ANAEROBIC CO-DIGESTION OF COW MANURE, FOOD WASTE AND WASTE ACTIVATED SLUDGE WITH *Trametes versicolor* PRE-TREATMENT UNDER MESOPHILIC CONDITION

by

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ABSTRACT

ANAEROBIC CO-DIGESTION OF COW MANURE, FOOD WASTE AND WASTE ACTIVATED SLUDGE WITH *Trametes versicolor* PRE-TREATMENT UNDER MESOPHILIC CONDITION

Biological pre-treatment with fungal species such as *Trametes versicolor* using their extracellular enzymes leads to an improvement in biodegradation of lignocellulosic substrates and increases biogas production. In this study, cow manure (M), food waste (F) and waste activated sludge (W) were co-digested under mesophilic conditions with and without pre-treatment with *T. versicolor* captured in Ca-alginate beads. *T. versicolor* was incubated in the medium for 10 days and then it was encapsulated in Ca-alginate beads, and the pre-treatment process was conducted for the combination of substrates of MF, MW, FW and FMW. Following the biological pre-treatment, same amount of volatile solids-containing feedstock mixtures were inoculated with anaerobic seed sludge with an inoculum to substrate ratio of 2:1 (VS basis) and anaerobic co-digesters were set up. The results indicated that pre-treatment with *T. versicolor* led to an increase in methane yield for the combination of MF, MW, FW and FMW by 35%, 8%, 16% and 23%, respectively. Besides, the results showed that the food waste was the most significant substrate for improving methane yield. Moreover, volatile fatty acid concentrations in the digesters were at their highest level on the 3rd day of their operation and also significantly lower in the digesters that did not include food waste. These results can also be interpreted that the acidogenic phase was successfully accomplished within the first three days; the food waste led to the acceleration of the acidification stage.

ÖZET

Trametes versicolor İLE ÖNARITIM YAPILMIŞ OLAN BÜYÜKBAŞ HAYVAN ATIKLARININ, YEMEK ATIKLARININ VE AKTİF ÇAMURUN MEZOFİLİK ORTAMDA BİRLİKTE ANAEROBİK PARÇALANMASI

Trametes versicolor gibi hücre dışı enzimlerini kullanan mantarlarla yapılan biyolojik önarıtma, lignoselülozik substratların biyolojik parçalanmasında gelişmeye ve biyogaz üretiminin artmasına sebep olmaktadır. Bu çalışmada, büyükbaş hayvan atığı (M), yemek atığı (F) ve aktif çamur (W) mezofilik ortamda kalsiyum-alginat kürelerinin içine hapsedilmiş *T.versicolor* ile önarıtım yapılmış olarak ve önarıtım yapılmadan mezofilik ortamda birlikte anaerobik parçalanmıştır. *T. versicolor*, 10 gün boyunca besiyerinde büyütülmüş ve kalsiyum-alginat kürelerinin içine hapsedilmiştir. Önarıtma işlemi substratların bütün ikili ve üçlü kombinasyonlarına uygulanmıştır. Bu işlemden sonra anaerobik çürütücüler, aynı miktarda uçucu katı miktarı olacak şekilde ve inokülüm /substrat oranı 2:1 olacak şekilde (uçucu katı miktarına göre) kurulmuştur. Sonuçlara göre *T. versicolor* ile önarıtım, MF, MW, FW ve FMW kombinasyonlarının metan verimliliğini sırasıyla %35, %8, %16 ve %23 oranında artırmıştır. Ayrıca yemek atığı metan verimliliğine katkı açısından en önemli substrat olduğu görülmüştür. Çürütücülerdeki uçucu yağ asitleri konsantrasyonu en yüksek 3.günde görülmüş ve yemek atığı içermeyen çürütücüler, yemek atığı içeren çürütücülere göre daha az miktarda uçucu yağ asitleri konsantrasyonuna sahipti. Sonuçlar, asidojenik fazın ilk 3 günde başarıyla gerçekleştiği şeklinde yorumlanabilir; yemek atığı anaerobik parçalanmada asitleşmeyi hızlandırmıştır.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Unit
CH_4	Methane	mL
CO_2	Carbon Dioxide	
$CaCO_3$	Calcium Carbonate	mg/L
$CaCl_2$	Calcium Chloride	
$CuSO_4.5H_2O$	Copper Sulfate Pentahydrate	
HCl	Hydrochloric Acid	
H_2O	Water	
$FeSO_4.7H_2O$	Iron (II) Sulfate Heptahydrate	
KH_2PO_4	Monopotassium phosphate	
ml	Milliliter	
$MgSO_4.7H_2O$	Magnesium Sulfate Heptahydrate	
Na_2HPO_4	Disodium Hydrogen Phosphate	
µl	Microliter	
$ZnSO_4.7H_2O$	Zinc Sulfate Heptahydrate	

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
dH2O	Distilled Water	
DNA	Deoxyribonucleic Acid	
FW-	Food Waste and Waste Activated Sludge without Pre-treatment	
FW+	Food Waste and Waste Activated Sludge with Pre-treatment	
GC	Gas Chromatography	
GC-MS	Gas Chromatography-Mass Spectrometry	

MF-	Manure and Food Waste without Pre-treatment	
MF+	Manure and Food Waste with Pre-treatment	
MFW-	Manure, Food Waste, and Waste Activated Sludge without Pre-treatment	
MFW+	Manure, Food Waste, and Waste Activated Sludge with Pre-treatment	
MW-	Manure and Waste Activated Sludge without Pre-treatment	
MW+	Manure and Waste Activated Sludge with Pre-treatment	
PCR	Polymerase Chain Reaction	
PDA	Potato Dextrose Agar	
QIIME	Quantitative Insights Into Microbial Ecology	
Q-PCR	Quantitative PCR (Real-Time PCR)	
sCOD	Soluble Chemical Oxygen Demand	mg/L
SSYE	Soluble Starch Yeast Extract	
TKN	Total Kjeldahl Nitrogen	ppm
TS	Total Solids	mg/L
TVS	Total Volatile Solids	mg/L
VFA	Volatile Fatty Acid	mg/L

1. INTRODUCTION

Renewable energy sources are particularly important for securing long-term sustainable energy supplies and reducing local and global atmospheric emissions (Goldemberg and Teixeira, 2004). For this respect, biomass is the most common type of renewable energy which is produced from all organic material which comes from plants. It contains not only all land and water vegetation but also all organic wastes. Biomass is used for producing electrical/heat energy, chemical feedstock and transport fuel (McKendry, 2002).

Energy production is very important for Turkey because we are an energy importing country. Turkey has very limited petroleum-based fuel sources although she has rich biomass potential such as animal waste and agricultural waste. Besides, when the economical problems in Turkey are considered, renewable energy from biomass which is inexpensive energy source is important for Turkey to produce electrical/heat energy, chemical feedstock and transport fuel (Kaygusuz and Turker, 2002; Toklu, 2017; McKendry, 2002).

Anaerobic digestion is an important biotechnology to convert organic waste to valuable biogas (Neshat et al., 2017). Last decades, this technology has had rapidly growing because of new and stricter legislations on organic waste disposal, and the requirements of finding new energy sources in place of the fossil fuels (Lema and Omil, 2001; Lettinga, 2001). Anaerobic digestion not only reduces the volume of material to be disposed and prevents soil and groundwater pollution but also produces a renewable and inexpensive energy such as biogas. Biogas is not harmful to environment although fossil fuels are harmful. Besides, it maintains the concentration of greenhouse gasses (Esposito et al., 2012).

In this study, cow manure (M), food waste (F) and waste activated sludge (W) were co-digested under mesophilic conditions with and without pre-treatment with *T. versicolor* captured in Ca-alginate beads. The aim of this study was to show the importance and the efficiency of pre-treatment process with *Trametes versicolor* to increase methane production by enhancing hydrolysis stage. To do so, dual and triple substrate mixtures in anaerobic co-digesters compared to that of digesters without it. Moreover, this study led to comparing different combinations of substrates of anaerobic co-digestions (MF, MW, FW, MFW) in terms of amount of biogas/methane production and VS removal under mesophilic condition.

2. LITERATURE REVIEW

2.1. Definition of Anaerobic Digestion

Anaerobic digestion is a microbial conversion from organic compounds into biogas which is a renewable energy source. Therefore, it provides not only waste disposal but also biogas production (Esposito et al, 2012). Moreover, electrical/heat energy, chemical feedstock, and transport fuel can be produced using anaerobic digestion (McKendry, 2002). Also, it is important for maintaining the concentration of greenhouse gases by using biomass in anaerobic digestion that is an alternative renewable source (Esposito et al, 2012).

A wide range of substrates/leftovers that are coming from industries, agriculture animal husbandries and forest residues etc. can be used. The most common substrates are animal manure, agricultural residues, sewage sludge, dedicated energy crops, organic fraction of municipal waste and digestable organic wastes from food and agro industries (Adekunle and Okolie, 2015). Some of the important substrates and final products in anaerobic digestion are shown in Figure 2.1.

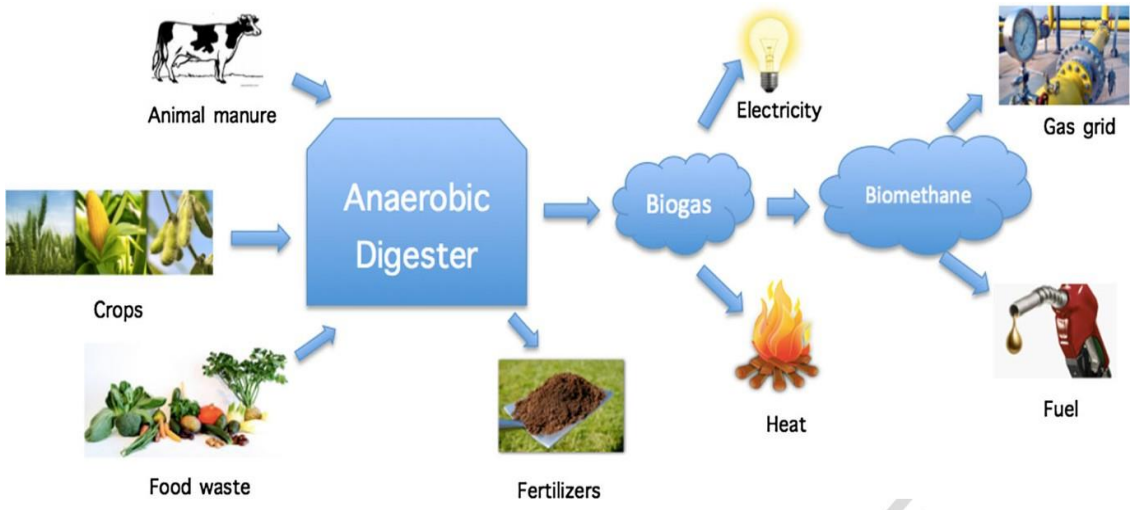

Figure 2.1. The important substrates and final products in anaerobic digestion process (Bozan et al., 2017).

Biogas that is coming from anaerobic digestion by microorganisms includes approximately 50-60% methane and 30-35% carbon dioxide with rare amounts of hydrogen, nitrogen, water vapor, and hydrogen sulfide (Fitzgerald, 2013; Tsavkelova and Netrusov, 2012). Ultimate methane yield is related to the biodegradability of the organic compounds (Pesta, 2007).

2.2. Biochemistry and Microbiology of Anaerobic Digestion

There are 4 stages in anaerobic digestion: hydrolysis, acidogenesis, acetogenesis and methanogenesis, as shown in Figure 2.2. Different groups of microorganisms work in each group (Gerardi, 2003). In anaerobic digestion process, fermentative bacteria, syntropic acetogens and methanogens are found, which is displayed in Figure 2.3. Besides, the diversity of microorganisms and their work rate depend on environmental conditions such as pH, alkalinity, temperature, ammonium concentration, hydraulic retention time, organic loading rate and the substrate characteristics.

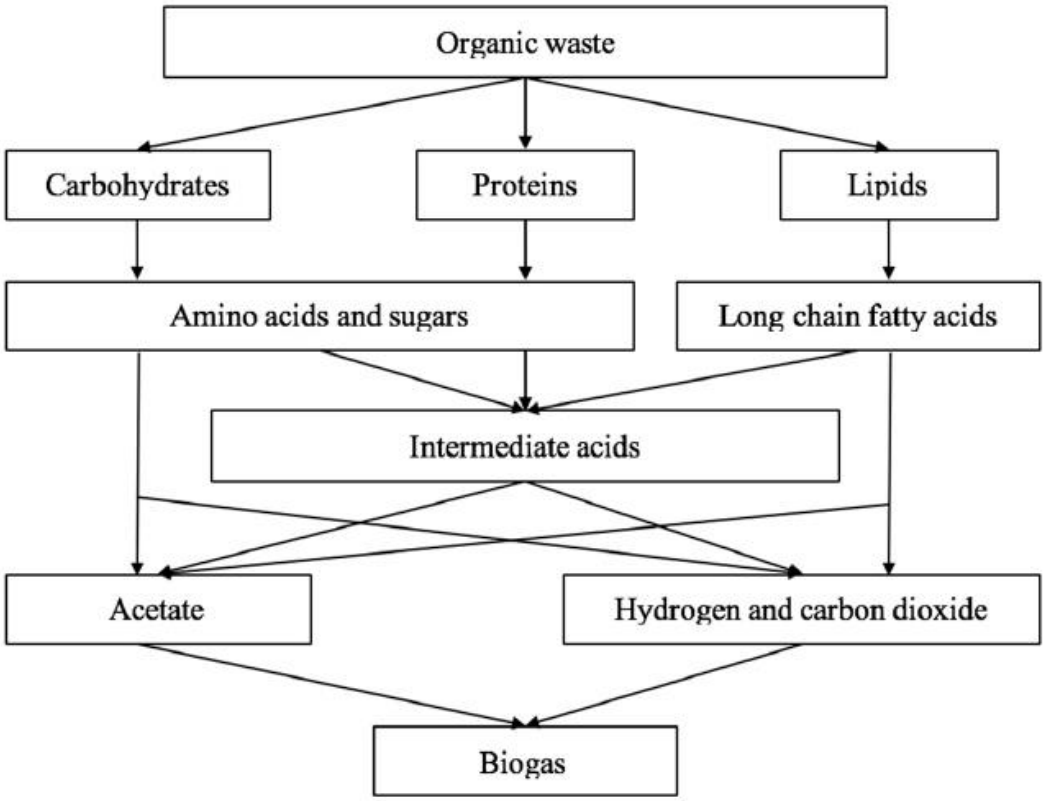

Figure 2.2. Four steps in the anaerobic digestion process (Meegoda et al., 2018).

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*

Figure 2.3. The diversity of microorganisms in anaerobic digestion (Korres, 2013).

2.2.1. Hydrolysis

In hydrolysis step, fermentative microorganisms hydrolyze the complex organic compounds such as carbohydrates, lipids, proteins into monosugars, fatty acids and amino acids by the extracellular enzymes of the hydrolytic bacteria. Hydrolysis is a very important because it can determine the rate of anaerobic digestion process. For methanogenic bacteria, the successful completion of hydrolysis step of anaerobic digestion is important (Pesta, 2007; Meegoda et al., 2018). Moreover, the degradation of lignocellulose is hard. Therefore, some pre-treatment methods can be used for the hydrolysis of lignocellulose efficiently (Jorgensen et al., 2007).

When we examine the hydrolysis step in terms of microbiology, hydrolytic bacteria which is also called fermentative bacteria hydrolyze the organic compounds. These fermentative bacteria can be strict anaerobic bacteria or facultative anaerobic bacteria. *Clostridium* spp. and *Bacillus* spp. are the example of hydrolytic bacteria (Manyi-Loh et al., 2013).

2.2.2. Acidogenesis

In acidification step, the intermediates come from hydrolysis are converted into volatile fatty acids, alcohols and carbon dioxide gas. This step is also related to fermentative microorganisms. Acetic acid, propionic acid and butyric acid are the most common volatile fatty acids in anaerobic

digestion (Pesta, 2007; Meegoda et al., 2018). When the concentration of VFA increases significantly, the pH value of digester decreases, which can damage the methanogens in anaerobic digesters. Therefore, methane production can be affected negatively (Franke-Whittle et al., 2014).

Acidogenic bacteria works in the acidogenesis part of anaerobic digestion, when the microbial community of acidogenesis is studied. These bacteria are also fermentative bacteria that can be strictly anaerobic or facultative anaerobic bacteria (Manyi-Loh et al., 2013). For example, *Clostridia* can produce acetate, acetone, butanol, butyrate, ethanol, lactate, carbon dioxide, and hydrogen is the one of the most important example of acidogenic bacteria. Moreover, *Butyrivibrio* can produce butyrate in anaerobic digestion (Gerardi, 2003).

2.2.3. Acetogenesis

In acetogenesis step, the end products of acidification process which are mainly volatile fatty acids are converted into carbon dioxide and short chained volatile fatty acids such as acetate. This step works at a low concentration of hydrogen which is produced from the acetogenesis step. Therefore, methanogenic bacteria and acetogenic bacteria live in symbiosis by transferring hydrogen to each other (Pesta, 2007).

In acetogenesis, acetogenic bacteria is very important for producing acetate. Most acetogens are in the phylum *Firmicutes* (Korres, 2013). Acetogens such as genera of *Syntrophomonas* and *Syntrophobacter* convert the volatile fatty acids into acetate and hydrogen. For example, *Methanobacterium propionicum* converts propionic acids into acetic acid. After acetogenesis, hydrogen that is toxic for some microorganisms is released. Therefore, symbiosis is very important for acetogenic bacteria, and methanogenic bacteria live in symbiosis with these acetogenic bacteria in anaerobic digestion (Pesta, 2007; Shah et al., 2017). Moreover, acetogenesis steps also show the efficiency of anaerobic digestion because approximately 70% of methane production generally comes from acetate (Shah et al., 2017; Manyi-Loh et al., 2013).

2.2.4. Methanogenesis

Methanogenesis is the final step of anaerobic digestion. In methanogenesis, methanogenic bacteria produce methane and carbon dioxide which are related to biogas from acetate. In this process, the level of oxygen inhibits the metabolisms of the microorganisms because these organisms are strictly anaerobic (Pesta, 2007). Moreover, methanogenic bacteria are very susceptible to pH range,

and the optimum pH for them at between 6.5 and 7.2. Although fermentative bacteria can live wider pH range such as pH of 5.0 and 8.0.(Boe, 2006; Appels et al., 2008).

When the methanogenesis part of anaerobic digestion is analyzed, the production of methane is done by methanogens that is belong to domain *Archaea*. These methanogens that are exclusively anaerobic play important role in anaerobic digestion by producing valuable methane. *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, Methanocellales,* and *Methanopyrales* are the six orders of methanogens (Manyi-Loh et al., 2013; Korres et al., 2013). Moreover, 70% of methane production comes from acetate (Shah et al., 2017; Manyi-Loh et al., 2013). Besides, these methanogens that are very susceptible of the pH change use hydrogen that comes from acetogenesis, which balances the pH for microorganisms in digester thanks to their symbiotic lives (Boe, 2006; Appels et al., 2008; Pesta, 2007; Shah et al., 2017).

2.3. Important Parameters of Anaerobic Digestion

Environmental conditions such as nutrients content, temperature, pH, carbon/phosphorus (C/P), carbon/nitrogen (C/N), inhibitors, typology of substrates, microelements and particle size affect the anaerobic digestion process (Esposito et al, 2012).

2.3.1. Digestion Time

The degradation of lignocellulosic substrates is hard because lignin, cellulose and hemicellulose are joined together. Therefore, the decomposition of lignin efficiently leads to producing higher methane from anaerobic digestion, and then the digestion time is an important parameter for microorganisms in anaerobic digestion (Jorgensen et al, 2007). The digestion time for lignocellulosic substrates are generally 40 days in anaerobic digestion process (Chen and Neibling, 2014; Ali et al., 2018). When it decreases, the reactions in digesters also decrease. For example, when digestion time is shorter than 5-8 days, the degradations of compounds especially lipids are not finished. Stable digestion can be gained after 10 days when all compounds are significantly reduced (Appels et al., 2008).

TVS consumption is also related to the digestion time. TVS consumption is an important parameter for the efficiency of anaerobic digestion because TVS shows the organic fraction of total solids, and this organic fraction is converted into biogas by microorganisms. Therefore, TVS

consumption increases when the digestion time increases, which supports that TVS consumption depends on the digestion time (Meegoda et al., 2018).

2.3.2. Solid Loading

Dry digesters have approximately 25-40 % TS, and wet digesters have approximately 25-40 % TS in anaerobic digestion (Luning et al., 2003). In addition to the amount of total solids, the amount of total volatile solids ratio which is the organic part of solids is also significant parameter because when the organic solid increases, microorganisms can degrade more organics. Therefore, more organic compounds or more total volatile solids (TVS) lead to an increase in biogas production in anaerobic digesters. Moreover, the stability of anaerobic digester is affected by a decrease in the organic solids (Appels et al., 2008; Meegoda et al., 2018). Therefore, some digester systems are fed with organics continuously (Gomez et al., 2006). Moreover, overloading a digester causes more accumulation of VFA, which affects methanogens negatively (Franke-Whittle et al., 2014).

2.3.3. Temperature

Temperature has vital role in anaerobic digestion process. It affects the microorganisms' growth rates and metabolisms. Some methanogens such as Acetotrophic methanogens are very sensitive to the change of the temperature. Besides, the degradations of some compounds that are found in the steps of anaerobic digestion such as propionate and butyrate are also negatively affected above 70 °C (Appels et al., 2008). In mesophilic digestion (35 °C) provides slower reaction rate and lower biogas production. However, it is cheaper than thermophilic digestion (Moset et al., 2015; Meegoda et al., 2018). On the other hand, when temperature increases, the solubility of organic compounds, biological and chemical reaction rate also increases (Appels et al., 2008; Hartmann and Ahring, 2006; Meegoda et al., 2018). Thermophilic digestion (55 °C) uses higher temperature. Therefore, reaction rates increase, and it triggers more biogas production (Hartmann and Ahring, 2006; Meegoda et al., 2018). Besides, removal of pathogen can be done thanks to higher temperature (Smith et al., 2005). Nevertheless, very high temperature causes higher free ammonia that is an inhibitor for microorganisms (Wu et al., 2006; Appels et al., 2008). Therefore, the stability and control process are more important for thermophilic digestion (55 °C) than mesophilic digestion (35 °C). In conclusion, stable temperature is a very significant condition because the fluctuation of temperature damages microorganisms, especially methanogens (Appels et al., 2008).

2.3.4. pH, Alkalinity and Volatile Fatty Acids

Microorganisms are affected by pH range in anaerobic digestion. For example, methanogenic bacteria that produce methane are very susceptible to pH range. The optimum pH for methanogenic bacteria is between 6.5 and 7.2. However, fermentative bacteria can adapt to between pH of 4.0 and 8.5 which is a wider range of pH (Boe, 2006; Appels et al., 2008). Low pH and high pH also affect the volatile fatty acid products. For example, main products are acetic acid and butyric acid at a low pH although acetic acid and propionic acid are the main products at a higher pH (Boe, 2006).

Volatile fatty acids lead to a decrease in pH because of acidification of anaerobic digestion. This condition reduces the biogas production as low pH affects methanogenic bacteria. However, alkalinity maintains the pH value thanks to carbon dioxide, ammonia and bicarbonate (Appels et al., 2008). Even if carbon dioxide level stays constant, the accumulation of bicarbonate alkalinity can increase the pH of the digesters (Appels et al., 2008; Turovskiy and Mathai, 2006). Therefore, alkalinity monitoring should be important for the stability of anaerobic digestion because it maintains the change of pH.

2.3.5. Carbon/Nitrogen Ratio

Carbon/Nitrogen ratio (C/N) is an important value for anaerobic digestion. It shows the characterization of substrates. Carbohydrates, lipids and proteins in substrates determine the C/N ratio. Carbon level is crucial for microorganisms for digestion. However, nitrogen level is also important to make protein formation for microorganisms (Meegoda et al., 2018). Moreover, the most efficient C/N ratio is near 15 (Zhang et al, 2013; Heo et al,2003; Heo et al, 2004; Liu et al, 2013). Therefore, co-digestion can be used for adjusting C/N ratio. Besides, mixing ratio is also important for the ideal C/N ratio (Zhang et al, 2013; Heo et al,2003; Heo et al, 2004; Liu et al, 2013).

2.3.6. Free Ammonia Nitrogen

Free ammonia nitrogen (FAN) is an inhibitory to anaerobic digestion. It can be produced from proteins and urea during biological hydrolysis of substrates. When the concentration of free ammonia nitrogen is higher the threshold level of it, anaerobic digestion process takes serious damages in terms of stability because microorganisms are affected badly by ammonia inhibition. Therefore, it leads to a decrease in biogas and methane yields, which causes the failure of anaerobic digestion. According to reports in the literature, total ammonium nitrogen (TAN) concentration from 1.7 g/L to 14 g/L

leads to a 50% decrease in methane production (Chen et al., 2008; Yenigün and Demirel, 2013). Moreover, using pre-treatment techniques before anaerobic digestion contributes to a decrease in the possible inhibitory effects of free ammonia nitrogen (Yenigün and Demirel, 2013).

2.4. Feedstocks and Digester Systems for Anaerobic Digestion

2.4.1. Feedstocks

There are lots of substrates for the biogas production during anaerobic digestion system. These substrates provide energy sources for microorganisms to make new cells. The compositions of substrates are very important for digester in terms of nutrients and buffer capacity. Moreover, substrates affect the process stability and biogas production. The most common substrates are animal manure, agricultural residues, sewage sludge, dedicated energy crops, organic fraction of municipal waste and digestable organic wastes from food and agro industries (Adekunle and Okolie, 2005).

Cow manure includes high organic matter, nitrogen and phosphorous concentration. Therefore, it can cause some environmental problems such as air pollution because of volatilization of ammonia and other compounds, eutrophication of water, and soil degradation (Won et al, 2017; Neshat et al, 2017). Cow manure can be used as a substrate for anaerobic digestion. Besides, it has high buffer capacity (Li et al, 2009). However, it has low C/N ratio that leads to insufficient biogas production. Therefore, co-digestion is important for compensating carbon deficiency (Neshat et al, 2017).

Food waste includes high organic water content, high biodegradability and lots of nutrient elements. It has high C/N ratio and abundant organic matters. However, it causes unbalanced fermentation because of high organic particulate matter content. Besides, at early digestion process, soluble organics are converted to volatile fatty acids rapidly (Li et al, 2016; (Liu et al, 2013). Besides, it has low buffer capacity and it can be easily acidified (Li et al, 2009).

Although the treatment and disposal of waste activated sludge is an important problem for wastewater treatment plants, it contains largely of water, microorganisms and organic and inorganic matters in waste activated sludge, Therefore, it can be used for biomass in anaerobic co-digestion (Liu et al, 2013). Moreover, anaerobic digestion is commonly used as a sludge stabilization technique. However, the hydrolysis step of waste activated sludge has limitations in terms of rate. Therefore, several pre-treatments can be used to increase hydrolysis and anaerobic digestion performance for lysing sludge cells (Athanasoulia et al, 2012). Moreover, hydrogen and methane yield are so low

when waste activated sludge is used because it has low C/N ratio. Therefore, co-digestion is important to increase C/N ratio and biogas production by adjusting nutrient balance (Liu et al, 2013).

The combination of different organic wastes causes an increase in methane production when the substrates mixture is prepared with appropriate mixing ratios. The advantage of the co-digestion is generally related to the optimization of the nutrient balance in the substrates between nitrogen rich substrates and carbon rich substrates, which shows positive synergism. Therefore, it can provide higher methane yield. Operation conditions are also important for bio-methane potential. When the cow manure is used as mono-substrate, it was unstable because of the low C/N ratio. C/N ratio is the important parameter for the digestion process. This ratio is acceptable range from 13.9 to 19.6. Therefore, co-digestion can enhance the stability of the anaerobic process because of better C/ N balance. Besides, co-digestion may decrease the inhibitory effect of high ammonia and sulfide concentrations, and co-digestion can produce more stable biogas production thanks to better buffer capacity (Esposito et al, 2012; Zhang et al, 2013; Li et al, 2009; Kumar et al, 2010; Hartmann et al, 2003; El- Mashad and Zhang, 2010; Nayono et al, 2010).

2.4.2. Anaerobic Digester Systems

There are various digester types for anaerobic digestion in terms of substrate feeding, operating temperature, the amount of total solids, scale of digester and anaerobic digestion process complexity (Korres, 2013).

Dry digesters have high solids (>20-40 % TS) although wet digesters have low total solids (<10-20 % TS) (Angelonidi and Smith, 2015). The advantages of dry digesters are higher biomass retention, simple pre-treatment and controlled feeding and spatial niches. However, complexity and expensiveness, difficult handling and mixing, and using only structured material are the disadvantages of dry digesters. Scum formation during crop digestion, high consumption of water and energy, sensitivity to shock and short-circuiting are the advantages of wet digesters although dilution of inhibitors are the advantage of wet digesters (Nizami and Murphy, 2010; Vandevivere et al., 2003; Korres, 2013).

Batch and continuous digesters are related to substrate feeding. In batch digesters, mixing, stirring and pumping are not necessity. Moreover, low cost and low input in terms of process and mechanical demands are the advantages of batch digesters. However, channeling and clogging, larger volume and lower biogas yield are the disadvantages of batch digesters. In continuous digester,

simplicity and lower cost are related to its advantages although rapid acidification and larger VFA production are the disadvantages of it (Nizami and Murphy, 2010; Vandevivere et al., 2003; Korres, 2013).

Single stage digester and two-stage digester are related to the complexity of anaerobic digestion process. Simpler design with less technical failure is the advantage of single stage digester although its higher retention time and foam and scum formation are the disadvantages of one-stage digester. However, in two-stage digester, increased overall degradation because of recirculation, constant feeding rate to methanogenic stage and more resistant and less susceptible to failure are the advantages of two-stage digester although complexity, expensiveness and removal solid particles from the feedstock in the second stage are the disadvantages of two-stage digester (Nizami and Murphy, 2010; Vandevivere et al., 2003; Korres, 2013).

2.5. Techniques for Improving Methane Production

2.5.1. Pre-treatment Techniques in Anaerobic Digestion

Plant biomass includes cellulose, hemicellulose, lignin, pectin, protein and ash. Lignocellulose that is composed of up to %75 carbohydrates will be important in terms of an essential source for the fermentation of carbohydrates (Jorgensen et al., 2007). Cellulose, hemicellulose and lignin are joined together to provide integrity and rigidity (Kuhad et al., 1997). The degradation of lignocellulose is hard because decomposition of lignin is very slow process, and the hydrolysis step of anaerobic digestion can be rate-limiting step (Jorgensen et al., 2007; Ariunbaatar et al., 2014). To solve this problem, there are some pre-treatments to remove and degrade the hemicellulose for accelerating the hydrolysis step in anaerobic digestion (Carlsson et al., 2012; Jorgensen et al., 2007; Ariunbaatar et al., 2014). Then, pre-treatment is a significant process for anaerobic digestion. It limits loss of sugars, use of energy and chemicals. Besides, it maximizes the enzymatic convertibility and the production of other valuable by-products such as lignin (Jorgensen et al, 2007).

2.5.1.1. Physical pre-treatment. Grinding and milling, ultrasonic, centrifugal grinding and extrusion pre-treatments are related to physical pre-treatments. These techniques are used for reducing particle size with an increase in external surface area (Ravindran and Jaiswal, 2015). It has been displayed that the radius of larger particle leads to lower chemical oxygen demand removal and lower methane production (Esposito et al., 2011).

2.5.1.2. Chemical pre-treatment. Dilute acid pre-treatment, acid-acetone pre-treatment, ionic liquids pre-treatment, alkaline potassium permanganate pre-treatment, organosolv pre-treatment and metal chloride pre-treatment are the types chemical pre-treatments that can be used for anaerobic digestion (Ravindran and Jaiswal, 2015). Chemical pre-treatment causes the removal of lignin significantly (Ariunbaatar et al., 2014; Ravindran and Jaiswal, 2015). However, it is not proper for easily biodegradable substrates that include high amounts of carbohydrates because high amounts of VFA coming from powerful hydrolysis step harm the microorganisms which are important for methanogenesis step to methane production (Wang, 2011).

2.5.1.3. Physico-chemical pre-treatment. Steam explosion, hot water pre-treatment, wet oxidation, ammonia fibre expansion, super critical CO_2 explosion, IHRW pre-treatment and plasma pre-treatment are the types of physico-chemical pre-treatment (Ravindran and Jaiswal, 2015). For example, in steam explosion, high pressure and temperature are used for decomposition of biomass especially lignin breakdown. (Ravindran and Jaiswal, 2015).

2.5.1.4. Biological pre-treatment and usage of Ca-alginate beads. Biological pre-treatment is related to biological agents to remove lignin from the biomass. Biological pre-treatment does not include high temperature or pressure, and it does not need any acids, alkali and reactive species although physical and chemical pre-treatments need them. Moreover, there are not any undesirable products thanks to biological pre-treatment (Ravindran and Jaiswal, 2015). Therefore, biological pre-treatment such as bacteria and fungi does not need any chemicals although the chemical and physicochemical pre-treatment methods have, which shows that biological pre-treatment is an environmental friendly pre-treatment that converts lignocellulosic mass to biogas production by fungi (Haghighi Mood et al, 2013). Moreover, the most important microorganisms in terms of cellulose removal are fungi (Madadi and Abbas, 2017; Kumar and Wyman, 2009). There are three main groups in fungi for biodegradation of biomass. These are white-rot fungi, brown-rot fungi and soft-roft fungi (Isroi et al., 2011).

White rod fungi which can be used for biological pre-treatment are so important for efficiently removing lignin from the biomass thanks to producing various extracellular enzymes such as laccase, manganese peroxidase and lignin peroxidase (Nagai et al., 2007; Madadi and Abbas, 2017). White rod fungi can remove lignin faster than other organisms (Madadi and Abbas, 2017). Therefore, this biological pre-treatment that uses white rod fungi is a very crucial technique for energy production from lignocellulose. Besides, it needs low energy, mild environmental conditions and less environmental damage (Zhang et al, 2007 and Haghighi Mood et al., 2013). Moreover, biological pre-treatment with fungal species such as *Trametes versicolor* by using their extracellular enzymes

leads to an improvement in the biodegradation of lignocellulosic substrates and increases biogas production (Isroi et al., 2011, Bozan, 2018).

Ca-alginate beads are used for the immobilization of microorganisms. It has linear copolymer that includes D-mannuronic acid and L-guluronic acid (Whistler and Kirby, 1959; Hirst and Rees, 1965), and it is displayed in Figure 2.4. Ca is used for biotechnological purposes because Ca is not toxic. The entrapment of alginate is a safe and simple method for immobilizing any cells such as yeast, fungi, animal cells, bacteria and higher plant cells. Besides, in this entrapment with Ca-alginate beads, maximum catalytic activity of cells maintains (Nussinovitch, 2010). Aluminum nitrate can be used to strengthen for beads (Rochefort et al,1986). This technique can be also used as color and dye problems. For example, Pallerla and Chambers (1997), Dominguez et al. (2007) and Li et al. (2015) used *T. versicolor* in Ca-alginate beads for removing dye and color thanks to the extracellular enzymes of *T. versicolor*. Moreover, Bozan (2018) used *T. versicolor* in Ca-alginate beads for the pre-treatment of the biomass that includes macroalgae and corn.

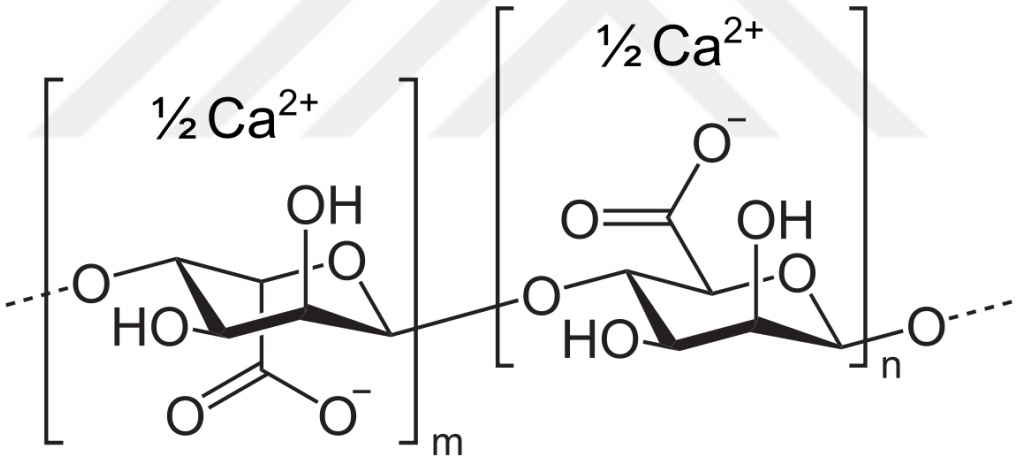

Figure 2.4. The chemical notation of Ca-alginate.

2.5.2. Bioaugmentation

Bioaugmentation is the addition of specific microorganisms to anaerobic system to improve the methane yield (Li et al., 2018). Previous studies showed that using bioaugmentation had some advantages for anaerobic digestion which were shorter start-up period (Lins et al., 2014), shorter hydraulic retention time (Baek et al., 2016; Lebiocka et al., 2018), and an increase in methane production (Öner et al., 2017; Nkemka et al., 2015; Lebiocka et al., 2018).

3. MATERIALS AND METHODS

3.1. Sampling and Characterization

Seed sludge was taken from a full-scale anaerobic digester fed with cattle manure at an Integrated Dairy Plant in Bursa, Turkey. Fresh cow manure was taken from a barn of Veterinary School, Istanbul University. Waste activated sludge was taken from Advanced Biological Wastewater Treatment Plant, Atakoy, Istanbul. Finally, food waste (including rice, haricot bean and zucchini hash browns and yoghurt) was taken from a dining hall at Boğaziçi University. Food waste was mixed throughly for being homogeneous before storage. All these substrates were stored at +4°C. Then, their characterization studies were carried out using Standard Methods (APHA, 2005).

3.2. Growth of *Trametes versicolor*

Trametes versicolor ATTC 42530 was bought from American Type Culture Collection (ATTC), and it was used as a white-rot fungus. *Trametes versicolor* ATTC 42530 was stored at -80°C.

Potato dextrose agar (PDA) (Bioshop, Canada Inc.) was used for the cultivation of *Trametes versicolor* ATTC 42530 for 10 days at 25°C. PDA plate is shown in Figure 3.1. Then, the optimum media was prepared for the growth of *T. versicolor*, and its ingredients were shown in Table 3.1. This media improved the production of laccase for fungi because of including starch and copper sulfate (Revankar and Lele, 2006; Kocyigit et al., 2012; Bozan, 2018). After the dissolution of soluble starch in distilled water completely thanks to microwave, the other materials were added according to Table 3.1. Then, the final pH was fixed to 5, and distilled water was added to the mixture to adjust water level to 1 L. Finally, the optimum media was sterilized by an autoclave at 121°C for 15 min.

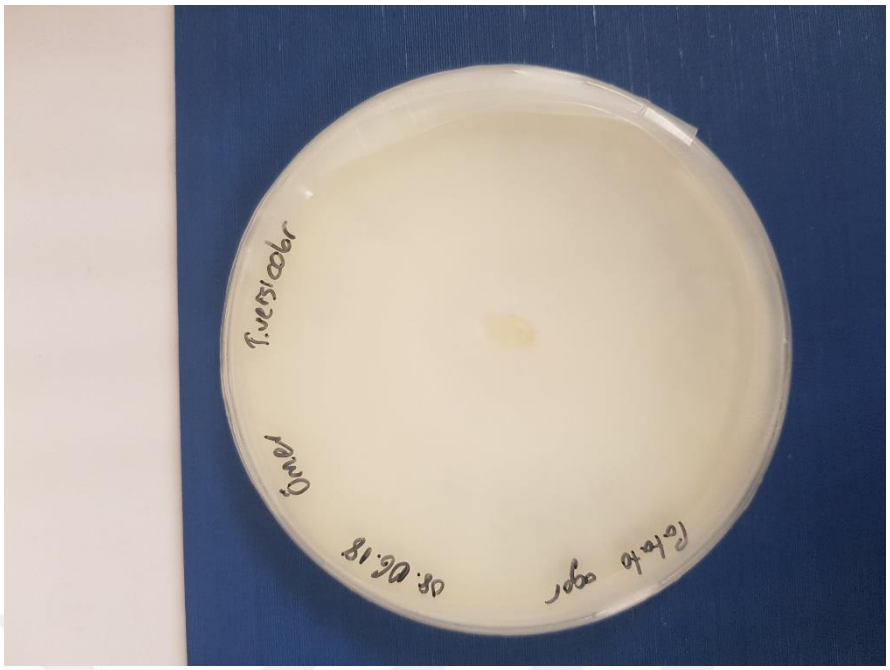

Figure 3.1. PDA plate for the cultivation of *T. versicolor*.

Table 3.1. Optimum medium for *T. versicolor*.

Components	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

After the preparation of the optimum medium, three discs were taken from PDA in a sterile area and they are shown in Figure 3.2. Then, they were inoculated to 1 L optimum medium in a sterile area. Then, the media was left for 10 days at 25°C at 120 rpm.

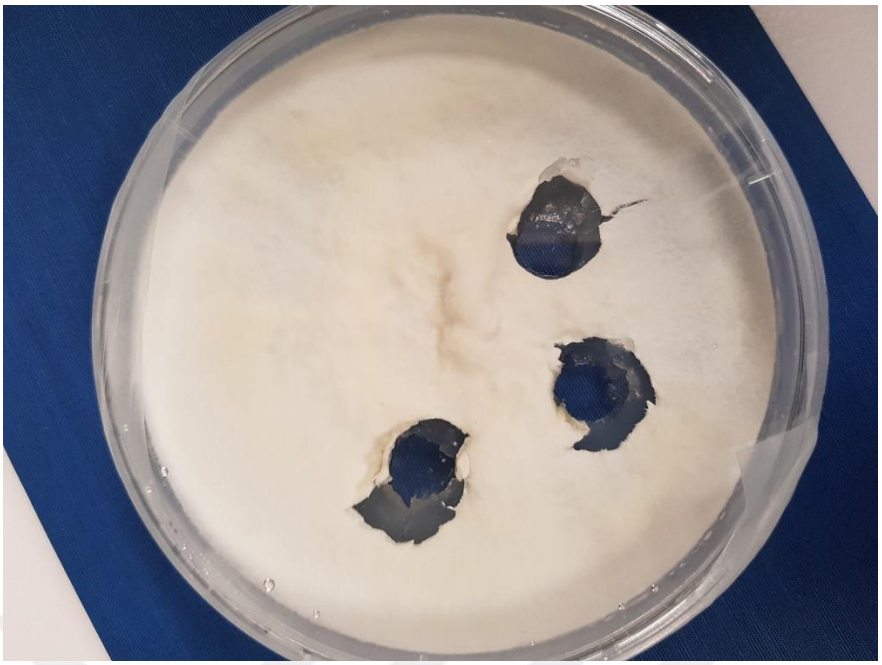

Figure 3.2. Three discs including *T. versicolor* from PDA.

3.3. Entrapment of *T. versicolor* into Ca-alginate Beads

The optimum medium with *T. versicolor* was homogenized by centrifugation at 10,000 rpm for 15 minutes. Then, it was washed by distilled water, and re-centrifuged at 10,000 rpm for 15 minutes. After the second centrifugation process, it was re-washed by distilled water then, it was mixed by a blender for 10 seconds under sterile conditions. Finally, the biomass was sterilized by autoclave at 121°C for 15 minutes (Bozan, 2018).

3g Na-alginate which was previously sterilized was added into 80 ml dH_2O, and this mixture was mixed with 20 ml biomass which was homogenized to adjust 3% Na-alginate concentration. The final mixture was pumped into 2% $CaCl_2$ solution by Pasteur pipette to make Ca-alginate beads. These beads including *T. versicolor* were left into 2% $CaCl_2$ solution for 1 hour. Besides, empty Ca-alginate beads were prepared without entrapment by using the same methods which included distilled water instead of homogenized fungi cells. Then, in a sterile condition, entrapment of *T. versicolor* into Ca-alginate beads was accomplished (Bozan, 2018). The sequence of work is shown in Figure 3.3.

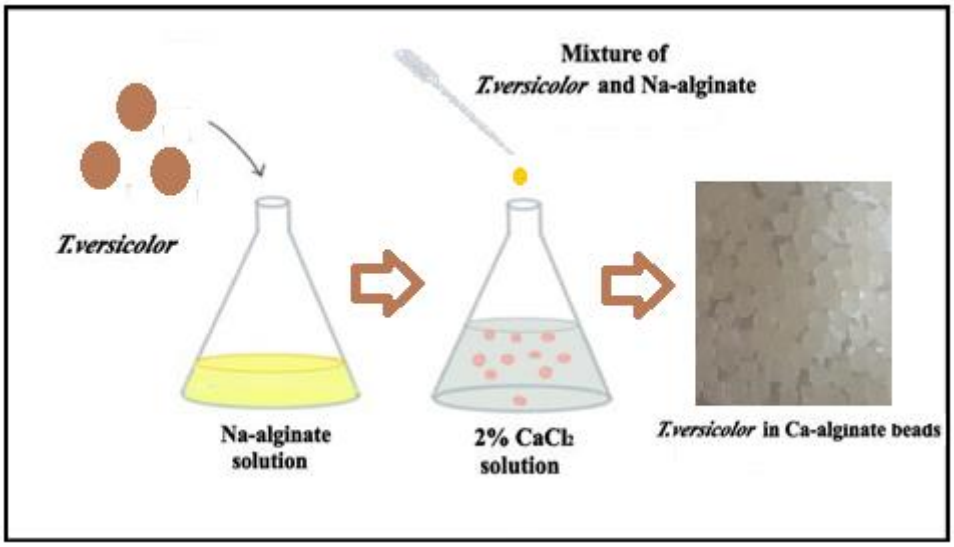

Figure 3.3. Entrapment of *T. versicolor* in Ca-alginate beads.

3.4. Pre-treatment of Substrates by Entrapped Cells

Firstly, beads containing *T. versicolor* was incubated in optimum medium at 25°C at 120 rpm for 3 days. Then, the amounts of cow manure, food waste and waste activated sludge were calculated for set-up of anaerobic batch digesters and pre-treatment process. This calculation was related to 7 replicates that were 80 ml for each anaerobic batch digester. Therefore, the amounts of substrates for pre-treatment were calculated and prepared, which are shown in Table 3.2 and Table 3.3.

Substrates were added into 1 L Erlenmeyer flask for pre-treatment by *T. versicolor* captured in Ca-alginate beads. 8 flasks were prepared according to Table 3.3., and they were labeled as MF- (Manure and Food Waste without Pre-treatment), MW- (Manure and Waste Activated Sludge without Pre-treatment), FW- (Food Waste and Waste Activated Sludge without Pre-treatment), MFW- (Manure, Food Waste and Waste Activated Sludge without Pre-treatment), MF+ (Manure and Food Waste with Pre-treatment by *T. versicolor*), MW+ (Manure and Waste Activated Sludge with Pre-treatment by *T. versicolor*), FW+ (Food Waste and Waste Activated Sludge with Pre-treatment by *T. versicolor*) and MFW+ (Manure, Food Waste and Waste Activated Sludge with Pre-treatment by *T. versicolor*). These flasks were autoclaved at 121°C for 15 minutes. Then, 20 ml Ca-alginate beads including *T. versicolor* were added into flasks which were related to pre-treatment process under sterile conditions. Besides, 20 ml empty Ca-alginate beads which did not include *T. versicolor* were added into the other flasks which were related to without pre-treatment under sterile conditions. Then, these flasks were incubated at 25°C at 120 rpm for 5 days. During this process, laccase enzyme activity in flasks which were related to pre-treatment was measured for 5 days.

Table 3.2. The amounts of substrates and seed sludge for each digester.

	Digesters	Cow Manure (g)	Food Waste (g)	Waste Activated Sludge (g)	Anaerobic Sludge (ml)
Without Pre-treatment	Manure and Food Waste	3.1	5.6	-	50.0
	Manure and Waste Activated Sludge	14.0	-	18.0	50.0
	Food Waste and Waste Activated Sludge	-	5.9	26.0	50.0
	Manure, Food Waste and Waste Activated Sludge	2.7	4.8	24.0	50.0
Pre-treatment by *T. versicolor*	Manure and Food Waste	3.1	5.6	-	50.0
	Manure and Waste Activated Sludge	14.0	-	18.0	50.0
	Food Waste and Waste Activated Sludge	-	5.9	26.0	50.0
	Manure, Food Waste and Waste Activated Sludge	2.7	4.8	24.0	50.0
Control	Seed Sludge	-	-	-	50.0

Table 3.3. The total amounts of each substrate for pre-treatment process.

	Digesters	Cow Manure (g)	Food Waste (g)	Waste Activated Sludge (g)	Water (ml)
Without Pre-treatment	Manure and Food Waste	21.9	39.2	-	168.0
	Manure and Waste Activated Sludge	98.0	-	126.0	-
	Food Waste and Waste Activated Sludge	-	41.6	182.0	-
	Manure, Food Waste and Waste Activated Sludge	18.9	33.7	168.0	-
Pre-treatment by *T. versicolor*	Manure and Food Waste	21.9	39.2	-	168.0
	Manure and Waste Activated Sludge	98.0	-	126.0	-
	Food Waste and Waste Activated Sludge	-	41.6	182.0	-
	Manure, Food Waste and Waste Activated Sludge	18.9	33.7	168.0	-

3.5. Set-up of Anaerobic Batch Digesters

Inoculum to substrate ratio was adjusted to 2:1 (g TVS) in all batch digesters (Raposo et al., 2006; Labatut et al., 2011; Akyol et al., 2016). Moreover, mixing ratios for substrates were determined according to the C/N ratio of anaerobic co-digestions (Esposito et al., 2012; Zhang et al., 2013; Li et al., 2009; Kumar et al., 2010; Hartmann et al., 2003; El-Mashad and Zhang, 2010; Nayono et al., 2010). The volume of serum bottles used were 120 ml with an active volume of 80 ml. According to these values, F:M was adjusted to 4:1, M:W was adjusted to approximately 8:1, F:W was adjusted to approximately 6:1, and F:M:W was adjusted to approximately 20:5:4. 7 replicate serum bottles were used for each digestion set, and every bottle included 3.43 g TVS seed sludge and 1.715 g TVS substrate. The amounts of substrates in bottles are shown in Table 3.2.

After pre-treatment process, each flask was transferred into new 7 bottles. These bottles including manure and food waste without pre-treatment; manure and waste activated sludge without pre-treatment; food waste and waste activated sludge without pre-treatment; manure, food waste and waste activated sludge without pre-treatment; manure and food waste with pre-treatment; manure and waste activated sludge with pre-treatment; food waste and waste activated sludge with pre-treatment; manure, food waste and waste activated sludge with pre-treatment were labeled as MF-, MW-, FW-, MFW-, MF+, MW+, FW+ and MFW+, respectively. Then, 50 ml of seed sludge was added into all serum bottles. Therefore, 80 ml of serum bottles were prepared, and pH value of each bottle was set to 7.2± 0.2. The lids of serum bottles were closed, and the metal crimpers were closed over the lids for bottles. Finally, N2 was given to all bottles for 2 minutes to supply anaerobic condition to microorganisms. These bottles were incubated at 37°C at 120 rpm for 40 days. On the 0th, 3rd, 6th, 10th, 20th, 30th, 40th days, one bottle was opened and discarded from the 7 digesters of each set. The combinations of substrates are shown again in Table 3.4.

Table 3.4. The combinations of substrates in digesters.

Components of Digesters	BIOREACTORS								
	MF-	MW-	FW-	MFW-	MF+	MW+	FW+	MFW+	Seed Sludge (Control)
Pre-treatment	-	-	-	-	+	+	+	+	-
Seed Sludge	+	+	+	+	+	+	+	+	+
Waste Activated Sludge	-	+	+	+	-	+	+	+	-
Food Waste	+	-	+	+	+	-	+	+	-
Cow Manure	+	+	-	+	+	+	-	+	-

3.6. Analytical Methods

Analysis of alkalinity, Total Solids (TS), Total Volatile Solids (TVS), Soluble Chemical Oxygen Demand (sCOD), Total Kjeldahl Nitrogen (TKN) and Carbon:Nitrogen ratio (C:N) were carried out using Standard Methods (APHA, 2005).

Soluble COD (sCOD) of digesters were measured on the 0th, 3rd, 6th, 10th, 20th, 30th, 40th days. Firstly, samples were centrifuged at 14.000 rpm at 4°C for 30 minutes. After this centrifugation, 0.45 µm pore filters were applied to gain the supernatants. These supernatants were used for sCOD measurement for samples.

On the 0th, 3rd, 6th, 10th, 20th, 30th, 40th days, digesters were also examined in terms of their Volatile Fatty Acids (VFA) concentrations. Samples were centrifuged at 14.000 rpm at 4°C for 30 minutes. Then, 0.22 µm pore filters were used to gain the supernatants. 10 N phosphoric acid was added into the supernatants as 10% (v/v) in order to fix all biological activity. They were measured with gas chromatography-mass spectrometry (GC-MS) (Perichrom, France and Agilent Technologies 6890N, USA) for VFA analysis.

PM-9107 7000 mbar manometer (Lutron Electronic Enterprise Co., LTD, Taiwan) was used for gas measurement every day. After the measurements, all gases were produced in digesters were removed each time. HP Agilent 6850 Gas Chromatography with HP Plot Q column 30 m x 0.53 mm thermal conductivity detector was used for determination of the gas compositions. Every time, 2.5 ml syringe was used for the injection of 0.5 ml gas sample.

ECS 4010 CHNSO Analyzer (COSTECH Analytical Technologies, INC., USA) was used for determination of C:N ratios of seed sludge and substrates.

For the measurement of laccase enzyme activity, samples were centrifuged at 10.000 rpm at 4°C for 10 minutes. Then, the supernatants of the samples were used for determination of the laccase activity by spectrophotometric method (Kocyigit et al., 2012). According to this method, 200 µl 5 mmol ABTS was added into the cuvette, and then 600 µl glycine-HCl (pH: 3.0) was added into the cuvette. After this addition, 400 µl sample which was supernatant was added into the cuvette finally. were Samples taken from pre-treatment process were centrifuged at 10,000 rpm for 10 min at 4°C. The absorption changes at 420 nm for 5 minutes were noted. For this measurement, UV160U UV/Vis spectrophotometer (Shimadzu) was used. For the calculation of laccase enzyme activity, the formula that is shown in Equation (3.1) was used. ΔE is related to mean values of the absorbances at different minutes, ε is related to the extinction of coefficient of ABTS that is 36 mmol-1 cm-1 at 420 nm, d is related to the distance of light, Vt is related to the volume of cuvette, and Vs is related to the volume of enzyme.

$$\text{Enzyme activity (U/L)} = \frac{\Delta E \times Vt}{\varepsilon \times d \times Vs} \times 10^3 \times \text{Dilution Factor} \qquad (3.1)$$

4. RESULTS AND DISCUSSION

In this study, cow manure (M), food waste (F) and waste activated sludge (W) were co-digested under mesophilic conditions with and without pre-treatment with *T. versicolor* captured in Ca-alginate beads to overcome hydrolysis limitations in order to enhance methane production. Methane production from dual and triple substrates (manure and food waste; manure and waste activated sludge; food waste and waste activated sludge; manure, food waste and waste activated sludge) of co-digestions under mesophilic conditions were compared to that of the digesters without pre-treatment. This study also showed that which co-digestion set produces highest amount of methane production and solids removal.

4.1. Characterization Results

Alkalinity, pH, Total Solids (TS), Total Volatile Solids (TVS), Total Kjeldahl Nitrogen (TKN) and Carbon/Nitrogen ratio (C/N) were measured for substrates and seed sludge using Standard Methods (APHA, 2005). Their characterization results are shown in Table 4.1.

Table 4.1. The characterization results for substrates and seed sludge.

Samples	TS (% w/w)	TVS (% w/w)	TVS/TS (% w/w)	C/N	TKN (ppm)	pH	Alkalinity (mg $CaCO_3$/L)
Cow Manure	13.60	10.95	80	21.3	2720 ± 192	7.5 ± 0.2	3875 ± 80
Food Waste	29.80	24.50	82	16.7	8940 ± 620	4.3± 0.2	-
Waste Activated Sludge	2.10	1.00	48	7.2	80 ± 4	5.8 ± 0.2	1000± 53
Seed Sludge	11.50	6.90	60	15.6	2473±166	8.7± 0.2	28000+250

4.2. Laccase Enzyme Activity during Pre-treatment

Extracellular enzymes of *Trametes versicolor* in Ca-alginate beads were used during pre-treatment process. Laccase enzyme activities in flasks were measured for 5 days, and were calculated by formula that is shown in in Equation (3.1). Results of laccase enzyme activities for all digesters are shown in Figure 4.1.

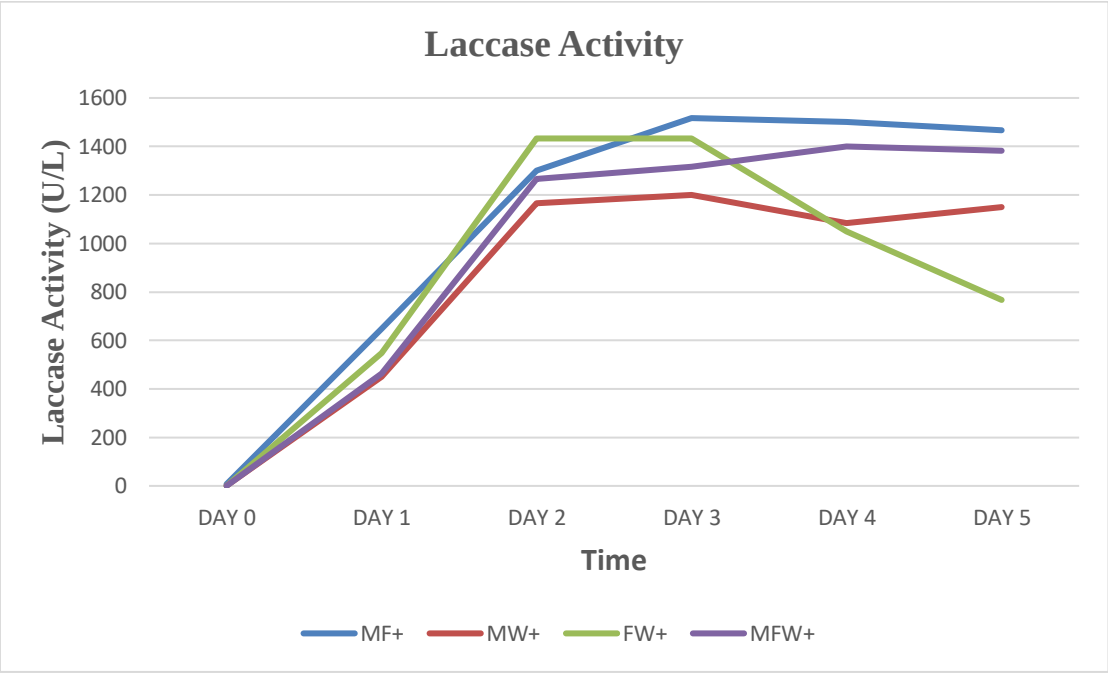

Figure 4.1. Laccase enzyme activity results of digesters during pre-treatment process for 5 days.

It can be seen that laccase enzyme activities increased in the first 2 days for all digesters. The maximum laccase activity which was 1517 U/L for MF+ whereas that of was for MW+ and FW+ were 1200 U/L and 1433 U/L respectively, all were observed on the 3rd day. However, the maximum laccase activity of 1400 U/L for MFW+ reached on the 4th day. An increase in laccase activity for each digester during pre-treatment showed that all digesters induced the laccase activity of *T. versicolor* positively because of the ingredients of substrates used. Moreover, starch concentration in digesters, especially in food waste might have affected the production of laccase positively. Also, other carbon sources, nitrogen sources and aromatics might have led to induce the laccase activity of *T. versicolor* (Revankar and Lele, 2006; Kocyigit et al., 2012; Bozan, 2018). The optimum media for *T. versicolor* had already starch and copper sulfate to induce the laccase production (Revankar and Lele, 2006; Kocyigit et al., 2012).

4.3. Batch Digester Performance

4.3.1. CODs Removal and VFA Production/Removal

Because of the limitations of substrates used in the digesters having high TVS concentrations, sCOD of digesters were measured instead of total COD. sCOD concentrations of all digesters were measured on the 0th, 3rd, 6th, 10th, 20th, 30th, 40th days, and are shown in Figure 4.2 and Table 4.2. According to the sCOD results, digesters which were pre-treated had higher sCOD values than digesters without pre-treatment. Moreover, digesters without food waste had lower sCOD values than digesters including food waste. It can be seen the downward trend of sCOD concentrations of all digesters from the 0th day. Seed sludge had the lowest sCOD concentration as expected. In terms of their rate of sCOD consumptions, digesters which contained food waste also had higher rate of sCOD consumptions than the sCOD consumptions of digesters which did not contain food waste (MW). Overall, it can be seen that pre-treatment led to higher initial sCOD concentrations in a range of 2-18 % in all digesters.

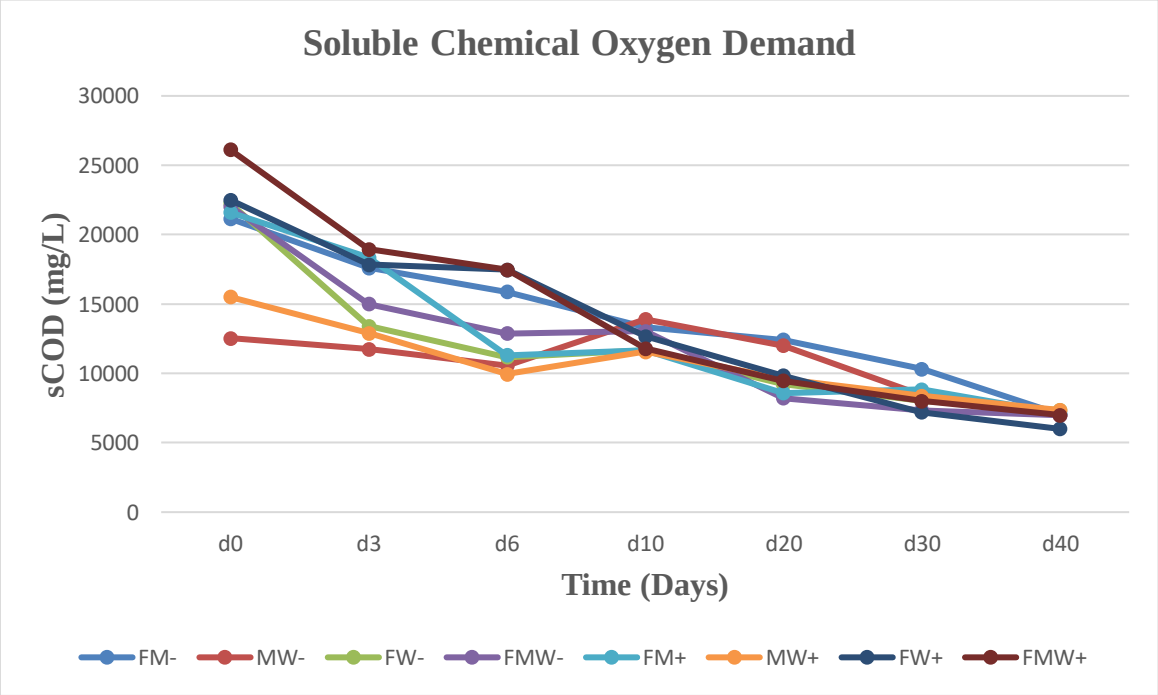

Figure 4.2. Changes in sCOD concentrations in all digesters with the digesting time.

Table 4.2. sCOD concentrations and consumptions in all digesters during anaerobic digestion.

	sCOD(mg/L)							
Days	0	3	6	10	20	30	40	Consumption (%)
MF-	21151	17599	15871	13342	12416	10318	7053	67
MW-	12539	11744	10565	13897	12000	8428	6986	50
FW-	22250	13403	11182	11675	9207	8042	7356	67
MFW-	22042	15007	12884	13033	8220	7345	6986	68
MF+	21600	18339	11305	11675	8590	8837	7110	67
MW+	15501	12909	9948	11552	9577	8379	7356	53
FW+	22512	17846	17476	12663	9824	7204	5999	73
MFW+	26114	18956	17476	11799	9454	8012	6989	73
Seed Sludge	4210	3874	5014	5256	3321	2989	3012	43

Total VFA concentrations of the digesters were measured on the 0th, 3rd, 6th, 10th, 20th, 30th, 40th days, and calculated as total acetic acid (mg/L) which are shown in Figure 4.3 and given in Table 4.3. Firstly, the most common volatile fatty acid in the digesters was acetic acid, and the second was propionic acid. According to Figure 4.3., it can be seen that acetic acid concentrations in the digesters were at their highest level on the 3rd day, which showed that the acidification phases of anaerobic digesters were completed on the 3rd day, and acetic acid concentrations were also significantly lower in the digesters that did not include food waste than the ones contained food waste; similar results were obtained with methane yields. The results also showed that the food waste led to the acceleration of the acidification phase of anaerobic co-digestion, and consequently the concentrations of volatile fatty acids increased. Moreover, the acetic acid concentrations for all digesters were almost depleted after the 20th day, which are also shown in Figure 4.3.

In terms of their VFA consumptions, digesters including food waste had higher VFA consumptions (96-98 %) than the VFA consumptions (89-91 %) of digesters which did not contain food waste (MW), which is shown in Table 4.3. Moreover, higher VFA consumptions contributed to increases in the methane yields. Besides, high VFA concentrations did not cause any sign of inhibition during the anaerobic co-digestion in fact high VFA concentrations were neutralized by co-digestion (Franke-Whittle et al., 2014). Therefore, pH values of the digesters were not affected by VFA concentrations due to the higher buffer capacity of co-digestion (Zhang et al., 2013).

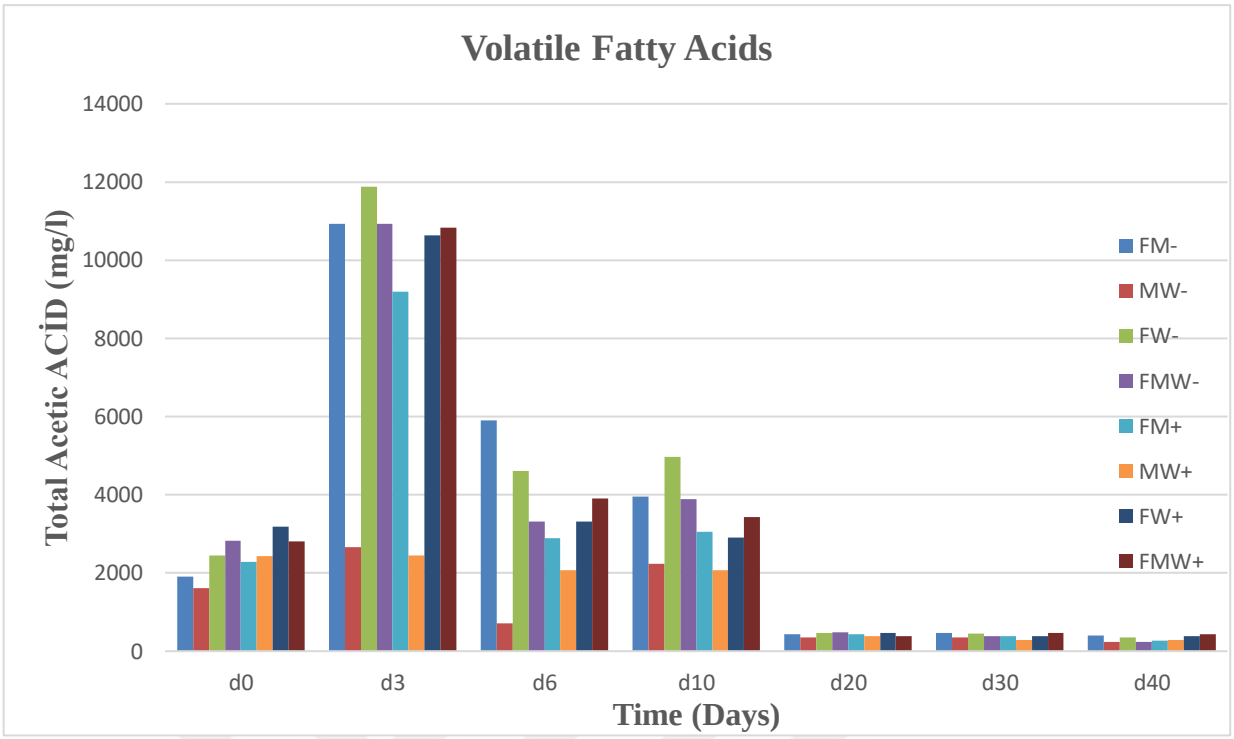

Figure 4.3. Results of VFA concentrations of the digesters during co-digestion.

Table 4.3. VFA concentrations and consumptions for digesters during co-digestion.

	Total Acetic Acid (mg/L)							
Days	0	3	6	10	20	30	40	Consumption (%)
MF-	1914	10931	5902	3949	427	462	400	96
MW-	1619	2659	714	2232	354	357	237	91
FW-	2451	11874	4613	4977	459	456	358	97
MFW-	2814	10928	3309	3886	477	390	237	98
MF+	2286	9199	2884	3049	430	377	273	97
MW+	2436	2439	2063	2065	382	289	281	89
FW+	3176	10627	3321	2905	470	389	387	96
MFW+	2806	10840	3909	3430	389	473	432	96

4.3.2. Biogas/Methane Production

It can be seen that biogas productions for all digesters were stabilized approximately on the 25th day although biogas measurements were recorded till 40 days, which are shown in Figure 4.4. After the 25th day, the amount of biogas production significantly reduced for all digesters and that of were nearly ceased after the 30th day.

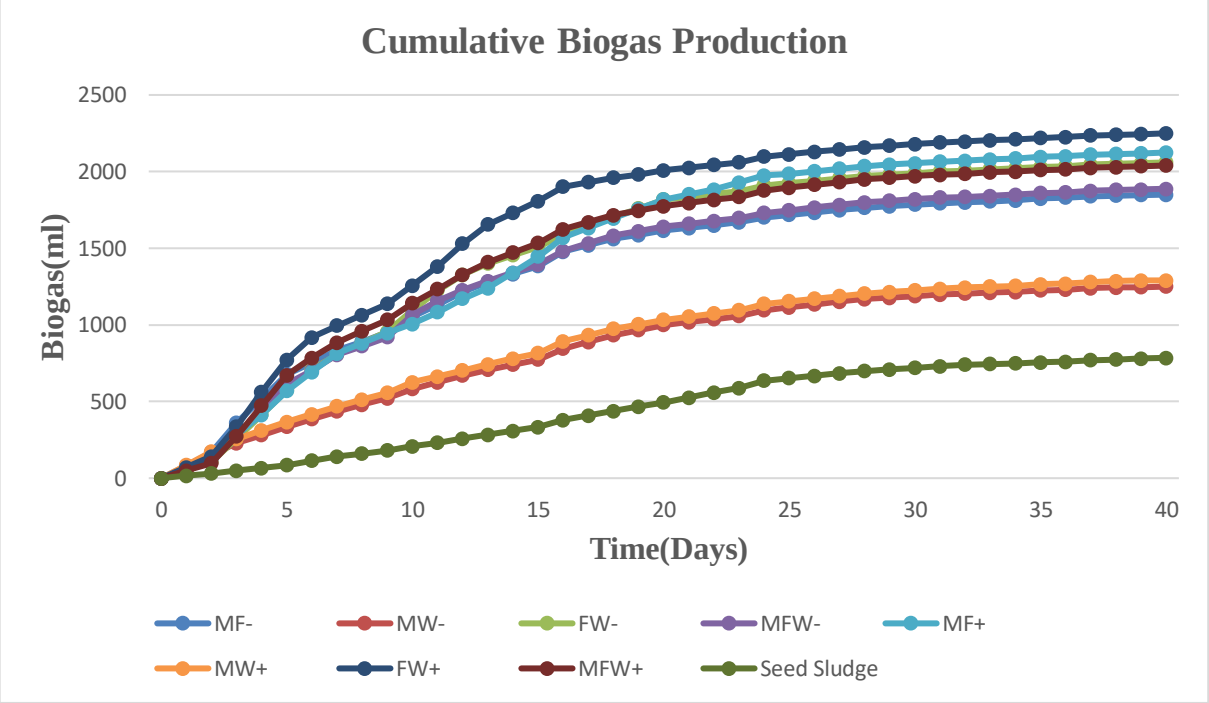

Figure 4.4. The cumulative biogas productions during anaerobic co-digestion for all digesters.

The cumulative biogas productions during anaerobic co-digestion for 25 days were noted as MF- (1717 ml biogas), MW- (1115 ml), FW- (1925 ml), MFW- (1748 ml), MF+ (1985 ml), MW+ (1154 ml), FW+ (2113 ml), MFW+ (1895 ml) and seed sludge (652 ml). It can be realized that all digesters including pre-treatment with *T. versicolor* captured in Ca-alginate beads were higher biogas production than digesters without pre-treatment, which are shown in Figure 4.5 to Figure 4.8. The results indicated that the pre-treatment led to an increase in biogas production. In the Figure 4.4., the highest biogas production obtained was from FW+. Besides, the digesters (MW) which did not include food waste contributed to significantly lower biogas productions than the ones contained food waste, which showed that food waste was the most significant substrate in this study in terms of biogas production.

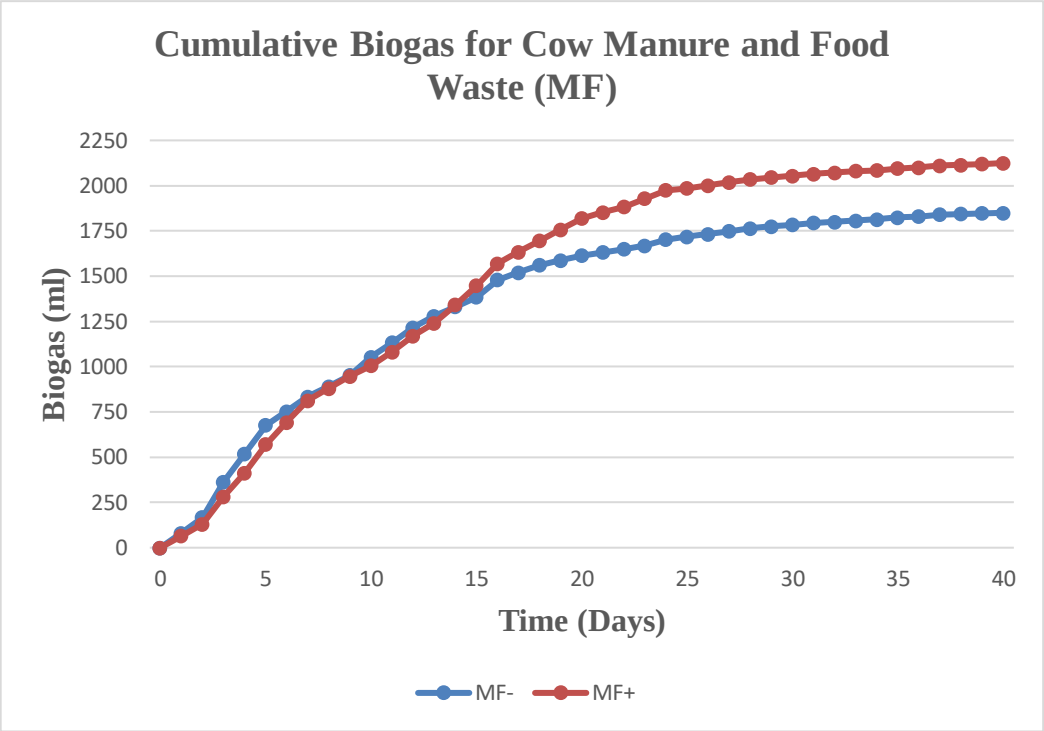

Figure 4.5. Comparison of biogas productions in MF- and MF+ digesters.

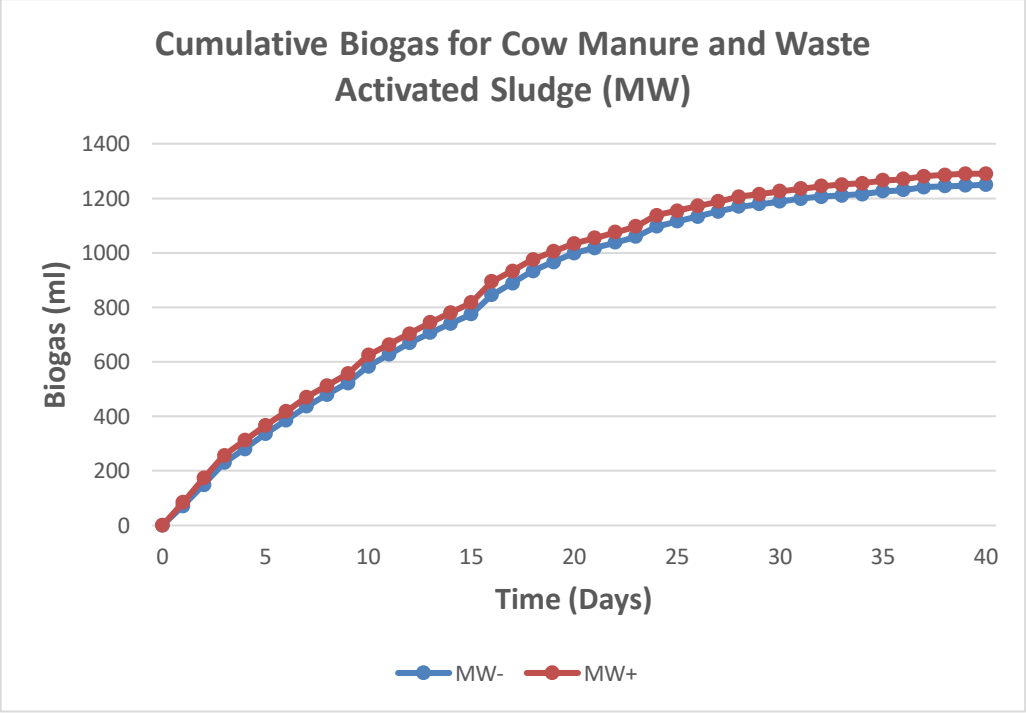

Figure 4.6. Comparison of biogas productions in MW- and MW+ digesters.

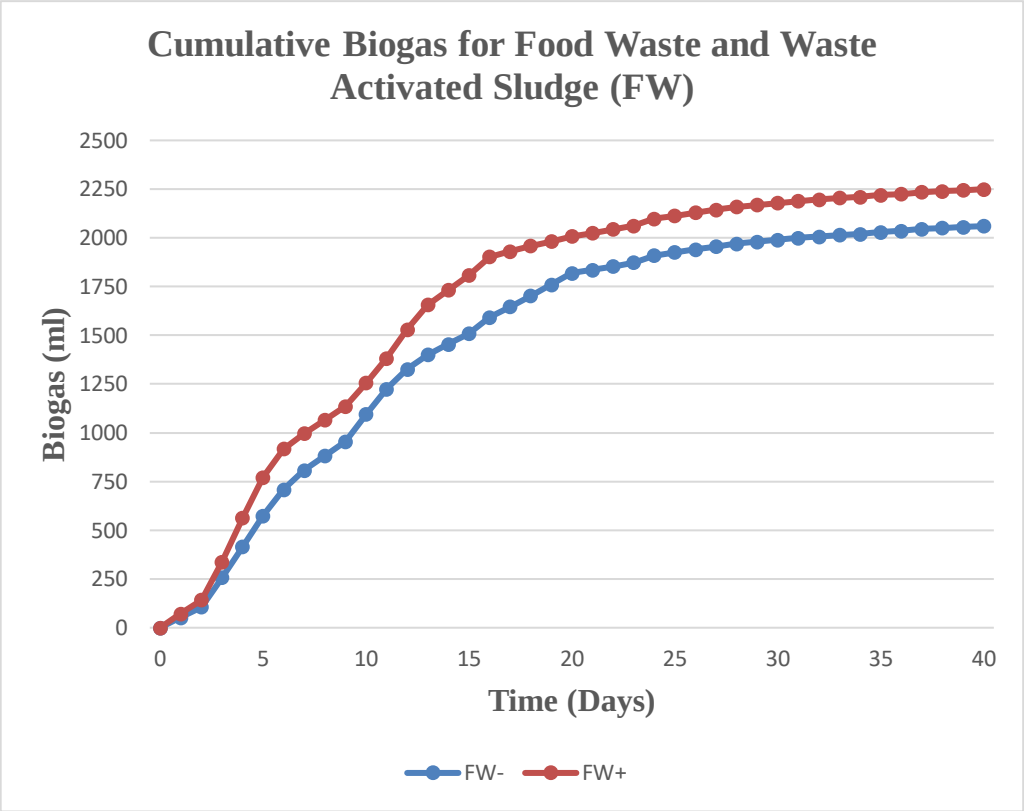

Figure 4.7. Comparison of biogas productions in FW- and FW+ digesters.

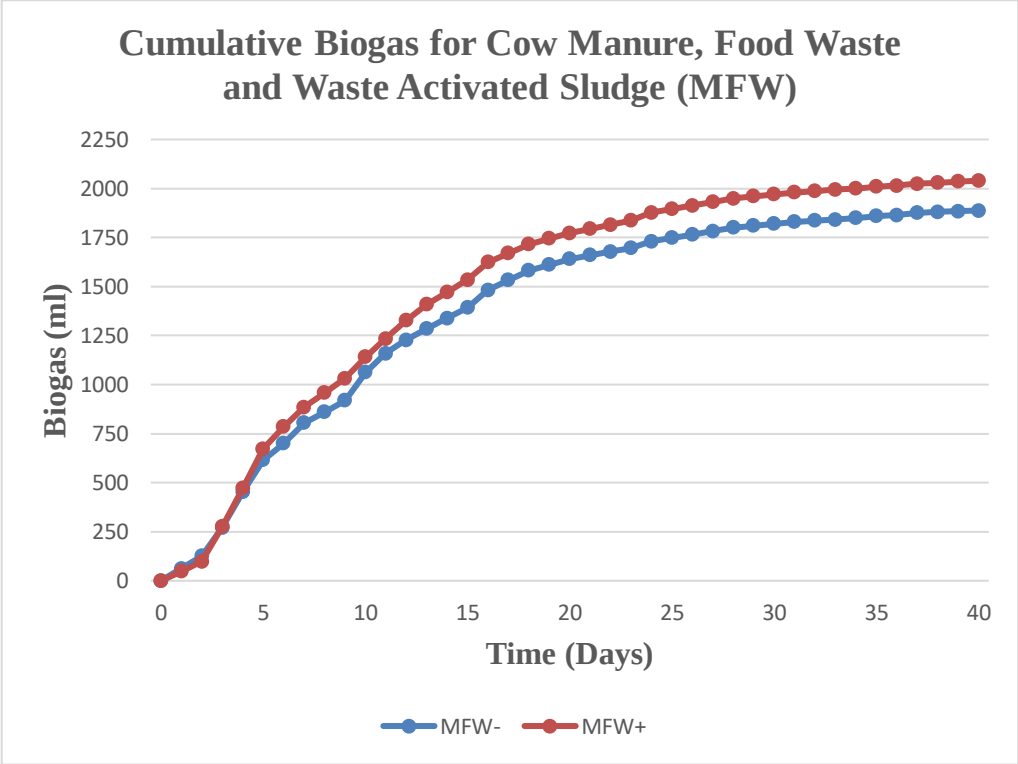

Figure 4.8. Comparison of biogas productions in MFW- and MFW+ digesters.

The biogas yield results are shown in Figure 4.9. As seen, the pre-treatment with *T. versicolor* led to an improvement in biogas yield for all digesters. The changes in the biogas yields were MF, MW, FW and FMW by 25%, 8%, 15% and 13%, respectively, which are shown in Table 4.4. These results were also consistent with VFA and sCOD consumption ratios of the digesters.

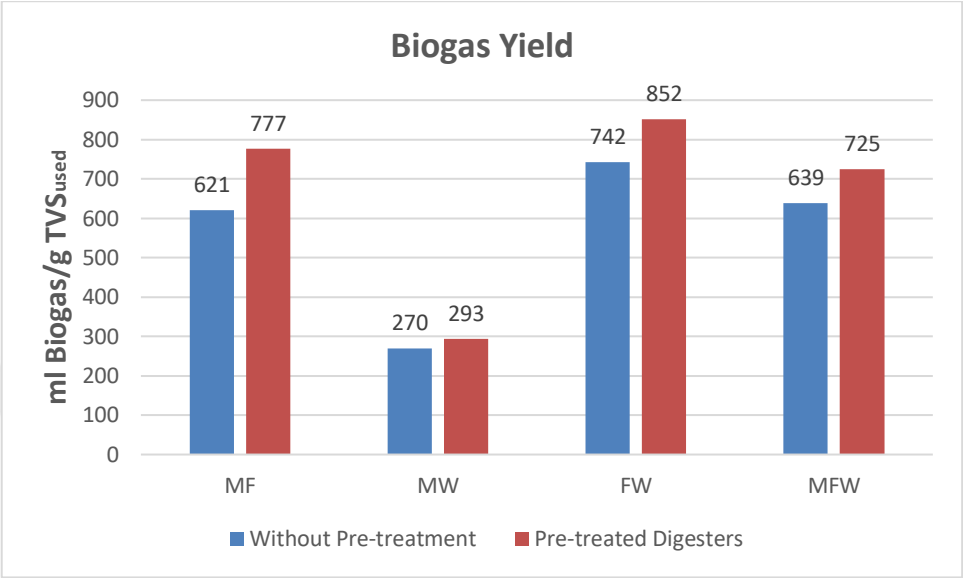

Figure 4.9. The results of biogas yields for MF, MW, FW and MFW with and without pre-treatment by *T. versicolor*.

Table 4.4. The percentage of improvement of biogas yield for digesters.

Digesters	Without Pre-treatment (ml biogas/g TVS_{used})	Pre-treated digesters (ml biogas/g TVS_{used})	Biogas yield increase
MF	621	777	25%
MW	270	293	8%
FW	742	852	15%
MFW	639	725	13%

Methane productions for all digesters were measured for 40 days during digestion periods. It can be realized that methane productions were almost ceased after 25th day for all digesters, are shown in Figure 4.10. The average methane ratios for all digesters during 25 days are shown in Table 4.5.

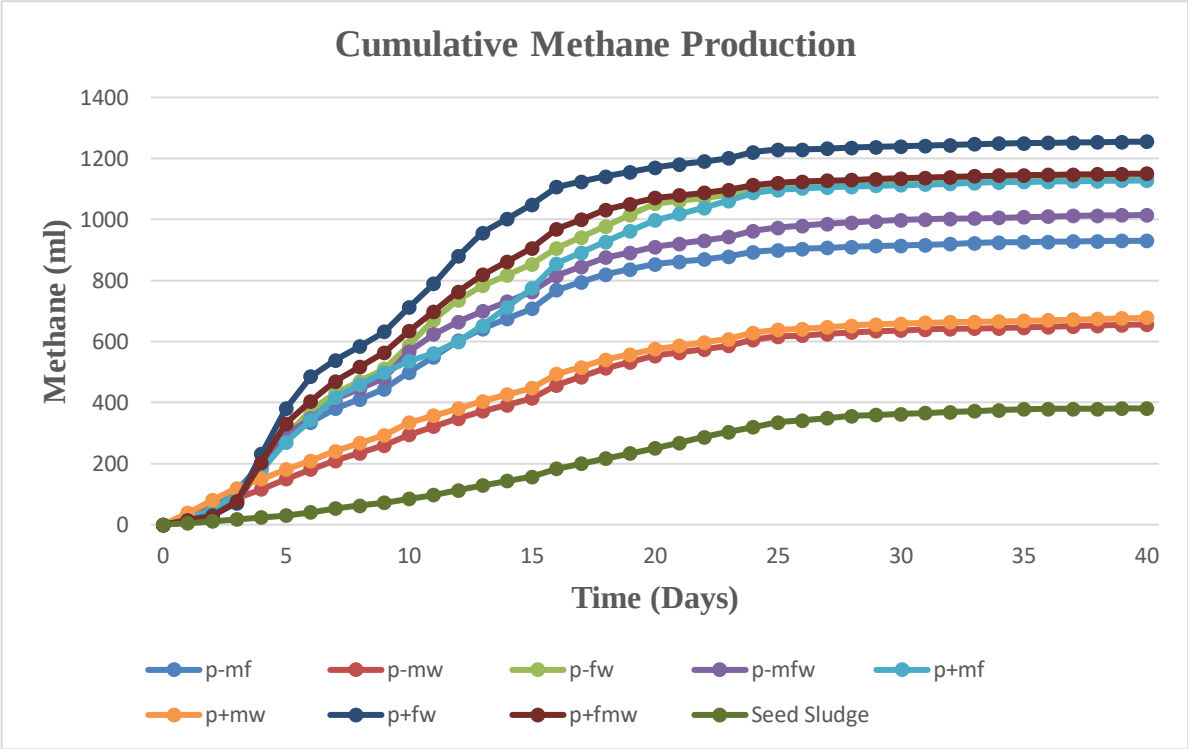

Figure 4.10. The cumulative methane production of digesters during digestion period.

Table 4.5. The average CH4 ratios for all digesters during 25 days.

Digesters	Average CH_4 ratio	CH_4 ratio intervals
MF-	52%	50-62 %
MW-	55%	53-65 %
FW-	58%	55-68 %
MFW-	56%	54-64 %
MF+	55%	54-63 %
MW+	55%	53-64 %
FW+	58%	53-66 %
MFW+	59%	55-68 %

The cumulative methane productions during anaerobic digestion for 25 days were noted as MF- (900 ml methane), MW- (616 ml methane), FW- (1108 ml methane), MFW- (973 ml methane), MF+ (1097 ml methane), MW+ (638 ml methane), FW+ (1229 ml methane), MFW+ (1120 ml methane) and seed sludge (339 ml methane). It can be seen that all digesters with pre-treatment by *T. versicolor* produced higher methane productions than digesters without pre-treatment, which are shown in Figures 4.11-4.14. Moreover, these results showed that pre-treatment improved methane production. Besides, the highest methane production resulted from FW+ digester. The digester (MW) which did

not have food waste led to significantly lowest of all. This showed that food waste was the most significant substrate in this study.

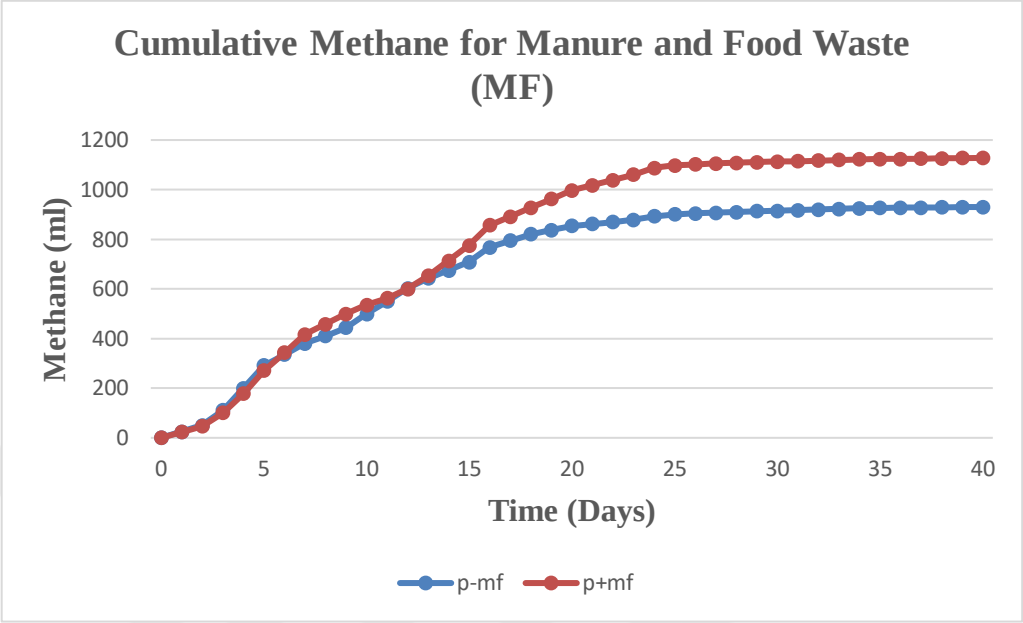

Figure 4.11. Comparison of methane productions in MF- and MF+ digesters.

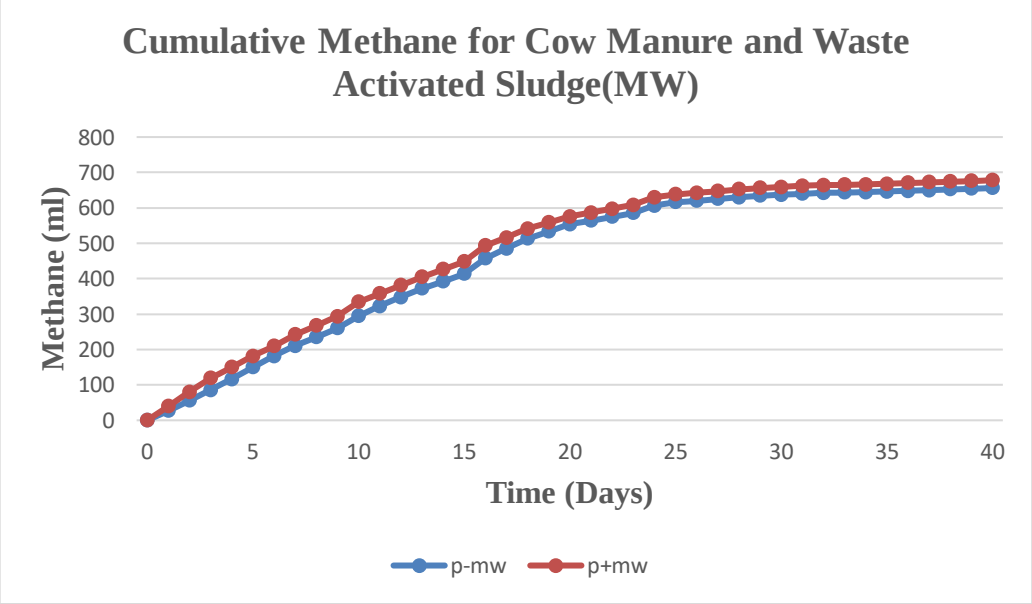

Figure 4.12. Comparison of methane productions in MW- and MW+ digesters.

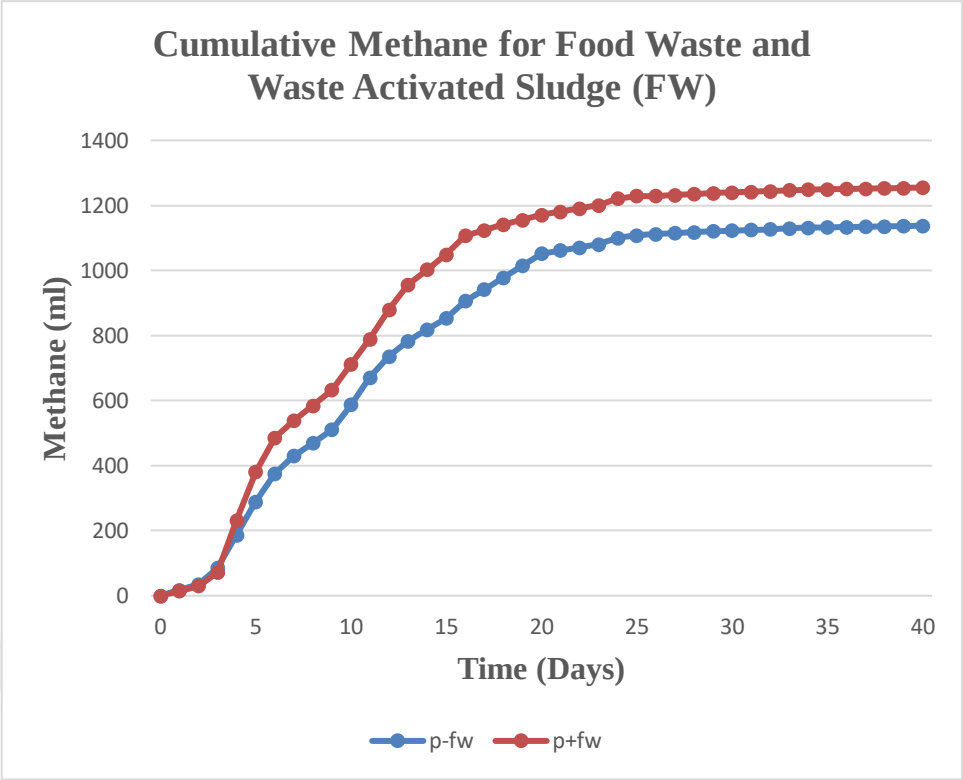

Figure 4.13. Comparison of methane productions in FW- and FW+ digesters.

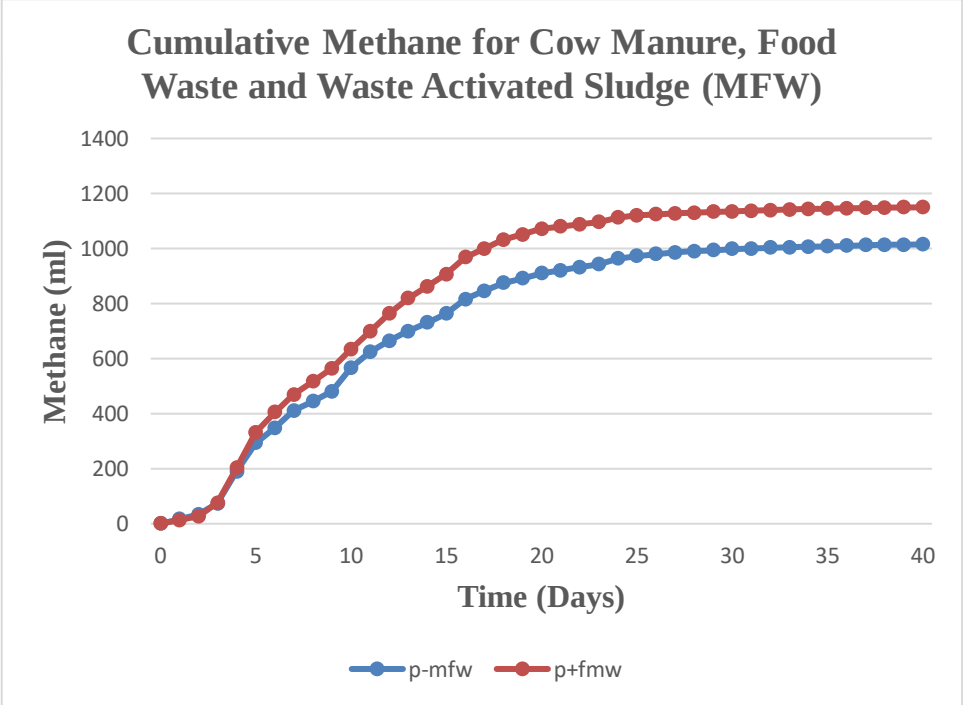

Figure 4.14. Comparison of methane productions in MFW- and MFW+ digesters.

The methane yield results are shown in Figure 4.15. The results of methane yield for all digesters were observed as MF- (327 ml methane/g TVS_{used}), MW- (161 ml methane/g TVS_{used}), FW- (448 ml methane/g TVS_{used}), MFW- (370 ml methane/g TVS_{used}), MF+ (442 ml methane/g TVS_{used}), MW+

(174 ml methane/g TVS_{used}), FW+ (519 ml methane/g TVS_{used}) and MFW+ (455 ml methane/g TVS_{used}).

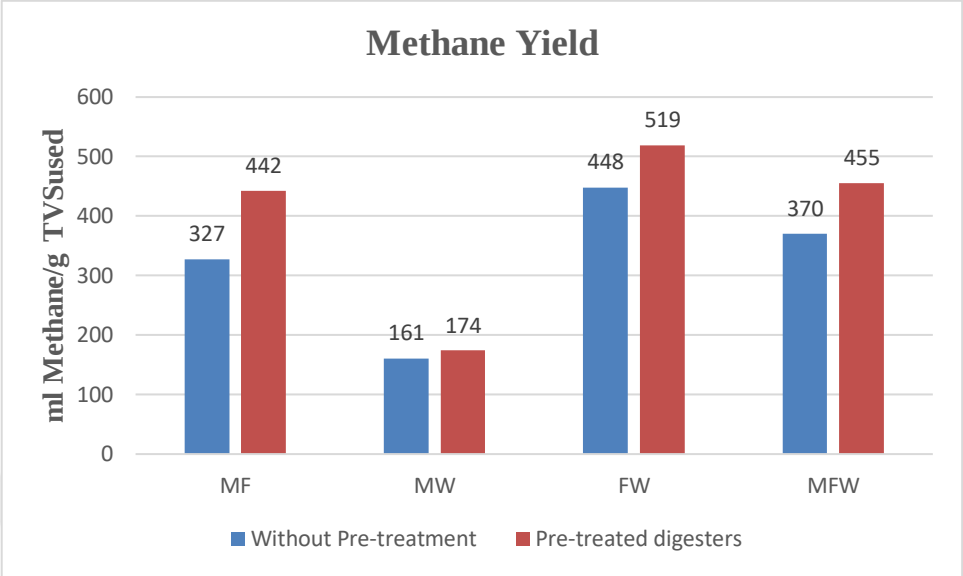

Figure 4.15. Overall results of methane yield for digesters.

It is realized that pre-treatment with *T. versicolor* contributed to enhance methane yield for all digesters, which is displayed in Figure 4.15. Therefore, this pre-treatment led to an increase in methane yield for the combination of MF, MW, FW and FMW by 35%, 8%, 16% and 23%, respectively, which is also displayed in Table 4.6. These methane yields were also correlated with VFA and sCOD consumption ratio for digesters. FW+ had the highest methane yield which was 519 ml methane/g TVSused. Digesters which did not include food waste (MW) had the two lowest methane yields which were 161 ml methane/g TVSused and 174 ml methane/g TVSused for MW- and MW+, respectively.

Table 4.6. The percentage of improvement of methane yield for digesters.

Digesters	Without Pre-treatment (ml methane/g TVS_{used})	Pre-treated digesters (ml methane/g TVS_{used})	Methane yield increase
MF	327	442	35%
MW	161	174	8%
FW	448	519	16%
MFW	370	455	23%

Moreover, the digesters (MW) which did not include food waste contributed to significantly lower methane yield than the ones included food waste, which displayed that food waste was the most significant substrate in this experiment in terms of methane yield. Therefore, MW was not suitable for methane production according to results. Besides, the digester (MF+) which contained the combination of manure and food waste was the most affected digester from the pre-treatment process because it had the highest laccase activity during pre-treatment, and pre-treatment led to not only improving biogas production but also improving the methane ratio in biogas significantly. Moreover, it might be concluded that waste activated sludge did not need any pre-treatments. However, this pre-treatment was important for food waste and cow manure because of their lignocellulosic contents. In future studies, this technique should be improved and/or used in pilot-scale studies and could be used to improve methane yields of full-scale co-digesters containing cellulosic and/or hemi-cellulosic substrates.

5. CONCLUSIONS AND RECOMMENDATIONS

Pre-treatment with *T. versicolor* in Ca-alginate beads contributed to increases in biogas yields of the co-digestions of the combinations of manure and food waste (MF); manure and waste activated sludge (MW); food waste and waste activated sludge (FW); and manure, food waste and waste activated sludge (MFW) by 25%, 8%, 15% and 13%, and also increases in that of methane yields by 35%, 8%, 16% and 23%, respectively. Moreover, digesters that did not include food waste (MW) produced lowest methane yields than digesters containing food waste, which showed that food waste was the most significant substrate increasing methane yield.

The concentrations of acetic acid in the digesters were at their highest level on the 3rd day, and also significantly lower in the digesters that did not include food waste (MW) than the ones contained food waste, which was correlated with methane yield. Moreover, food waste contributed to the acceleration of the acidification stage of anaerobic co-digestion, and that caused increases in volatile fatty acids' concentrations. Therefore, food waste led to higher methane yields.

Digesters without food waste (MW) had lower sCOD values than digesters containing food waste, which were also correlated with those of methane yields. Moreover, all sCOD concentrations decreased after the 10th day. In terms of their COD consumptions, digesters which contained food waste also had higher COD consumptions than the COD consumptions of digesters which did not include food waste (MW).

Moreover, *Trametes versicolor* pre-treatment led to an increase in VS removal and TS removal for all digesters. VS and TS removal could increase if the digestion time increased. However, methane could not be produced in the extra time. Therefore, composting can be recommended for improving VS and TS removal after anaerobic digestion process.

An increase in laccase enzyme activity for each digester during pre-treatment showed that all digesters induced the laccase activity of *T. versicolor* positively because of the contents of digesters, which confirmed the pre-treatment process worked properly.

Although digestion times in including lignocellulosic substrates were generally maintained at 40 days, in this study, the anaerobic digestion period was almost completed in the 25th day, which was a significant result in terms of shorter digestion time of anaerobic digestion.

The recommendations of this study can be summarized as, (1) in order to apply these promising results in to full-scale digesters, first of all pilot scale studies including hydraulics, optimum solids loading, characteristics of solid/liquid digestate, etc., must throughly be covered; (2) composting could be used after anaerobic digestion for improving VS and TS removal; (3) the Ca-alginate beads from digesters after the pre-treatment process was extremely hard. In future studies, using specific bags for collecting the Ca-alginate beads could make this step easier, and also the collected Ca-alginate beads can be reused in digesters many times; (4) Ca-alginate beads could be improved in terms of their strength, and they could be examined during reuse in terms of the possibility of reduced permeability of Ca-alginate beads; (5) molecular techniques could be used in future studies to identification of microbial diversity in digesters. Then, the efficiency and stability of anaerobic digestion could also be analyzed in terms of microorganisms.

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