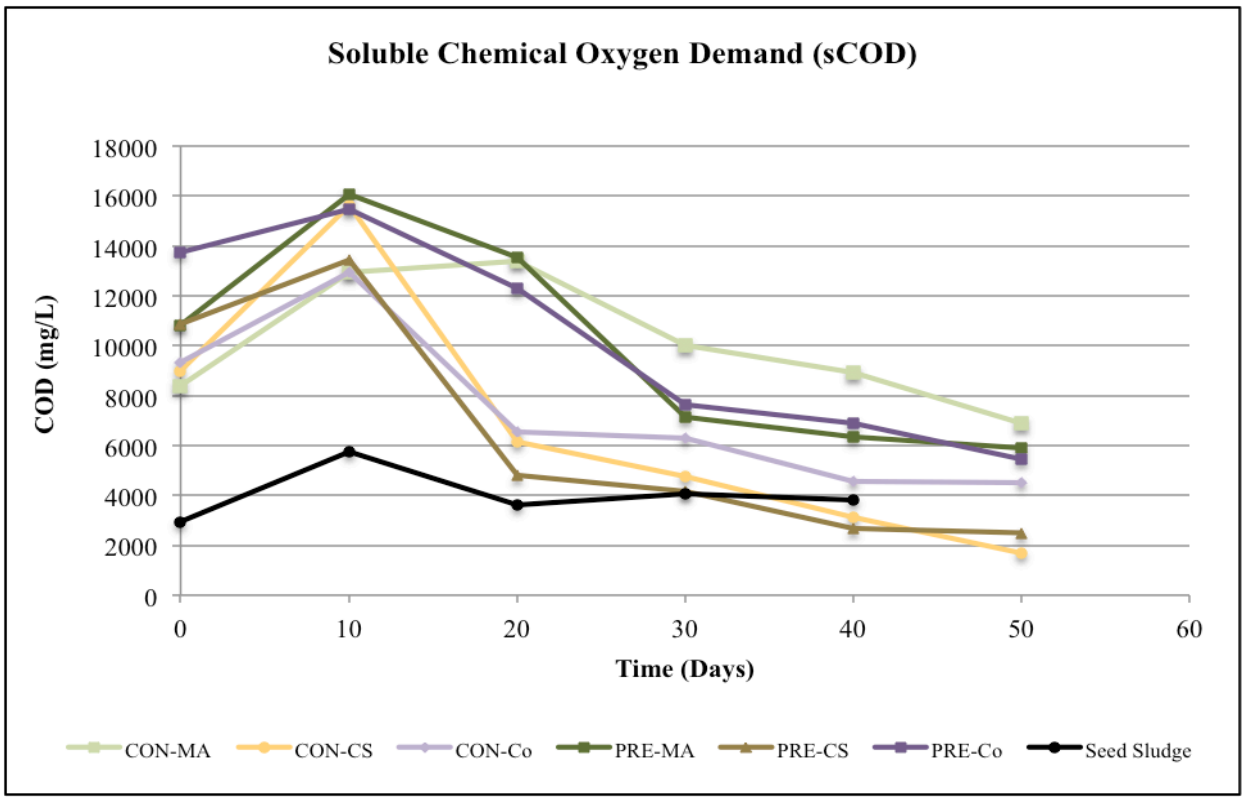

Figure 4.7. sCOD alterations during anaerobic batch digestion.

Digesters consisting of lignocellulosic substrate and seed sludge, which was also fed with manure and lignocellulosic substrate, can be difficult to analyse COD value because of their high viscosity. Therefore, soluble COD measurement was applied to overcome problem in this kind of situations. In this study, seed sludge was taken from an industry processing dairy products; thus, it was fed also with lignocellulosic biomass and manure. Because of high viscosity of samples, sCOD method was applied in this study.

4.4.2. VFAs Consumption

Most abundant VFAs in all digesters were measured as acetic acid, propionic acid and butyric acid. VFAs concentrations for all digesters during AD process were illustrated in Figure 4.8. In industrial large-scale anaerobic digestion plants, acetic acid, propionic acid and butyric acid were generally observed as most abundant VFAs. Short-chain fatty acids have not a negative effect on the metabolism of methanogens; however, methanogens can be more sensitive to some VFAs such as butyric acid and propionic acid (Azman, 2016). Therefore, accumulation of VFAs can create inhibitory effect on AD systems. In this study, there was no accumulation in VFAs concentration as it can be seen in Figure 4.8.

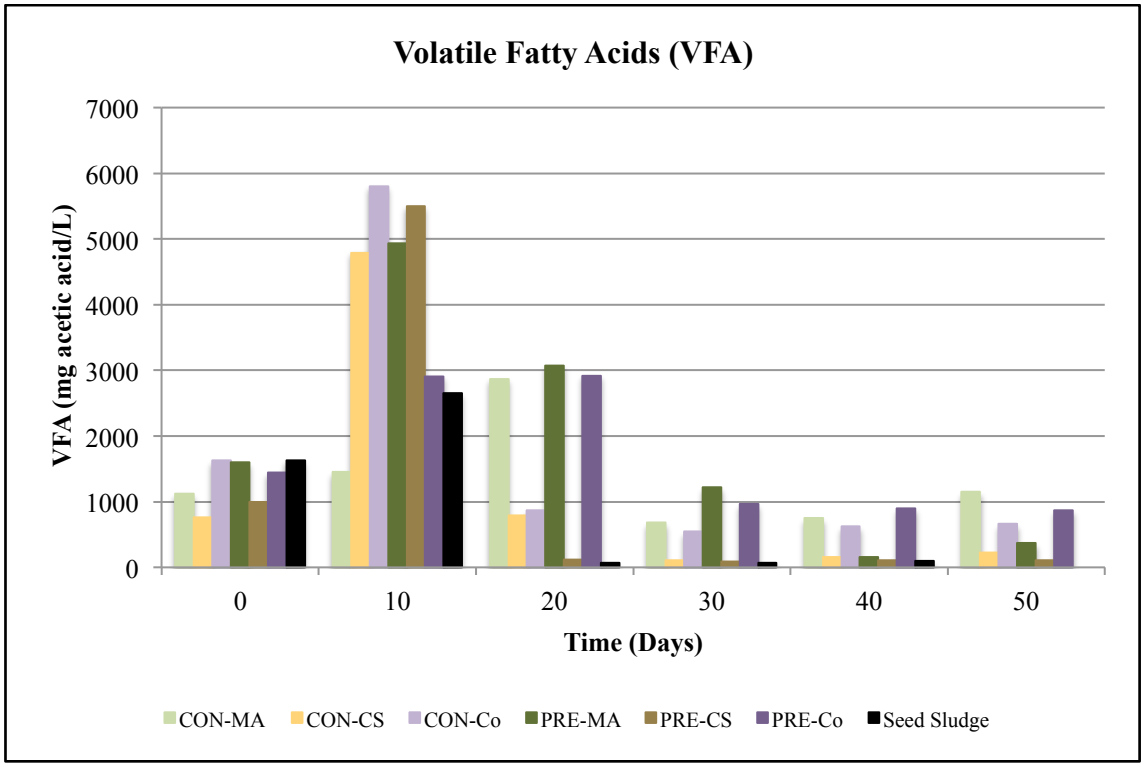

Figure 4.8. VFA alterations during anaerobic batch digestion

Average VFAs concentration in the beginning of digestion process was measured as 1303 mg acetic acid/L. It has shown that VFAs concentration bigger than 1800 mg/L in the beginning of AD process can have inhibitory effect on hydrolysis step (Romsaiyud et al., 2009). VFAs concentrations in all digesters were observed no bigger than 1627±20 mg acetic acid/L that was the value measured in control co-digestion reactors. Initial conditions were suitable for hydrolysis step in AD process.

VFAs consumption during AD processes was the one of the parameters that should be followed to see the efficient conversion of them into biogas formation. VFAs accumulation in anaerobic digester can have inhibition effect on AD systems. As it can be seen in Figure 4.8, All VFAs concentrations increased on the 10^{th} day of the digestion except the digester containing macroalgae only. After hydrolysis step of macromolecules, acidogenic microorganisms convert products coming from hydrolysis step into VFAs in acidogenesis step. Therefore, it was expected to see an increase on the 10^{th} day of digestion in the concentration of VFAs. However, control macroalgae digestion did not show dramatic increase in VFAs concentration that might be result of harder digestion of macroalgae and a delay in VFAs production. Figure 4.8 shows an increase in VFAs concentration of control macroalgae digestion in the 20^{th} day of digestion. It can also be seen from Figure 4.8 that pre-treatment process might increase the hydrolysis efficiency because higher VFAs concentration were observed in digesters containing corn stover only and macroalgae only

compared to control digesters. These digesters, containing macroalgae and corn stover only, showed also 96.7% and 98.0% VFAs consumption, respectively. Control macroalgae digestion showed 73.9% VFAs consumption that was much lower than pre-treated digester of macroalgae (96.7%).

4.4.3. Biogas Production

The produced biogas in digesters was measured by manometer every day and manometer was giving the values with mbar unit; therefore, conversion of mbar to ml was accomplished after measurements. After the 40th day of digestion, biogas production was almost stabilized for all digesters as seen in Figure 4.9. Digester containing only seed sludge was ended after the 40th day of digestion because of undetectable biogas production. All batch digesters were ended after 50 days of digestion.

Total biogas produced in digesters after 50 days of digestion were CON-MA (1368 ml biogas), CON-CS (1805 ml biogas), CON-Co (1705 ml biogas), PRE-MA (1481 ml biogas), PRE-CS (1913 ml biogas), PRE-Co (1801 ml biogas), and seed sludge (768 ml biogas). The highest biogas was obtained from the digester containing pre-treated corn stover only. Biogas production of digesters containing pre-treated substrate and non-treated substrates was compared in Figure 4.10.

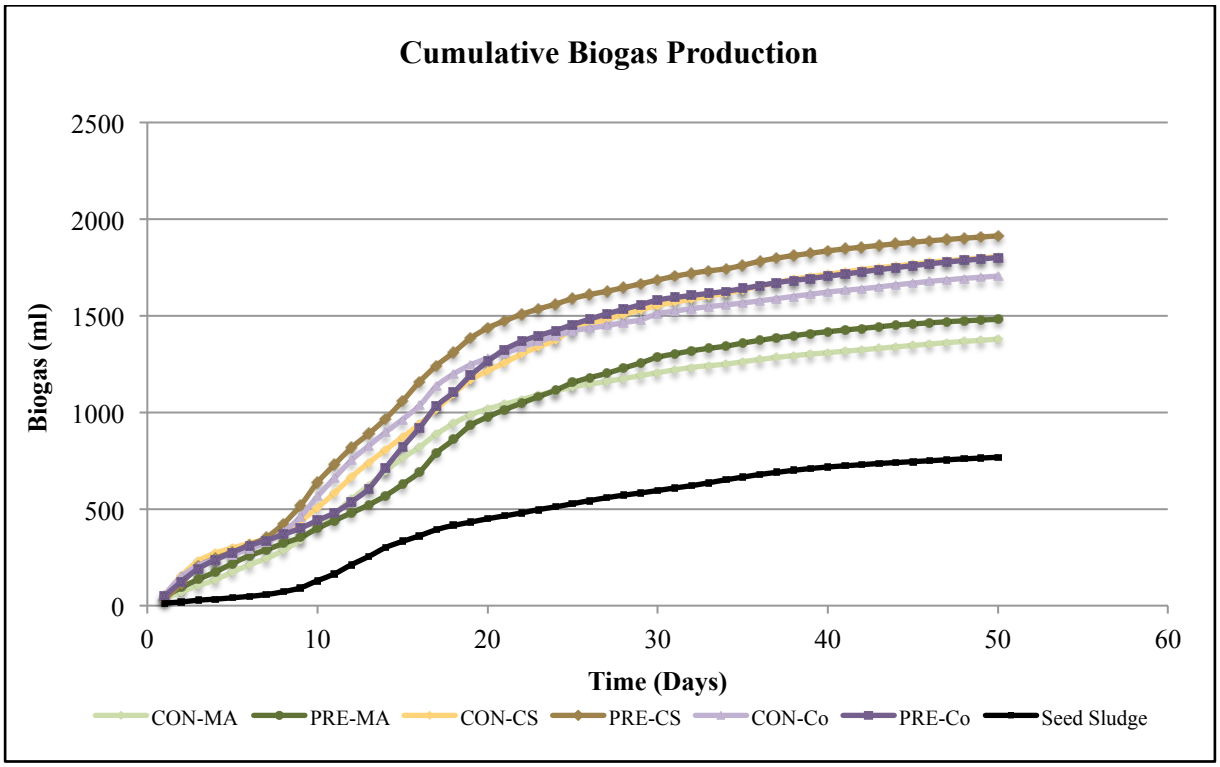

Figure 4.9. The cumulative biogas production during AD process.

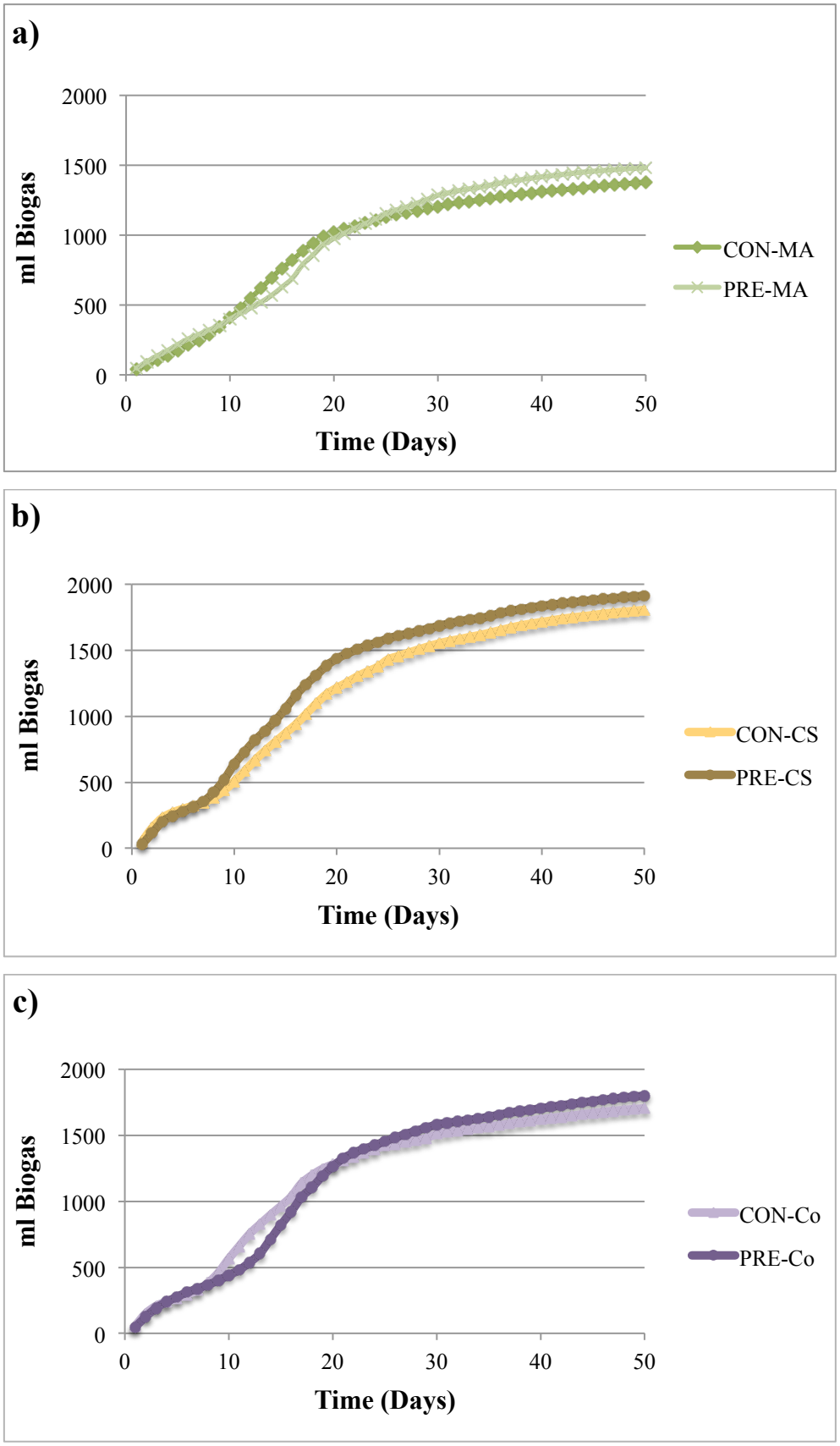

Figure 4.10. The cumulative biogas production of pre-treated and non-treated digesters: a) the AD of macroalgae only, b) AD of corn stover only, c) anaerobic co-digestion of both substrates.

Biogas yield was calculated by dividing produced biogas, which the value was first subtracted from total biogas value coming from seed sludge digester (768 ml biogas), into total volatile solids (TVS) added when digesters were constructed (1.5 g TVS_{added}). Measured biogas yield for each digesters were indicated in the Figure 4.11. Biogas yield for each digester was observed as CON-MA (400 ml biogas/g TVS_{added}), CON-CS (691 ml biogas/g TVS_{added}), CON-Co (625 ml biogas/g TVS_{added}), PRE-MA (475 ml biogas/g TVS_{added}), PRE-CS (763 ml biogas/g TVS_{added}), and PRE-Co (689 ml biogas/g TVS_{added}).

The pre-treatment process had an increasing effect on the yield of biogas. As it can be clearly seen from Table 4.4, pre-treatment with Ca-alginate beads entrapped with *T. versicolor* affected digesters by increasing biogas yield to 19%, 10%, and 10% for macroalgae only, corn stover only, and co-digestion of both substrates, respectively. It can be correlated with the results obtained from the COD consumption ratio, which increased from 49% to 63%, and the VFAs consumption ratio increased from 73.9% to 96.7% for anaerobic digestion of macroalgae only.

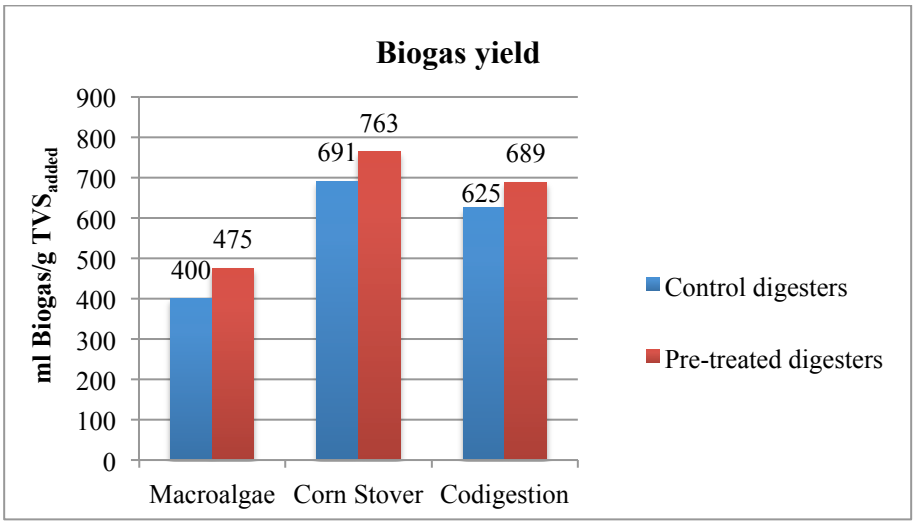

Figure 4.11. The calculated biogas yield per gram volatile solids added. (ml biogas/g TVS_{added}).

Table 4.4. The percentage of increase in biogas yield for the each type of digester.

Substrates	Control digesters (ml biogas/g TVS_{added})	Pre-treated digesters (ml biogas/g TVS_{added})	Biogas yield increase
Macroalgae	400	475	19%
Corn Stover	691	763	10%
Codigestion	625	689	10%

4.4.4. Methane Production

Methane ratio in the produced biogas was determined by applying gas chromatography (GC) method every day. Methane ratios of digesters had fluctuations during 50 days of digestion and these fluctuations generally ranged between some values signified in Table 4.5. Wirth and his colleagues (2015) showed that methane range was 52-56% in co-digestion of microalgae and maize silage (1:1 ratio).

Table 4.5. The CH_4 intervals during 50 days of digestion and average CH_4 ratio for each digester.

Digesters	CH_4 ratio intervals	Average CH_4 ratio
CON-MA	50-62%	52%
CON-CS	50-61%	51%
CON-Co	53-64%	51%
PRE-MA	53-65%	54%
PRE-CS	55-59%	53%
PRE-Co	53-61%	53%

The obtained methane ratios supported to create corresponding cumulative methane graphs for each digester as shown in Figure 4.12 and Figure 4.13. After 50 days of AD process, methane production was observed as CON-MA (713 ml CH_4), CON-CS (875 ml CH_4), CON-Co (862 ml CH_4), PRE-MA (797 ml CH_4), PRE-CS (991 ml CH_4), PRE-Co (934 ml CH_4), and seed sludge (393 ml CH_4).

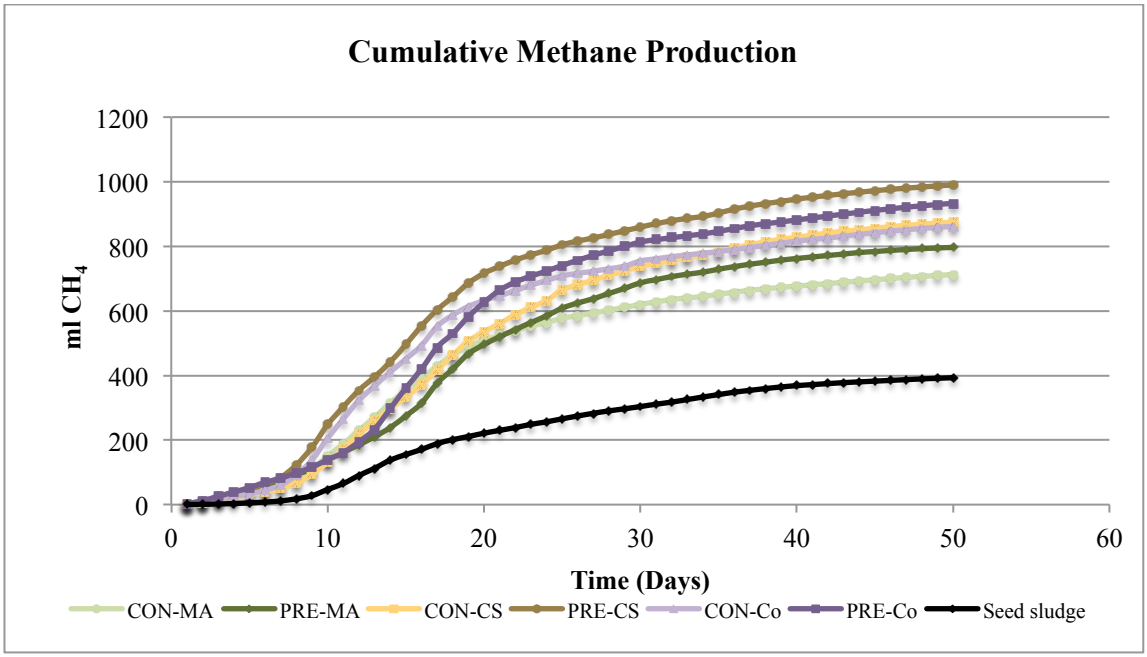

Figure 4.12. The cumulative methane production during AD process.

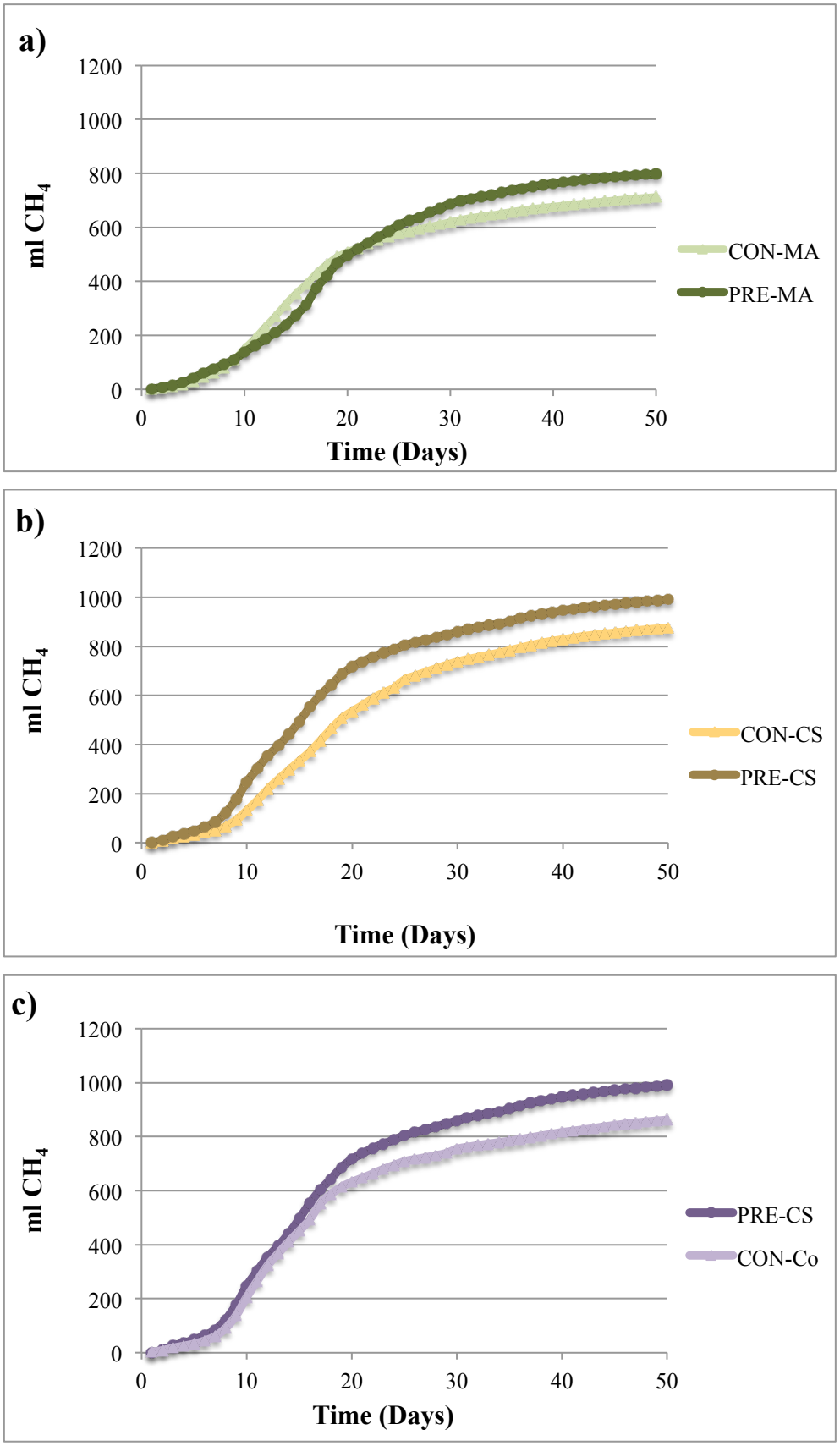

Figure 4.13. The graph of cumulative methane production of each digester type: a) the AD of macroalgae only, b) AD of corn stover only, c) anaerobic co-digestion of both substrates.

In the study conducted by Wirth and his colleagues (2015), biogas yield was the highest value in digester having only maize silage and lowest in digester consisting of microalgae only. Co-digestion of both substrates (1:1) gave the average value compared to mono-digestions.

Similarly with biogas yield calculations, methane yield was also obtained with dividing total produced methane, which the value was first subtracted from the produced methane amount obtained from seed sludge digester (393 ml CH_4), to total added volatile solids in the set up of digesters (1,5 g TVS). Methane yields found by this calculation were illustrated in Figure 4.14. The highest methane yield was obtained from the digester consisting of pre-treated corn stover only.

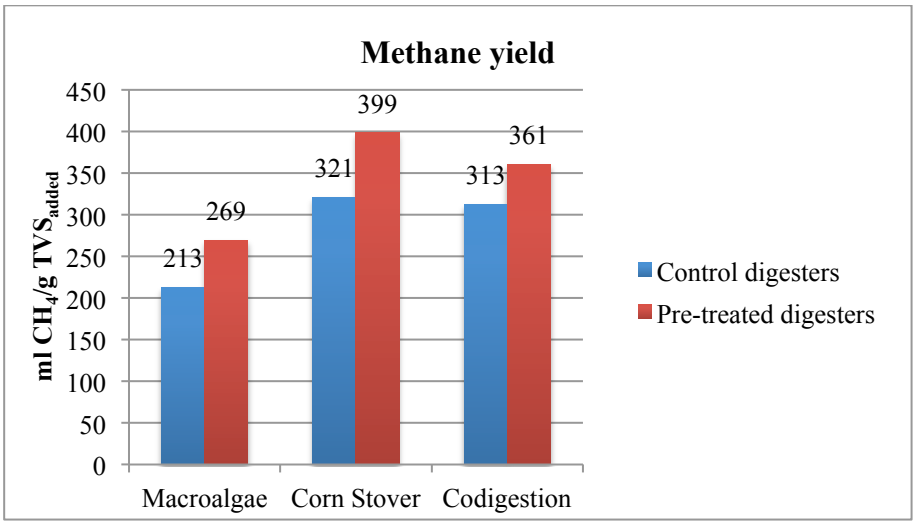

Figure 4.14. The calculated methane yield per gram volatile solids added (ml CH_4/g TVS_{added}).

Table 4.6. The percentage of methane yield increase in each type of digester.

Digesters	Control digesters (ml CH_4/g TVS_{added})	Pre-treated digesters (ml CH_4/g TVS_{added})	Methane yield increase
Macroalgae	213	269	26%
Corn Stover	321	399	24%
Codigestion	313	361	15%

As indicated in Table 4.6, Pre-treatment of substrates by Ca-alginate beads entrapped with *T. versicolor* increased methane yield by 26%, 24%, and 15% for digesters having macroalgae only, corn stover, and both substrates, respectively. Methane yield for each digester was recorded as CON-MA (213 ml CH_4/g TVS_{added}), CON-CS (321 ml CH_4/g TVS_{added}), CON-Co (313 ml CH_4/g TVS_{added}), PRE-MA (269 ml CH_4/g TVS_{added}), PRE-CS (399 ml CH_4/g TVS_{added}), and PRE-Co (361 ml CH_4/g TVS_{added}). Results were comparable with the data collected from the sCOD consumption

and VFAs consumption. It can be deduced that reduction of sCOD and VFAs in pre-treated digesters compared to control reactors can be complementary results.

Pre-treatment with *T. versicolor* entrapped in Ca-alginate beads can be an efficient technique to increase biomethane potential of lignocellulosic biomass especially for macroalgae considering biogas and methane yields. Corn stover can pre-treated with this technique also to increase biomethane potential because they have showed high laccase activity during pre-treatment process. However, the methane yield did not show high results as obtained for macroalgae digestion. Therefore, appropriate substrates should be defined to co-digest corn stover to obtain more methane yields. Macroalgae was not the best option to proceed co-digestion with corn stover regarding the results obtained from this study.

4.5. Microbial Community Analyses

Next-generation sequencing technologies started to be applied to determine microbial diversity in AD systems in recent years. In this study, microbial communities found in digesters were assessed and listed by applying NGS methodology for the samples taken from the 20th day of digestions. Digester containing only seed sludge was a control reactor for all reactors and microbial diversity of this reactor was also determined, which is indicated in Figure 4.15 and Figure 4.16, to compare with other digesters. As it can clearly seen in Figure 4.15, *Firmicutes* was the most abundant phylum (68%) followed by *Proteobacteria* (15%) and *Bacteroidetes* (11%) in the 20th day of sample gathered from the seed sludge digester. It was an expected outcome since it was proven that the most abundant phylum found in anaerobic digesters was found as *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* (Treu et al., 2016, Krause et al., 2008). *Fibrobacter*, *Spirochaetes*, and *Thermotogae* were also observed but in less amounts (Treu et al., 2016, Bozan et al., 2017). As it can be understood from Figure 4.15, microbial communities found in seed sludge digester were complementary with the literature. VFA, proteins, and carbohydrate degradation were mostly conducted by *Firmicutes* and *Bacteroidetes* in anaerobic digesters (Krause et al., 2008). Phyla *Bacteroidetes* contributes the degradation of cattle manure, which contains plant residues (De Francisci et al., 2015).

Most abundant bacterial species found in seed sludge digester was *Clostridium* sp. (43.2%), which is a highly cellulolytic bacterium. Other species, shown in Figure 4.16 were *Pseudomonas* sp. (12.9%), *Bacillus* sp. (8.6%), *Petrotoga* sp. (5.4%), *Bacteroides* spp. (5.0%), *Halocella*

cellulosilytica (3.8%), *Lewinella nigricans* (3.6), *Acetivibrio* spp. (3.5%), *Garciella garciaella* sp. (2.0%), and so on.

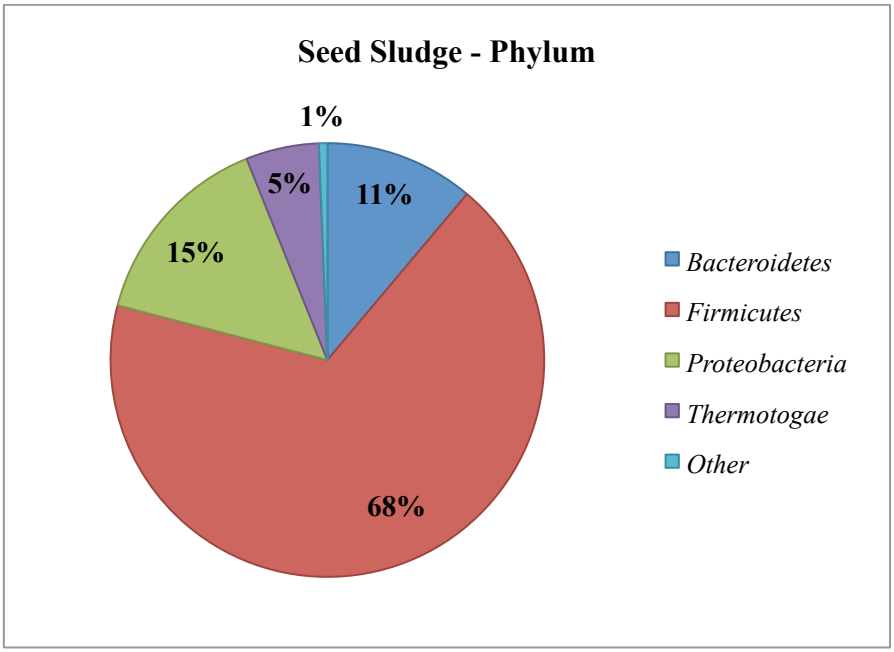

Figure 4.15. Bacterial communities in the seed sludge indicating phylum diversity in the 20th day of AD.

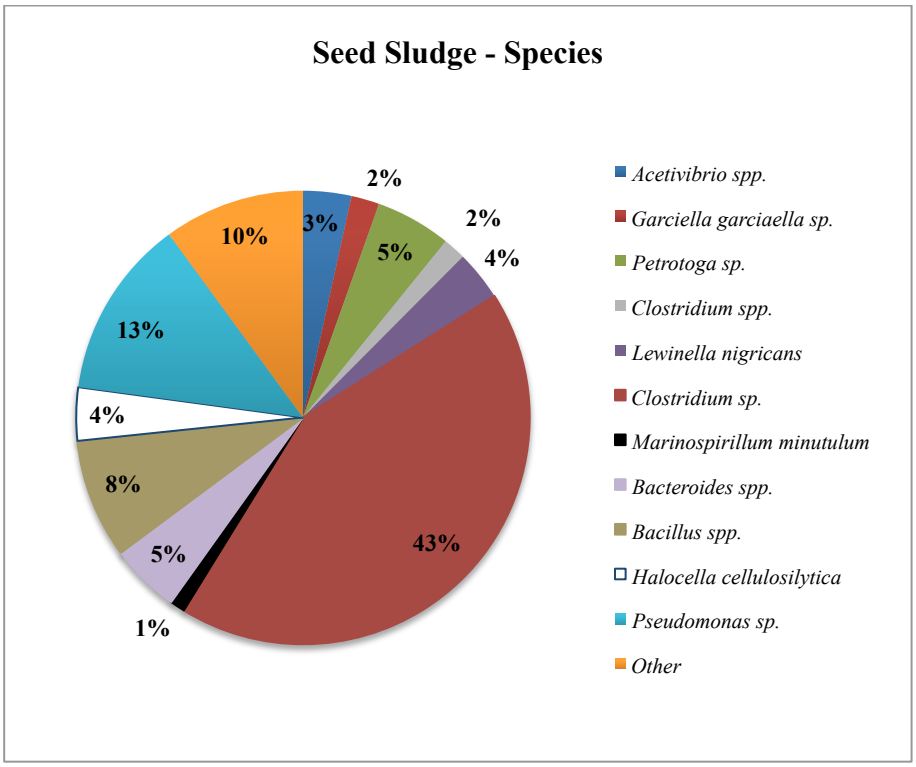

Figure 4.16. Bacterial communities in the seed sludge highlighting species diversity in the 20th day of AD.

Archaeal species detected in the seed sludge digester at the 20th day of incubation were shown in Figure 4.17. Most abundant archaeal species were found as *Methanoculleus* sp. (75.7%), *Methanoculleus bourgensis* (16.1%), and *Methanomassiliicoccus* sp. (7.2%) as emphasized in Figure 4.17. It was an expected result because it was shown that reactors containing manure in AD systems are dominated by *Methanoculleus* genus (Campanaro et al., 2016).

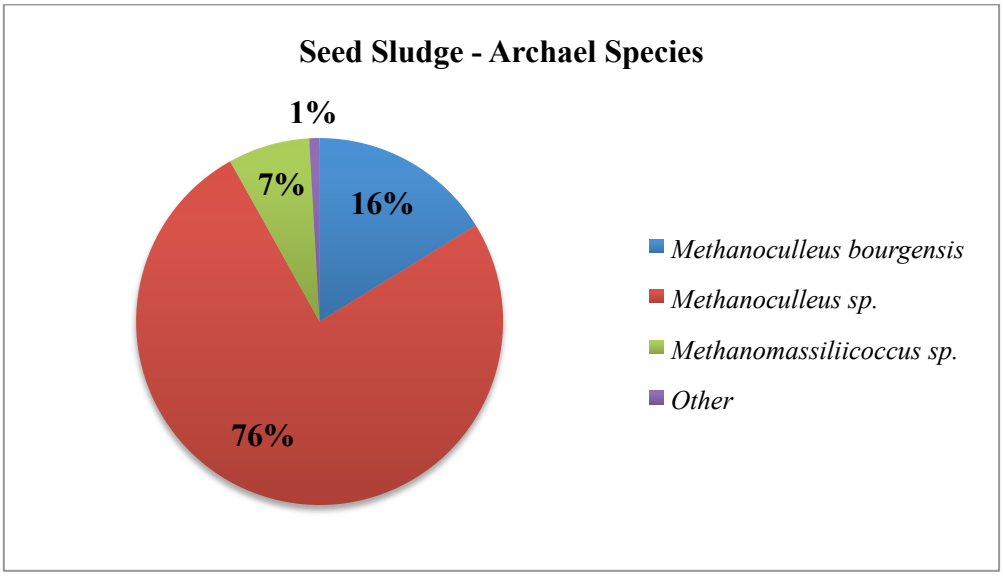

Figure 4.17. Archaeal communities in the seed sludge showing species diversity in the 20th day of AD.

Most abundant bacterial phyla in all digesters were *Bacteroidetes* (35-48%), *Firmicutes* (33-44%), *Synergistetes* (5-11%), and *Thermotogae* (0.2-12%) as it can seen from Table 4.7 and Figure 4.18. *Bacteroidetes* ratio was more than *Firmicutes* in all digesters except control macroalgae and seed sludge digesters. Seed sludge digester was used as control for all digesters and the only difference between seed sludge digester and other digesters was lignocellulosic biomass amount. It can be concluded that *Bacteroidetes* ratio increased after the addition of lignocellulosic biomass into the seed sludge according to the data obtained from NGS results (Figure 4.18 and Table 4.7).

Comparing control macroalgae and pre-treated macroalgae digesters can show that pre-treatment process of macroalgae might cause an increase in *Bacteroidetes* ratio and decrease in *Firmicutes* ratio. It has to be correlated with further studies. However, both *Bacteroidetes* and *Firmicutes* ratio in pre-treated digesters were higher than control digesters (See Table 4.7); Furthermore, *Thermotogae* ratio in pre-treated digesters was lower than control digesters.

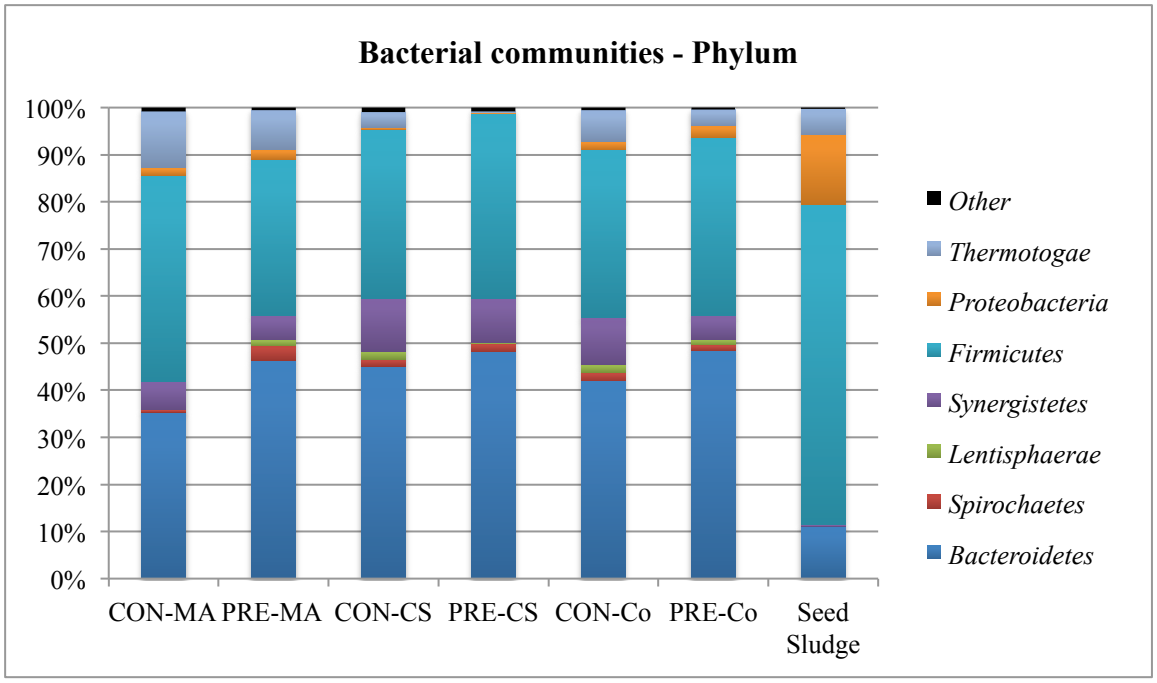

Figure 4.18. The relative abundance of bacterial phyla in the digesters in the 20th day of AD.

Table 4.7. The abundance of bacterial phyla found in the digesters.

Species	CON-MA	PRE-MA	CON-CS	PRE-CS	CON-Co	PRE-Co	Seed Sludge
Bacteroidetes	35.37%	46.41%	45.12%	48,26%	41,96%	48,48%	11,13%
Spirochaetes	0,49%	3,08%	1,50%	1,62%	1,89%	1,18%	0,04%
Lentisphaerae	0,00%	1,22%	1,69%	0,26%	1,58%	1,17%	0,00%
Synergistetes	5,96%	5,16%	11,25%	9,34%	9,94%	4,98%	0,29%
Firmicutes	43,81	33,12%	35,78%	39,22%	35,66%	37,82%	67,93%
Proteobacteria	1,67%	2,15%	0,51%	0,20%	1,89%	2,47%	14,86%
Thermotogae	11,86%	8,31%	3,15%	0,25%	6,49%	3,55%	5,45%
Other	0,85%	0,55%	1,00%	0,85%	0,61%	0,35%	0,30%

It can be remarked from Figure 4.19 that most abundant bacterial orders in digesters were *Bacteroidales* (26-38%), *Clostridiales* (29-36%), *Sphingobacteriales* (3-20%), *Synergistales* (5-11%), *Thermotogales* (0.2-12%), *Bacillales* (3-5%), and *Spirochaetales* (0.5-3%).

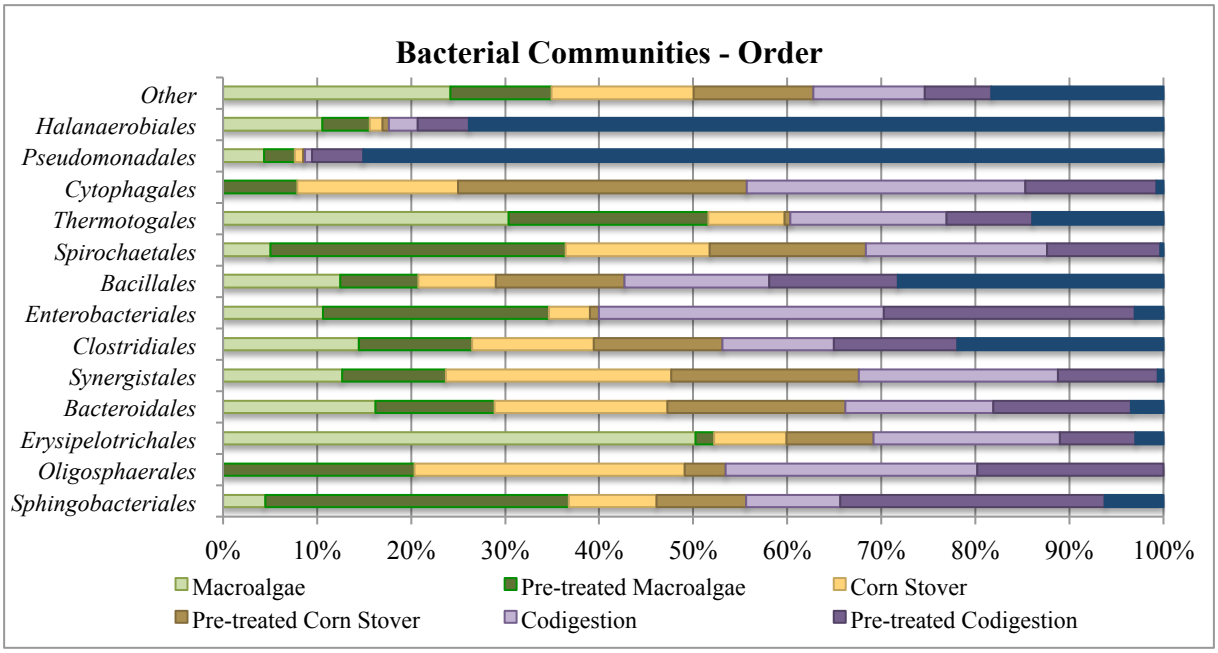

Figure 4.19. Bacterial communities indicating order diversity and abundance ratios of each order in the anaerobic batch digesters at the 20th day of incubation.

Archaeal diversity in digesters is listed in Table 4.8 according to their abundance ratios in digesters and it is highlighted in Figure 4.20 by comparing each species abundance ratio in all digesters. Most abundant archaeal species seen in digesters were *Thermoplasma* sp. (60%) for CON-MA, *Methanomassiliicoccus* sp. (55%) for PRE-MA, *Methanobacterium* sp. (29%) for CON-CS, *Methanobacterium* sp. (41%) for PRE-CS, *Methanoculleus bourgensis* (42%) for CON-Co, and *Methanomassiliicoccus* sp. (54%) for PRE-Co. It can be clarified that pre-treated macroalgae containing digesters showed high abundance in *Methanomassiliicoccus* sp. differently when they were compared with the other digesters. Digesters having corn stover only showed significant high abundance in *Methanobacterium* sp. distinctly from other anaerobic digesters. Methanogen found high in seed sludge digester, which was *Methanoculleus* sp. (75%), did not show abundance higher than 14% in other digesters.

Table 4.8. The abundance of archaeal species in the digesters.

Species	CON-MA	PRE-MA	CON-CS	PRE-CS	CON-Co	PRE-Co	Seed Sludge
Methanoculleus thermophilus	%10,09	%0,35	%1,22	-	%0,63	-	-
Thermoplasma sp.	%59,62	%18,34	%4,88	%3,13	%16,98	%11,76	%0,45
Methanoculleus bourgensis	%0,94	%11,07	%28,05	%3,13	%42,14	%14,38	%16,22
Methanobrevibacter sp.	%0,23	%0,35	%1,22	-	%0,63	-	-
Methanobrevibacter woesei	%1,88	-			-	-	-
Methanoculleus sp.	%3,05	%9,69	%4,88	%6,25	%10,69	%14,38	%75,68
Methanobacterium bryantii	-	-	%1,22	-	-	-	-
un.c. Nitrosocaldus sp.	-	-	-	-	%1,26	-	-
Methanosarcina sp.	%0,23	%2,08	-	-	-	-	-
Methanomassiliicoccus sp.	%6,81	%55,02	%9,76	%18,75	%15,72	%54,25	%7,21
Methanolinea sp.	%0,47	-	%1,22	-	-	%1,31	-
Methanosarcina spp.	%0,47	-	%15,85	%25,00	%5,66	-	-
Methanobacterium sp.	%12,91	-	%29,27	%40,63	%6,29	%2,61	%0,45
Methanosarcina siciliae	%0,47	%2,08	-	%3,13	-	%1,31	-
c. Nitrosocaldus yellowstonii	-	-	%1,22	-	-	-	-
c. Thermoplasmata archaeon	%2,82	%1,04	%1,22	-	-	-	-

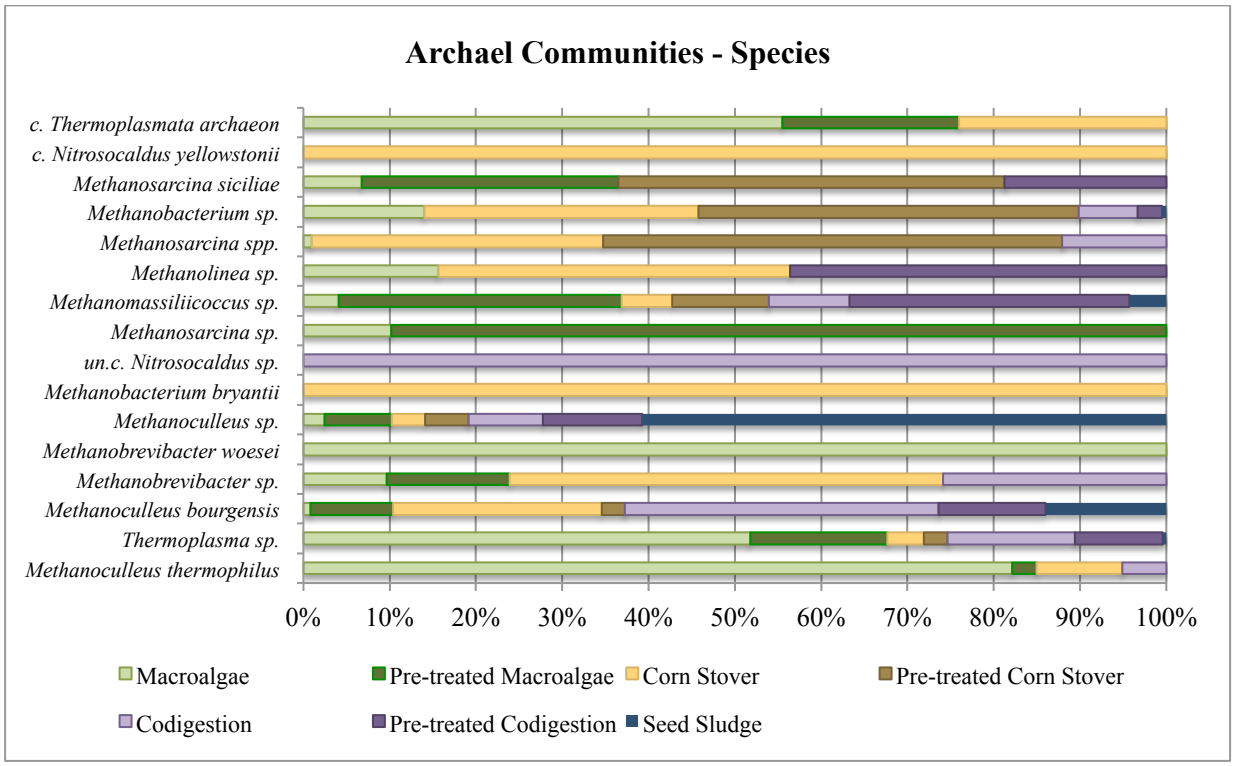

Figure 4.20. Archaeal communities indicating species diversity and abundance ratios of each species in the anaerobic batch digesters at the 20th day of incubation.

Bioinformatics analyses were conducted to show the alpha and beta diversities in digesters. Alpha diversity shows diversity of microorganisms within a digester type and beta diversity indicates the diversity between digesters. Obtained data were shown in Table 4.9 and Table 4.10. Shannon index is one of the diversity indices and it can be interpreted easily by taking its exponential value that gives the Effective Number of Species (ENS). When ENS increases, diversity increases also. As it can be interpreted from the Table 4.9 by using Shannon index, pre-treated digesters had higher microbial diversity compared to their control reactors except the digesters processing co-digestion that were similar with each other and had the highest diversities.

Table 4.9. The measured alpha diversity indexes in the digesters

Digester	Shannon index	Effective number of species (ENS)	Invsimpson index	Pielou's evennes
CON-MA	2,9543	19	8,9339	0,55299
CON-CS	2,8525	17	8,9312	0,53443
CON-Co	3,2626	26	14,7777	0,61126
PRE-MA	3,0259	21	10,6949	0,57554
PRE-CS	3,1327	23	13,7840	0,60325
PRE-Co	3,2736	26	14,1360	0,61613
Seed sludge	2,3478	10	4,5994	0,45766

Invsimpson index is also another index giving information about diversity and an increase in its number means an increase in diversity because it shows a decrease in the amount of dominancy. It can be observed from the data given in Table 4.9, the results gathered by Shannon index were complementary with those of Invsimpson index. It can be interpreted also that an increase in substrate diversity might also cause increase in species diversity. Pielou's evenness results suit with both Shannon index and Invsimpson index that show an increase in microbial diversity by the pre-treatment and also substrate diversity.

Table 4.10. The results of bioinformatics analysis for beta diversity.

	CON-MA	CON-CS	CON-Co	PRE-MA	PRE-CS	PRE-Co
CON-CS	0,276	-	-	-	-	-
CON-Co	0,290	0,221	-	-	-	-
PRE-MA	0,322	0,305	0,290	-	-	-
PRE-CS	0,373	0,268	0,258	0,296	-	-
PRE-Co	0,330	0,304	0,265	0,235	0,243	-
Seed Sludge	0,397	0,401	0,416	0,396	0,427	0,414

Beta diversity is an indicator to show microbial similarity and differences between different communities as conducted in this study. It can be deduced that all digesters were highly diverse from the seed sludge digester as shown in Table 4.10. Pre-treated corn stover digester showed highest diversity against seed sludge digester. High microbial diversity inside co-digesters was observed after completing alpha diversity analysis; therefore, it was expected to see high values in beta diversity to have more microbial diversity compared to seed sludge digester also. Expected results were observed in the meaning of microbial diversities (Table 4.10) .

Microorganisms play crucial role in the degradation of macromolecules into smaller accessible compounds in biogas plants. Hydrolytic microorganisms were used as seed culture in order to start AD process in big-scale AD plants. It is important to have knowledge about microbial diversity and abundances of microorganisms in digesters to get precautions and continue to AD process. Some of the hydrolytic bacterial phylum can be listed as: *Firmicutes* (species: *Clostridium*, *Ruminococcus*, *Caldicellulosiruptor* etc.), *Bacteroidetes*, *Fibrobacter*, *Spirochaetes* (*Spirochaeta*), and *Thermotogae* (species*: Fervidobacterium* and *Thermotoga*). Hydrolytic bacterial groups found in digesters depend on the type of seed used. It is shown that *Firmicutes* play important role in the degradation of VFAs in anaerobic digestion systems and activated sludge. *Bacteroidetes* have the ability to degrade complex molecules like proteins and they convert amino acids into acetate. *Synergistetes* similarly can consume amino acids and produce short fatty acids; moreover, propionate, acetate, and butyrate can be utilized by *Proteobacteria* (Riviere et al., 2009).

As it is indicated in Table 2.3, co-digestion of *Scenedesmus obliquus*, which a type of microalgae, and maize silage showed *Methanosarcina* as dominant methanogen in a previous study conducted by Wirth and his colleagues (2015); however, in this study, dominant methanogens were observed differently as *Methanoculleus bourgensis* (42%) and *Methanomassiliicoccus* sp. (54%) for control co-digesters and pre-treated co-digesters, respectively.

In previous studies, macroalgae batch digestions gave sensibly a methane yield ranging between 94 ml CH_4/g TVS to 330 ml CH_4/g TVS under mesophilic conditions and methane percentage in produced biogas was ranging between 49% to 79% (Briand and Morand, 1997). In this particular study, methane percentage was obtained as ranging between 50% to 65% of produced biogas in anaerobic batch digesters under mesophilic conditions as shown in Table 4.5. Furthermore, methane yield was measured as 213 and 269 ml CH_4/g VS for control macroalgae and pre-treated macroalgae digestion, respectively. Obtained results are matching realistically with the data obtained from previous studies.

One of the problems for macroalgae anaerobic digestion is inhibiting effect of sulphur existing in *Ulva* sp. and other macroalgae species. S content in *Ulva* sp. was detected as 3% of total algae biomass. That can be another reason why methane yield was lower in *Ulva* sp. digestion because it can cause a competition between species for methanization and sulphate-reducing (Briand and Morand, 1997). However, pre-treated macroalgae batch digestion showed higher methane yield compared to its control as shown in Table 4.6 and Figure 4.14. One of the studies showed that laccase produced by *T. versicolor* can increase the desulphurization of coal mass under optimal conditions (Aytar et al., 2011). Pre-treatment of *Ulva* sp. with *T. versicolor* entrapped in Ca-alginate beads might cause desulphurization prior to anaerobic batch digestion, and thus methane yield was observed higher compared to control digester.

Overall, the pre-treatment of macroalgea and corn stover biomass with *T. versicolor* entrapped in Ca-alginate beads prior to anaerobic batch digestion showed increase in biogas and methane yield for all substrates especially macroalgae digestion. Different microbial communities and diversities were observed in pre-treated digesters compared to their control digesters. This study shows that the newly developed pre-treatment techniques using cells entrapped beads for anaerobic digestion can be a promising method in order to reduce the cost and increase the efficiency of AD systems.

5. CONCLUSIONS AND RECOMMENDATIONS

Pre-treatment of lignocellulosic and macroalgae biomass with Ca-alginate beads entrapped with *T. versicolor* resulted in 19%, 10%, and 10% increase in biogas yield and 26%, 24%, and 15% increase in methane yield for anaerobic digesters consisting of macroalgae only, corn stover only, and both substrates as substrates, respectively. These results were obtained for small-scale digesters; thus, this kind of researches should be carried to bigger scale to verify the obtained results.

VFA results were in parallel with the results obtained for biogas and methane yield. sCOD results showed that pre-treatment process increased the sCOD values for the first day of AD process. sCOD values in all samples increased dramatically in the 10^{th} day of AD process, and later sCOD consumption was ranged between 49-89%. However, the pre-treatment process might cause early increase in VFAs concentration and sCOD values. Therefore, VFAs concentrations and sCOD values should be followed carefully between 0-10 days in order to get more accurate information about VFAs and sCOD consumption for the future studies.

Reducing sugar concentration of pre-treatment samples increased after 24 h incubation with beads, and later they showed tendency to decrease and fluctuate as expected. This can show that the shorter pre-treatment time would be enough to degrade lignocellulosic substrates; however, pre-treatment test should be extended to longer time (more than 1 week) in order to see the exact time of decrease in the degradation of lignocellulosic biomass and laccase enzyme activity.

Pre-treatment process showed different microbial communities compared to control digesters according to NGS results. Furthermore, microbial diversity was higher in pre-treated digesters based on bioinformatics analyses. Especially *Methanomassiliicoccus* sp. was clearly observed higher ratio in pre-treated digesters containing macroalgae compared to their control digesters. The main reason behind this should be investigated in future researches.

It is known that S content in *Ulva* sp. can be problematic for AD process. In this study, methane yield of macroalgae digestion increased probably by the help of desulphurization effect of laccase enzyme produced by *T. versicolor*. More studies should be carried out by using macroalgae containing high amount of S and laccase enzyme in order to prove the effect and show the mechanism of desulphurization effect of laccase.

One of the problems was the collection of Ca-alginate beads that was very difficult. A new collection methodology should be developed to make the process easier such as putting beads inside mesh bag. Also, composite material of Ca-alginate beads with other polymers can be developed to increase mechanical stability of beads for very long time. Then, they can be used repeatedly.

It can be concluded that pre-treatment of lignocellulosic and macroalgae biomass by Ca-alginate beads entrapped with *T. versicolor* can be a novel approach to increase the efficiency of anaerobic digestion process.

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