ANAEROBIC MONO-DIGESTION AND CO-DIGESTION OF AGRICULTURAL WASTES AND COMPARISON OF VALORIZATION POTENTIALS

by

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ABSTRACT

ANAEROBIC MONO-DIGESTION AND CO- DIGESTION OF AGRICULTURAL WASTES AND COMPARISON OF VALORIZATION POTENTIALS

Anaerobic digestion is an immense technology for not only waste disposal but also biogas production for agricultural-based residues, however the economic value of the biogas is limited. The transition of anaerobic digestion to produce carboxylates can provide a sustainable future and better valorization. In this study, Turkish delight (D), sunflower head (S), and tea waste (T) were mono-digested and co-digested at neutral pH under mesophilic conditions to determine the best set of substrates that can produce maximum methane gas. Methane yield for each digester was recorded as D (388 ml CH_4/g TVS_{used}), S (206 ml CH_4/g TVS_{used}), T (69 ml CH_4/g TVS_{used}), DS (312 ml CH_4/g TVS_{used}), DT (213 ml CH_4/g TVS_{used}), ST (173 ml CH_4/g TVS_{used}), and DST (234 ml CH_4/g TVS_{used}) after 30-day in anaerobic digestion. According to the results, the three sets D, DS, and DST were the ones that produced the highest amount of methane gas within their groups. Bacterial community composition for these three sets was determined using MinION^{TM}. The family *Pseudomonadaceae* was observed more numerous in D digester that producing the highest methane than the digesters of DS and DST. Moreover, acidification was also conducted to the three sets D, DS, and DST to determine VFA productions under mesophilic condition at pH 5.5 ± 0.2. According to the valorization results, market values of VFAs (sum of acetic and butyric acid) were 6-13 times higher than methane.

ÖZET

TARIM ATIKLARININ TEKLİ VE ÇOKLU ANAEROBİK ÇÜRÜTÜLMESİ VE DEĞERLEME POTANSİYELLERİNİN KARŞILAŞTIRILMASI

Anaerobik çürütme, tarımsal kaynaklı atıklar için yalnızca atık bertarafı için değil, aynı zamanda biyogaz üretimi için de muazzam bir teknoloji olmasına rağmen, biyogazın ekonomik değeri sınırlı kalmaktadır. Anaerobik çürütmenin karboksilat üretimine geçişi, sürdürülebilir bir gelecek ve daha iyi ekonomik değerleme sağlayabilir. Bu çalışmada, maksimum metan gazı üretebilen en iyi substrat setini belirlemek için Türk lokumu (D), ayçiçeği başı (S) ve çay atığının (T) mezofilik koşullar altında nötr pH'da tekli ve çoklu substratlı çürütmesi gerçekleştirildi. 30 gün sonra anaerobik sindirimde metan verimleri D (388 ml CH_4/g $UKM_{eklenen}$), S (206 ml CH_4/g $UKM_{eklenen}$), T (69 ml CH_4/g $UKM_{eklenen}$), DS (312 ml CH_4/g $UKM_{eklenen}$), DT (213 ml CH_4/g $UKM_{eklenen}$), ST (173 ml CH_4/g $UKM_{eklenen}$), ve DST (234 ml CH_4/g $UKM_{eklenen}$) olarak bulundu. Sonuçlara göre, üç set D, DS ve DST, kendi grupları içerisinde en fazla metan gazı üreten setler oldu. Bu üç set için bakteri topluluğu analizi MinION^{TM} cihazı kullanılarak belirlendi. En yüksek metan üreten D sindiricisinde *Pseudomonadaceae* familyasının, DS ve DST sindiricilerinden daha fazla sayıda olduğu gözlendi. Ayrıca, yine D, DS ve DST setlerine mezofilik koşullar altında ve pH 5.5 ± 0.2 olacak şekilde uçucu yağ asiti üretimlerini belirlemek için asidifikasyon çalışması yapıldı. Değerleme sonuçlarına göre uçucu yağ asitlerinin piyasa değerleri (asetik ve bütirik asit toplamı) metandan 6-13 kat daha yüksek çıktı.

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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
HCl	Hydrochloric Acid	
H_2O	Water	
H_2O_2	Hydrogen Peroxide	
H_2S	Hydrogen Sulfide	
H_2SO_4	Sulfuric Acid	
$K_2Cr_2O_7$	Potassium Dichromate	
ml	Milliliter	
NaOH	Sodium Hydroxide	
NH_3	Ammonia	
NO_3^-	Nitrate	
N_2	Nitrogen	
SO_4^{-2}	Sulphate	
µl	Microliter	

Abbreviation	Explanation	Unit
AMPTS II	Automated Methane Potential Test System II	
BMP	Biomethane Potential	
BC01	Seed Sludge on the 0^{th} day	
BC02	Seed Sludge on the 30^{th} day	
BC03	Turkish Delight Waste on the 0^{th} day	
BC04	Turkish Delight Waste on the 30^{th} day	
BC05	Turkish Delight and Sunflower Head Waste on the 0^{th} day	
BC06	Turkish Delight and Sunflower Head Waste on the 30^{th} day	

BC07	Turkish Delight, Sunflower Head, and Tea Waste on the 0^{th} day	
BC08	Turkish Delight, Sunflower Head, and Tea Waste on the 30^{th} day	
AD	Anaerobic Digestion	
cDNA	Complementary DNA	
C:N	Carbon:Nitrogen	
COD	Chemical Oxygen Demand	mg/L
dH_2O	Distilled Water	
D	Turkish Delight Waste	
DGGE	Denaturing Gradient Gel Electrophoresis	
DNA	Deoxyribonucleic Acid	
DS	Turkish Delight and Sunflower Head Waste	
DST	Turkish Delight, Sunflower Head and Tea Waste	
FISH	Fluorescence in situ Hybridization	
GC	Gas Chromatography	
I:S	Inoculum:Substrate	
NGS	Next-Generation Sequencing	
OTUs	Operational Taxonomic Units	
PCR	Polymerase Chain Reaction	
Q-PCR	Quantitative PCR (Real-Time PCR)	
RNA	Ribonucleic Acid	
S	Sunflower Head Waste	
sCOD	Soluble Chemical Oxygen Demand	mg/L
ST	Sunflower Head and Tea Waste	
T	Tea Waste	
TS	Total Solids	mg/L
TVS	Total Volatile Solids	mg/L
VFA	Volatile Fatty Acid	mg/L

1. INTRODUCTION

Huge amounts of organic residues are generated due to the agricultural industry every year. If they are released into the environment without proper disposal procedures, residues can lead to environmental pollution and cause detrimental effects on human and animal health (Sadh et al., 2018). Agricultural wastes can be converted into sustainable biological products such as fertilizers and energy using different transformation processes. This transformation is significant for economic growth, human health, biodiversity, and global food security (Gontard et al., 2018).

Improper disposal of the wastes generated from agro-industrial occurs in economic issues. In this case, the development of innovative approaches is needed for the sustainable management of them. Agricultural industry residues offer tremendous potential to produce sustainable products and bioenergy (Beltrán-Ramírez et al, 2019). One of the most widely applied biological processes is anaerobic digestion (AD) in which biomass is converted to bioenergy (Chen et al., 2018). Several microbial communities convert raw material to biogas, through digestion steps such as hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Shah et al., 2017).

Circular economy is related to the concept of biorefinery and the emphasis to reduce, reuse and recycle waste to recover the obtained materials by accepting waste as a renewable resource (Velis, 2015). Obviously, biogas production added value to the anaerobic digestion process. However, there are also studies suggesting that biogas production is not the best way to anaerobic digestion. Producing organic acids such as acetic and butyric acids and alcohols may be more logical than biogas (Kleerebezem et al., 2015). These high-value end products can be used in biofuels, bulk chemicals, bioplastics, nutrient removal from wastewater, and food additives. Residues from industries and agricultural activities have high availability, making anaerobic digestion a sustainable path for the biosynthesis of these metabolites (Wainaina et al., 2019).

Turkey is the seventh largest agricultural producer in the world; therefore, it is expected that it has a lot of agricultural residue. The analysis shows that one of the most available crop residue types is sunflower head that has the highest availability at 1 million tons per year in Turkey in terms of collected residues (FAO, 2016). Turkey is also the world's sixth-biggest producer of tea (FAO, 2016). Moreover, Turkey is famous for delight production and has many delight factories that produce millions of tons every year. Therefore, sunflower head and tea residues which have high

lignocellulosic content along with wasted delights made of high amount of starch and sugar were chosen in this research as the substrates.

In this study, Turkish delight (D), sunflower head (S), and tea waste (T) were mono digested and co-digested at neutral pH under mesophilic conditions to determine the best set of substrates that can produce maximum amount of methane gas. This study aimed to show difference between mono and co-digestion and led to a comparison between different combinations of substrates under anaerobic mono and co-digestions (D, S, T, DS, DT, ST, DST) in terms of the methane yield under the mesophilic condition. According to the results, the substrates which have higher methane yield were D, DS, DST, and bacterial community compositions for these three sets were determined by MinION^{TM} device of Oxford Nanopore Technologies followed by bioinformatics analyses. Besides, acidification was applied to these three sets that produced the highest amount of methane gas within their groups at pH 5.5 ± 0.2, under mesophilic conditions. A comparison between valorization of bio methane and VFA (as acetic and butyric acids) productions by D, DS, DST digestion sets were studied.

2. LITERATURE REVIEW

2.1. Anaerobic Digestion

Anaerobic digestion is microbial process that complex organic materials are broken down gradually by various enzymes in the absence of oxygen, to obtain a digestate and production of biogas. Recently, the utilization of anaerobic treatment has increased due to the need to find new energy sources as an alternative to fossil fuels. Anaerobic digestion provides a reduction in the volume of material to be disposed of, prevents soil and groundwater from pollution, and produces renewable energy sources like biogas (Esposito et al, 2012).

Biogas production by anaerobic digestion offers advantages when compared to other bioenergy production methods (Fehrenbach et al., 2018). Several substrates can be used in anaerobic digestion systems, such as animal waste, 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 the biogas yield of anaerobic digesters (Weiland, 2010). Anaerobic co-digestion process produce biogas that is a renewable energy source as shown in Figure 2.1.

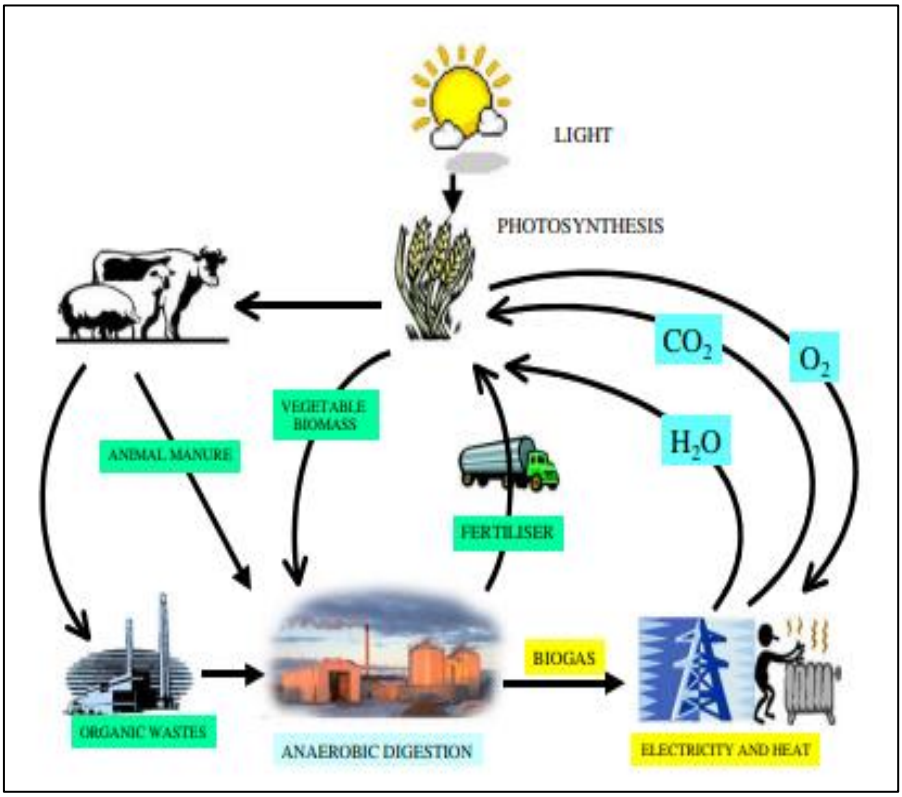


Figure 2.1. Schematic representation of anaerobic co-digestion process (Holm-Nielsen et al., 2009).

The carbon oxidation-reduction state of the organic matter in the waste determines the composition of the biogas (Muñoz et al., 2015). For instance, the biogas from the anaerobic degradation of sewage sludge, livestock manure or agro-industrial bio-wastes contains: CH_4 (53–70 %), CO_2 (30–47 %), N_2 (0–3 %), H_2O (5–10 %), O_2 (0–1 %), H_2S (0–10.000 ppm_v), NH_3 (0–100 ppm_v), hydrocarbons (0–200 mg m^{-3}) and siloxanes (0–41 mg m^{-3}) (Persson et al., 2006; Bailón and Hinge, 2012; Muñoz et al., 2015).

2.2. Biochemistry of Anaerobic Digestion

Biogas production by anaerobic digestion has four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Besides, different groups of microorganisms work in each step (Gerardi, 2003). Figure 2.2 shows a scheme of the steps involved in anaerobic digestion.

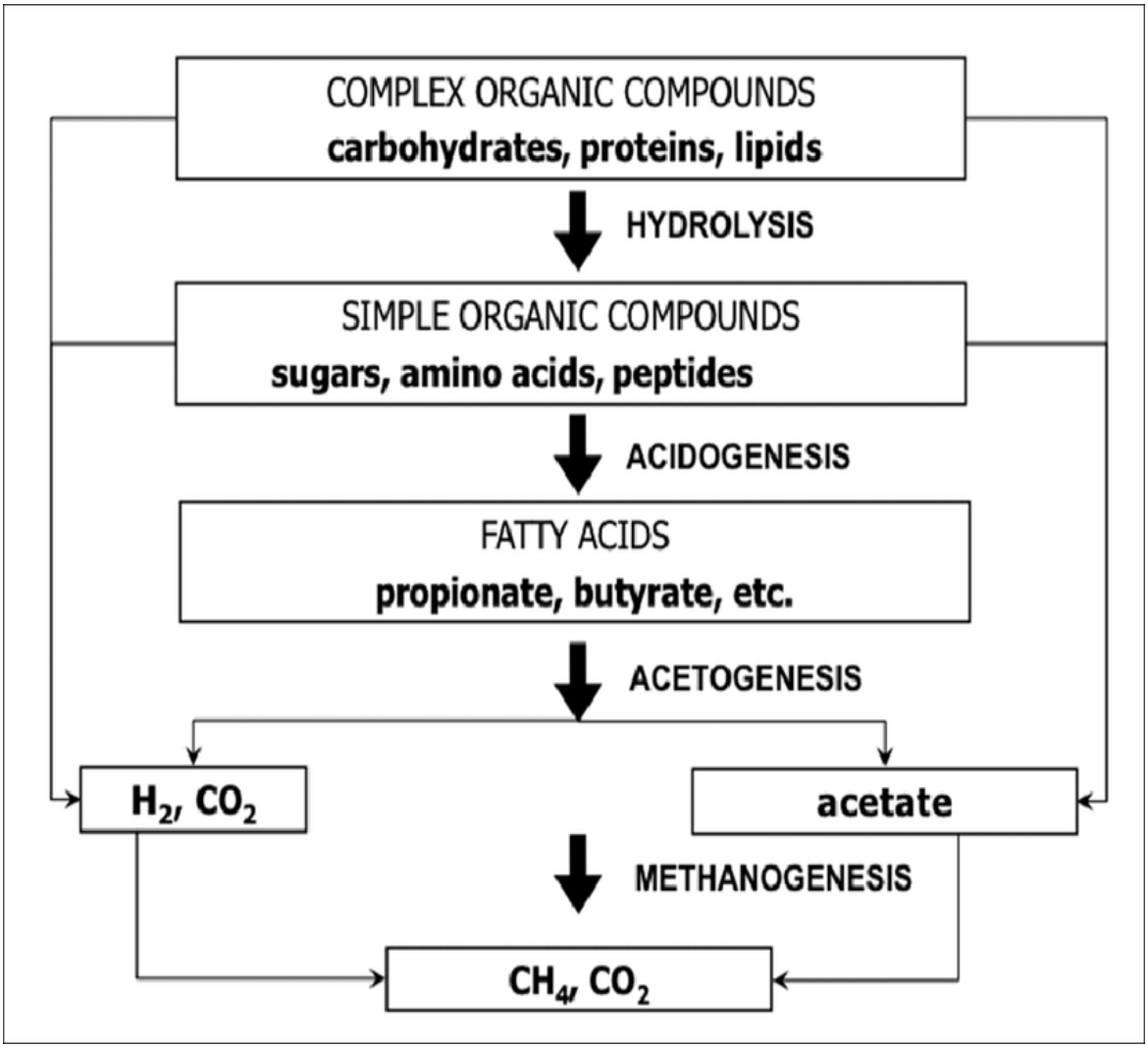


Figure 2.2. Four steps involved in the anaerobic digestion process (Drosg, 2013).

2.2.1. Hydrolysis

Hydrolysis is the first step of the anaerobic digestion process, in which microorganisms hydrolyze the complex substrate such as carbohydrates, lipids, proteins into mono sugars, fatty acids, and amino acids, respectively (Pesta, 2007). The cellulose and hemicellulose derived from lignocellulosic biomass may not be easily hydrolyzed and may require pretreatment (Jørgensen et al., 2007). Various pretreatment options are being researched in order to optimize hydrolysis especially for lignocellulosic wastes. Crystallinity and accessible surface area are some of the important processes for pretreatment. Different pretreatment methods can be used to increase biogas production, such as milling, irradiation, microwave, alkaline hydrolysis, steam explosion, ozonolysis, liquid hot-water pretreatment, wet oxidation, organosolv processes, dilute and concentrated-acid hydrolyses, and biological pretreatments (Taherzadeh and Karimi, 2008).

2.2.2. Acidogenesis

In this step, products coming from hydrolysis are converted into water-soluble organic end products such as fatty acids, alcohols, and carbon dioxide gas. Besides, these conversions are carried out by the same fermentative bacteria that are responsible for hydrolysis (Pesta, 2007). Ammonia (NH_3), CO_2, H_2S, and other by-products along with volatile fatty acids (VFAs) are produced by acidogenic bacteria (Appels et al., 2008). A significant increase in VFA concentration can damage methanogens in the anaerobic digesters, as increasing VFA concentrations can cause a decrease in the pH. Therefore, process monitoring is very important at these stages (Franke-Whittle et al., 2014).

2.2.3. Acetogenesis

Acetogenesis is a connection between the degradation of water-soluble compounds and methane formation. In this step, end products of microbial metabolisms are converted into short-chained volatile fatty acids such as acetate and CO_2 (Pesta, 2007). Acetogenic bacteria are in a symbiotic relationship with methane-forming bacteria (Chandra et al., 2012).

2.2.4. Methanogenesis

Methanogenesis is the last step of anaerobic digestion in which bacteria produce methane and carbon dioxide. Methanogenic bacteria belong to the strictly anaerobic microorganism group;

therefore, they can live only in the absence of oxygen (Pesta, 2007). Although the fermentative microorganisms are less sensitive and can live in a wider range of pH between 4.0 and 8.5; methanogenic bacteria are extremely sensitive to pH and can function with an optimum pH between 6.5 and 8 (Hwang et al., 2004; Boe, 2006). As methanogens are sensitive to environmental changes, this step is the rate-limiting step (Bozan, 2018).

2.3. Microbiology of Anaerobic Digestion

The stages of hydrolysis, acidogenesis, acetogenesis, and methanogenesis proceed with microbial processes in anaerobic digestion and each of these stages is conducted by metabolically related microorganisms. Therefore, stable digestion can be achieved if a balanced reaction rate is maintained between the stages and microbial guilds (Venkiteshwaran et al., 2015). Hydrolytic fermentative bacteria, acidogenic microorganisms, hydrogen-producing acetogens, hydrogen-utilizing acetogens, methanogenic organisms are included in these processes (Lettinga et al., 1996; Chernicharo, 2007). Details of the microbial profile are shown in Table 2.1.

Table 2.1. The microbial profile during different anaerobic digestion stages 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 fermentative bacteria hydrolyze complex organic substances into monomers. Parameters like pH, production of enzymes, and diffusion affect the rate of hydrolysis process. Although hydrolytic bacteria are diverse, the two phyla, namely *Bacteroidetes* and *Firmicutes*, contain most of the known species (Venkiteshwaran et al., 2015). The hydrolysis step is dominated by the species of these two phyla.

Hydrolysis products are converted to VFAs, which include acetate, propionate, isobutyrate, butyrate, valerate and isovalerate in acidogenesis. Alcohols, lactate, formate, CO_2 and H_2 are other produced products along with VFAs. *Bacteroidetes, Chloroflexi, Firmicutes,* and *Proteobacteria* are the well-known acidogenic bacteria phyla (Venkiteshwaran et al., 2015). Acetate and hydrogen can be produced by the metabolic activity of fermentative microorganisms for instance, *Clostridium* spp. can produce butyric acid, butanol, isopropanol, and acetone (Gerardi, 2003). Furthermore, the genus Clostridium are the most used for butyrate production (Zigová and Šturdík, 2000).

In acetogenesis, *Syntrophomonas* and *Syntrophus* which are the acetate bacteria convert the volatile fatty acids into acetate and hydrogen. Synthrophic acetogenesis is a critical step for the stable AD process because methanogenesis is inhibited by some of the VFAs, at high concentrations even at a pH of 7 (Venkiteshwaran et al., 2015). At the end of acetogenesis, hydrogen is released, and hydrogen has detrimental effects on the microorganisms that achieve this process (Pesta, 2007).

Methanogens belong to the domain *Archaea* and can be sensitive to changing conditions such as pH changes, VFA concentrations, ammonium ion and free ammonia concentrations (Westerholm et al., 2012; Manyi-Loh et al., 2013). Since methane is produced in this last step of the process, methanogens play an important role in the anaerobic digestion process. Methanogens are currently classified into six classes: *Methanopyrales*, *Methanobacteriales*, *Methanosarcinales*, *Methanococcales*, *Methanomicrobiales* and *Methanocellales*. *Methanosarcinales* consist of two families *Methanosarcinaceae* and *Methanosaetaceae*. Although these two families described as acetoclastic methanogens, they vary in terms of their physiology, biokinetics, and growth environment depending on the acetate concentration. Besides, more than half of methane production comes from acetate (Manyi-Loh et al., 2013).

2.4. Molecular Methods Applied for Microbial Analyses in Anaerobic Digesters

Anaerobic digestion (AD) contains diverse amounts of microorganisms that convert organic wastes into biogas (Venkiteshwaran et al., 2015). These microorganisms have a balanced relationship with each other (Bozan, 2018). Moreover, if an adequate balance is achieved between all microbial populations, the anaerobic process performs well (McMahon et al., 2004). As it can be seen in Figure 2.3., there are some methods to determine the microbial ecology of digesters: Real-time polymerase chain reaction (Q-PCR), denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH), metagenomics, and genomic sequencing (Bozan et al., 2017).

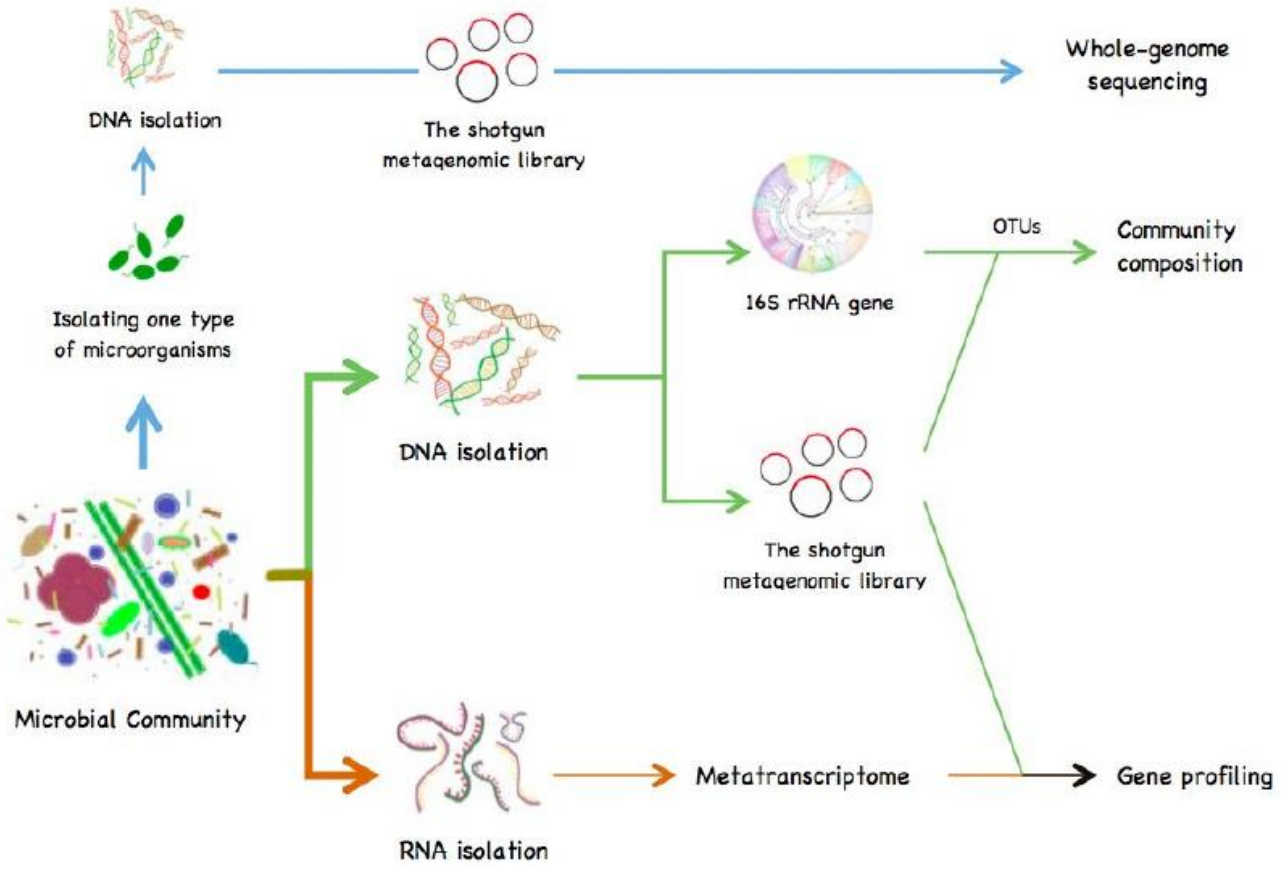


Figure 2.3. Applications of some special methods to determine the microbial ecology of digesters (Bozan et al., 2017).

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

Quantitative polymerase chain reaction (Q-PCR) is a useful technique for enhancing functional genomics, and helpful for analyzing gene expression. There are different types of Q-PCR; the basic

Q-PCR can be used to detect the gene without quantifying its expression. The data generated during the reaction can be used to monitor the amount of PCR product over time and by the effect of parameters such as melting temperature can be determined. Reverse transcriptase PCR can be used to study the expression, you need to extract the RNA and convert it into cDNA before the Q-PCR reaction (Maddocks and Jenkins, 2017).

2.4.2. Fluorescence in situ Hybridization (FISH)

Fluorescence in situ hybridization (FISH) is a technique that determines specific groups of microorganisms and it provides information about both the culturable and the unculturable microorganisms. The microorganisms in the samples can be defined as domain, family, genera, and species. Besides, it can give some ideas of the function and structure of complex microbial communities (Kumar et al., 2011). However, *Archaea* might not be observed under fluorescence microscopy, because of the special characteristics of the cell wall (Dinova et al., 2018).

2.4.3. Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is one of the most widely used techniques, especially in environmental science research areas (Muyzer et al., 1993; Curtis and Craine, 1998). It has high reliability; therefore, this approach is useful for comparison of microbial communities. Changes in community structures over time can be followed, where the stability of the methanogenesis process is highly dependent on complex microbial interactions (Muyzer et al., 1993; Liu et al., 2002).

2.4.4. Metagenomics

Metagenomic analysis is generally used to analyze complex microbial communities. Amplicon based method and whole metagenomic shotgun sequencing are the two most used methods for high throughput data. Shotgun metagenomic analysis can identify most of the organisms in the environmental sample and it can be divided into two types: sequence-based screens and functional screens. Amplicon based method includes 16S ribosomal RNA for bacteria, internal transcribed spacer and 18S region for fungi and eukaryotes, respectively (Ghosh et al., 2018).

2.4.5. Genomic Sequencing

The first-generation automated DNA sequencers based on the Sanger method with fluorescent dye-terminator reagents provided the sequencing of DNA populations and these sequencers were developed by adding computers in order to gather, store and analyze sequencing data (Smith et al., 1986). Next-generation sequencing (NGS) technologies have changed genomic research, parallel sequencing was massively increased in the second-generation (Miyamoto et al., 2014). Third-generation sequencing technologies enable longer read sequencing than second-generation allow direct sequencing of single DNA molecules (Heather and Chain, 2016).

One of the most expected areas for third generation DNA sequencing is the promise of nanopore sequencing. Oxford Nanopore Technologies was the first company to offer nanopore sequencers. The nanoporous platforms GridION and MinION created great excitement. MinION is a small, cell phone-sized USB device, it is used to generate bacterial genome reference sequences and targeted amplicons. Due to its small size and fast run times MinION device is very usable (Heather and Chain, 2016).

2.5. Important Parameters of Anaerobic Digestion

In the anaerobic environment, various parameters affect the stability of the process such as temperature, pH, alkalinity, VFAs concentration (Khanal, 2008).

2.5.1. Temperature

Temperature has a significant influence on the microorganisms in the anaerobic digestion processes and there are two optimal temperature ranges: one at 30-40 °C (optimum 37 °C) for mesophilic and one at 45-60 °C (optimum 55 °C) for thermophilic microorganisms (Mata-Alvarez, 2003). Increasing temperature has many benefits such as increased solubility of organic compounds, increased biological and chemical reaction rates and pathogen deaths. However, high temperatures also have negative effects, for example, increasing temperature increases the fraction of free ammonia and it causes inhibition for the microorganisms. Therefore, the thermophilic process more susceptible to inhibition (Boe, 2006). On the other hand, mesophilic process provides a slower reaction rate and lower biogas production but also it is less expensive than thermophilic process (Moset et al., 2015; Meegoda et al., 2018).

2.5.2. pH, Alkalinity and Volatile Fatty Acids

While methanogenic bacteria that produce methane are very sensitive to the pH range, fermentative bacteria can adapt to a wider pH range. (Boe, 2006; Appels et al., 2008). VFAs are also affected by pH values, products are mainly acetic and butyric acid at low pH, acetic and propionic acid at high pH values. (Boe, 2006).

The pH decreases as VFAs are produced during anaerobic digestion. This reduction has a negative effect on biogas production, but alkalinity protects the pH value in the form of carbon dioxide, ammonia, and bicarbonate (Appels et al., 2008). Since the system pH is controlled by the CO_2 concentration in the gas phase and the HCO_3 alkalinity of the liquid phase, if CO_2 levels stays constant, the possible addition of HCO_3 alkalinity may increase the pH of the digester (Turovskiy and Mathai, 2006).

2.5.3. Toxicity

There are some toxic elements and compounds that should not be in the anaerobic digestion process. Free oxygen elements and compounds containing oxygen such as NO_3^-, H_2O_2, and SO_4^{-2} are undesirable compounds in the anaerobic digesters. Sulfate-containing substrates can cause the growth of sulfate-reducing bacteria and the production of H_2S (Bozan, 2018).

Another toxic compound is ammonium which is toxic at high concentrations. Free ammonia nitrogen, produced from proteins and urea during biological hydrolysis of substrates, is an inhibitor for anaerobic digestion. Increasing the free ammonia nitrogen concentration above the threshold level has a detrimental effect on the anaerobic digestion process. (Chen et al., 2008).

2.6. Feedstocks

One of the most critical factors for the development of a biogas sector is the availability of feedstock potentials. There is an immense potential of a substrate, especially in the agricultural sector, such as livestock manure and crop residues (Al Seadi et al., 2018). As the substrates affect the process stability and biogas production the composition of the substrates is important for the digester (Adekunle and Okolie, 2005).

Turkish delight (lokum) is produced by heating a mixture of sugar, starch, and water at a certain temperature for a certain period. Starch is a polysaccharide, and it exists in two forms: straight chains and branched chains (Batu and Kırmacı, 2009). Starch is made up of glucose monomers and glucose is converted to higher volatile fatty acids, H_2, and acetic acid by microorganisms in the anaerobic digestion. Turkish delight waste includes high organic water and sugar content; therefore, it has high biodegradability. The delight production process is as shown detailed way in Figure 2.4 (Batu, 2006). Total candy and chocolate products were calculated to be 405,000 tons in 1999, 414,000 tons in 2000 and 429,000 tons in 2001. For the same years, Turkish delights production was 40,000 tons, 43,000 tons, 42,000 tons, respectively. In this case, delight production accounts for approximately 10% of the total candy production (Doyuran et al., 2004).

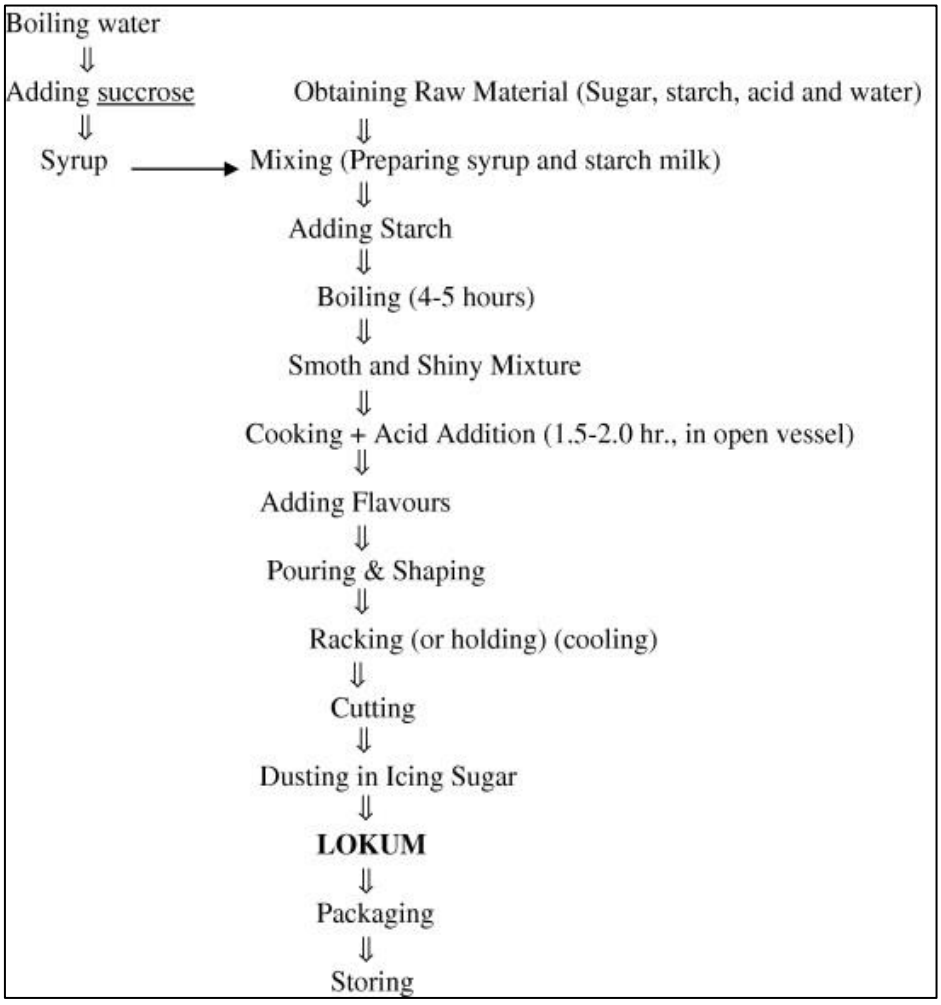


Figure 2.4. Turkish delight (lokum) production process (Batu, 2006).

Although there are large amounts of agro-industrial residue, it has very limited re-use, therefore, one of the promising feedstocks for anaerobic digestion is agricultural by-products (Rajput and Sheikh, 2019). The most common crop residue types are sunflower head, corn cob,

corn husk, rice husk and nutshell, among these residues, the sunflower head has the highest availability at 1 million tons per year Turkey. Edirne (Marmara), Adana (Mediterranean), Tekirdağ (Marmara), Konya (Central Anatolia) and Kirklareli (Marmara) provinces have the largest amount of collected residues (FAO, 2016). Sunflower residues are considered an important renewable resource for biogas production during anaerobic digestion. However, sunflower head waste lignin concentration is high, their rigid structure reducing the biodegradability (Zhurka et al., 2019).

One of the most consumed beverages in Turkey is the tea that settled in Turkish culture since old times. Tea waste can be used as an alternative raw material to cow dung in order to produce biogas which is a promising alternative energy source for the limited fossil fuels (Halder, 2016). Besides, it has been reported that when tea waste combined with other waste sources such as food waste, it increases efficiency by up to 30% and is a good substrate for biogas production.

2.7. Anaerobic Digester Systems

Currently, two types of AD processes are prominent for municipal solid waste organic wastes, commonly referred to as "wet" and "dry" anaerobic digestion processes. Wet digesters have lower total solids (<10-15 % TS) than dry digesters (25-40 % TS). Mechanical mixers are generally not applicable for dry AD processes. One of the main advantages of wet anaerobic digesters is that the dilution of the inhibitors with freshwater. The lower reactor volume, as well as less energy and water consumption, are the advantages of the dry combustion system (Luning et al., 2003).

The batch digester can operate significant amount of waste with little amount of the water and therefore it has low microbial activity and product yield. The continuous digester can process small amounts of residue with significant amount of water and therefore has a high microbial activity and product yield (Igoni et al., 2008).

Single-phase anaerobic systems have been the preferred reactor design for the majority of waste because all reactions (hydrolysis, acetogenesis, and methanogenesis) take place simultaneously in a single reactor. However, the operation of such systems at a high organic loading rate and for waste with biodegradable organic content becomes difficult. This type of waste undergoes rapid acidification, this result in the inhibition of methanogenic activity. On the other hand, two-phase systems can buffer the organic loading rate in the first stage, allowing a more constant feeding rate to the methanogenic stage (Bouallagui et al., 2005).

2.8. Bio-based Production from Anaerobic Digestion Processes

A transition to a circular economy that includes resource recovery, reuse, and recycling is essential for environmental sustainability. The demand for energy and raw materials is increasing day by day which must be provided by renewable resources (Atasoy et al., 2018). One of the methods used to generate renewable energy is anaerobic digestion; different types of wastes are used as substrates in bioreactors. The anaerobic digestion process consists of a series of biochemical reactions as it can be seen in Figure 2.5. Macromolecules are digested into monosaccharides, long-chain fatty acids, and amino acids during the hydrolytic reactions. VFAs, alcohols and hydrogen are produced during acidogenesis and acetogenesis (Wang et al., 2015). If biogas production is not desired in the system, VFA consumption should be prevented. As shown in the Figure 2.5, if biogas is not the end-product in anaerobic digestion, suitable system is necessary to extract acids from reactors (Wainaina et al., 2019).

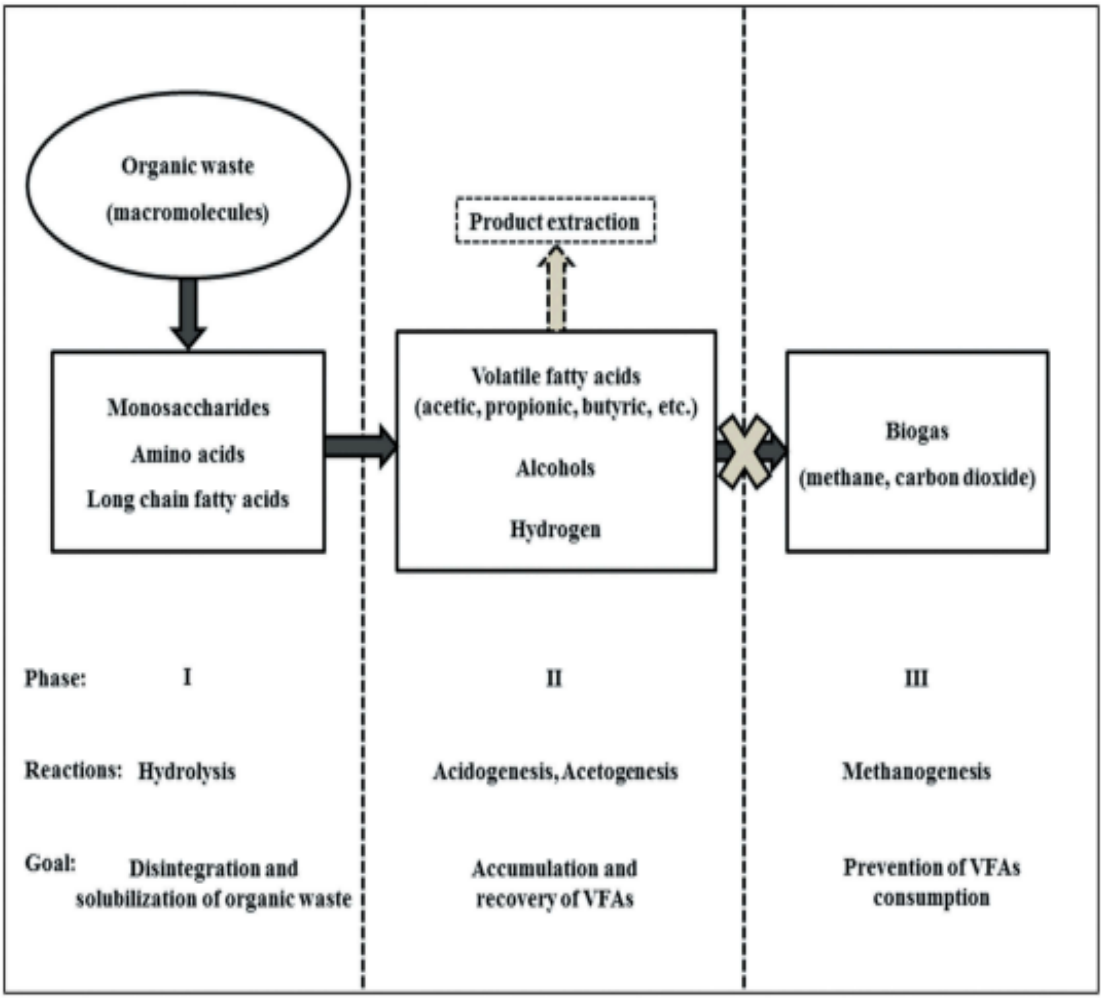
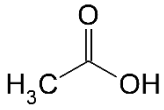
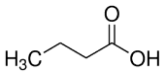


Figure 2.5. Formation of volatile fatty acids during anaerobic digestion (Wainaina et al., 2019).

Intermediates produced in the AD process such as hydrogen, carboxylic acids, VFAs are valuable for industries. For instance, essential fatty acids can provide valuable products through the microbial composition (Atasoy et al., 2018). These high value end products can be used in biofuels, bulk chemicals, bioplastics, nutrient removal from wastewater, and food additives (Wainaina et al., 2019). There are different types of VFAs such as formic, acetic, propionic, butyric, valeric, caproic acid. The most common VFAs are acetic, and butyric acids and they are summarized in terms of their general properties in Table 2.2.

Table 2.2. General properties of acetic and butyric acid (Atasoy et al., 2018).

VFAs	Chemical Formula	Market size (Kton/year)	Market Price (€/ ton)	Usage/Application	Production Methods	References
Acetic Acid		14000-17000	400-800	Vinyl acetate monomer (polymers, adhesives, dyes), Food additive, Solvent, Vinegar, Ester production, Chemicals	Chemical synthesis (carboxylation of methanol) and microbial fermentation (oxidative and anaerobic)	(Batia and Yang, 2017)
Butyric Acid		90-105	1500-1650	Animal and human food additive, Chemical intermediate, Solvent, Flavouring agent	Chemical synthesis (oxidation of butyraldehyde), Extraction from butter, microbial fermentation	(Zigová and Šturdík, 2000)

Acetic acid is one of the VFA production needed in industries such as chemistry, food, and beverage. Besides, it is the main components of aromas and acidity regulators. In food, drink or cellulose industries, acetic acid-producing bacteria are used as producers of certain species (Bhatia and Yang, 2017). Many types of bacteria can produce acetic acid such as *Acetobacter*, *Gluconacetobacter*, and *Gluconobacter*. Butyric acid is used as a valuable source of biodiesel. Since it has anti-pathogenic properties, it uses also as a food additive for human and animal. *Clostridium butyricum* are the main bacteria that produce butyric acid (Atasoy et al., 2018).

3. MATERIALS AND METHODS

3.1. Substrates and Inoculum Characterization

Seed sludge used for inoculant was supplied from Hurma municipal wastewater treatment plant in Antalya, Turkey. Sunflower heads were obtained from Sazlıdere village, Keşan, Edirne. The time for harvest is between 15 August and 15 September, depending on climatic conditions, substrate supply was coincided with harvest time and was provided in August. After the sunflower heads were provided, all developed seeds were removed from the head. Thus, only the head was used as the substrate. The tea waste substrate was provided by Unilever factory in which wastes were found during the production process. Samples from specific points of the process were taken by the factory employees and were sent to the laboratory with appropriate conditions. The sunflower head and tea waste were sieved to a maximum size of 0.5 mm to 1 mm as can be seen from Figure 3.1. The third substrate, the discarded Turkish delight was used. All these substrates were stored at +4 °C until used for bio methane production (BMP) tests. Before the BMP tests, all substrates were analyzed for their chemical characterization. After gathering all samples, the analyses of dry matter (TS), organic matter (VS), chemical oxygen demand (COD), pH, alkalinity, and C:N ratio were carried out according to Standard Methods (APHA, 2005).

Figure 3.1. The sieved substrates; sunflower head and tea waste.

3.2. Analytical Methods

Alkalinity, Total Solids (TS), Total Volatile Solids (TVS), Soluble Chemical Oxygen Demand (sCOD), and Carbon: Nitrogen ratio (C: N) were analyzed according to Standard Methods (APHA, 2005).

pH of the samples was measured with Hach, Pocket Pro^{+} pH meter. Alkalinity was determined by titration method with 0.1 N H_2SO_4, amount of consumed sulfuric acid was used for alkalinity calculation.

Soluble COD (sCOD) of digesters were measured on the 0th and 30th days. Samples taken from digesters were centrifuged at 14,000 rpm at 4°C for 30 min; supernatants were collected in a beaker. Then the supernatants of the samples were filtered through filters with 0.45-µm pore sizes before sCOD analyses, these supernatants were used for samples. For sCOD analyses, digestion solution was prepared using 10.216 g/L $K_2Cr_2O_7$ (dried at 150°C, 2 h), 167 ml/L Concentrated H_2SO_4 and 33.3 g/L $HgSO_4$. The mixture was prepared and filled with deionized water up to 1 L. Then, 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 a dark place for 1-2 days until it dissolved, after Ag_2SO_4 dissolved completely, solution was filled up to 1000 ml with concentrated sulphuric acid. 2.5 ml sample, 1.5 ml digestion solution and 3.5 ml concentrated sulphuric acid solution were added to the reactor tube. Samples were heated by thermoreactor (HACH, COD Reactor) at 150°C for 2 hours, and digested samples were cooled down. Absorbance of digested samples was measured by spectrophotometer (HACH, DR/2010) at 600 nm, the results were recorded, and Potassium hydrogen phthalate (KHP) was used to draw standard curve for the determination of sCOD.

On the 0th and 30th days digesters and on the 0th, 1st, 2nd, 3rd, 4^{th,} 7th, 8th, 10th, 13th, 15th, 17th days acidification reactors were analyzed in terms of their Volatile Fatty Acids (VFA) concentrations. As in sCOD measurement, samples were centrifuged at 14.000 rpm at 4°C for 30 minutes; supernatants were collected in beakers. The supernatants were filtered through 0.22-µm pore size membrane filters before VFA measurements. 10N phosphoric acid was added into the final filtrates as 10% (v/v) to fix all biological activity. The VFAs were determined by a gas chromatograph (GC-2025, Shimadzu Co., Japan) equipped with an auto injector (AOC-20i, Shimadzu Co., Japan). VFA composition was analyzed by a flame ionization detector, N_2 was the carrier gas connected to the instrument and 1 µl gas sample was injected by 0.5 ml syringe.

An automated elemental analyzer (ECS 4010 CHNS-O Analyzer, COSTECH Analytical Technologies, INC., USA) was employed to determine the amount of elemental C:N in the samples.

3.3. AMPTS II for Biogas Measurements

The methane yields of three substrates and combinations of three substrates were determined using a volumetric gas production method i.e. the Automated Methane Potential Test System II (AMPTS II). The system is shown in Figure 3.2.

Figure 3.2. The Automated Methane Potential Test System II (AMPTS II).

3.3.1. Guides to use: AMPTS II Test System

Before setting up of reactors for BMP tests make sure the followings are completed and ready. Firstly, the 3M NaOH solution was prepared for the CO_2 absorption unit. After the required amount of NaOH was weighed, it was mixed with about four-thirds of the total volume of the required distilled water. 0.4% Then, Thymolphthalein pH indicator solution was prepared. The NaOH solution containing the pH indicator, by mixing 5 ml of the 0.4 % Thymolphthalein solution per 1-liter 3M NaOH solution was prepared. Approximately 80 ml of the mixture containing NaOH

solution and Thymolphthalein pH indicator were added into each of the 15 glass bottles (100 ml) and the bottles' lid was sealed properly (Bioprocess Control Sweden AB, 2014).

After the CO_2 absorption unit is prepared, the sample (mixture of substrate and inoculum), and the blank (inoculum) was added into the reactors. Inoculum to substrate ratio was set to 2:1 (g TVS) in all batch digesters (Raposo et al., 2009; Labatut et al., 2011; Akyol et al., 2016; Uzun, 2019). 3 replicate BMP bottles were used for each digestion set for statistical significance, and every bottle included 3.30 g TVS seed sludge and 1.65 g TVS substrate. The volume of BMP bottles used was 500 ml with an active volume of 400 ml and headspace 100 ml. BMP bottles including Turkish delight waste, sunflower head waste, tea waste, Turkish delight, and sunflower head waste; Turkish delight and tea waste; sunflower head and tea waste; Turkish delight, sunflower head, tea waste were labeled as D, S, T, DS, DT, ST, DST, respectively. The substrates and combinations of substrates for mono and co-digestion are shown in Table 3.1.

Table 3.1. The substrates and combinations of substrates in digesters.

Component of Digesters	D	S	T	DS	DT	ST	DST	Seed Sludge (Control)
Seed Sludge	+	+	+	+	+	+	+	+
Turkish Delight Waste	+	-	-	+	+	-	+	-
Sunflower Head Waste	-	+	-	+	-	+	+	-
Tea Waste	-	-	+	-	+	+	+	-

Calculated amounts of substrates and combinations of substrates for mono and co-digestion are shown in Table 3.2. 150 ml of seed sludge and calculated amounts of substrates were added into all BMP bottles. The bottles were filled up to 400 ml with tap water. Then, the pH value of each bottle was set to 7.5 ± 0.2. Plastic glass lid was placed over the thermostatic water bath to minimize the evaporation of water during the experiment. Then, thermostatic water bath was filled with enough deionized water to completely cover the height of the content in the reactors and set to 37 °C. After placing all the reactors in the thermostatic water bath, motor cable was connected to digesters and the BMP system was set up as described below.

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

Digesters	Turkish delight waste (g)	Sunflower head waste(g)	Tea waste (g)	Seed Sludge (ml)
Turkish Delight Waste	4.75	-	-	150.0
Sunflower Head Waste	-	2.60	-	150.0
Tea Waste	-	-	3.54	150.0
Turkish Delight and Sunflower Head Waste	2.38	1.30	-	150.0
Turkish Delight and Tea Waste	2.38	1.77	-	150.0
Sunflower Head and Tea Waste	1.77	1.30	-	150.0
Turkish Delight, Sunflower Head and Tea Waste	1.58	0.87	1.18	150.0
Control (Seed Sludge)	-	-	-	150.0

3.3.2. Set up of AMPTS II System

First, tubes were disconnected from the gas volume measuring device. The digesters were flushed with a N_2 gas flow for 120 seconds to supply anaerobic condition to microorganisms. Then, the flushing gas was stopped, and the tube clamp was closed. Gas source was disconnected from the tube. The tubes were re-connected to the gas volume measuring device. The procedure was repeated for all reactors (Bioprocess Control Sweden AB, 2014).

All motor cables were connected to digesters. The long motor cable was connected to one of the digesters close to the motor controller. Motor controller and on/off switches on each motor unit were turned on. Finally, one end of the Ethernet cable was connected to the gas volume measuring device, and the other end of the Ethernet cable was connected to a computer that is not connected to a wireless network (Bioprocess Control Sweden AB, 2014). The set-up of AMPTS II is further described in the Bioprocess Control Sweden manual.

The AMPTS II systems continued for 30 d, using triplicate samples of each substrate, and using the same inoculum-to-substrate ratio, blanks, flushing with N_2 and incubation at 37 ± 1.0 °C.

Each AMPTS II bottle (500 ml total volume; 400 ml working volume and 100 ml headspace) was equipped with an individual mechanical mixer (112 revolutions per min; for 1 min after a 1 min pause; repeat). AMPTS II system was checked periodically, it was checked whether the motor was damaged, and the mixing was working properly. The water level in the thermostatic water bath was also checked and filled with deionized water as needed.

3.4. Molecular Techniques

3.4.1. DNA extraction

Genomic DNA was isolated from the 0^{th} and 30^{th} day of D, DS, and DST digestion samples that produced the highest methane yields in their groups under anaerobic conditions with the Soil Extraction Kit (Machenery-Nagel, Germany) according to the manufacturer's protocol. First, 5 ml sample was taken from digesters and centrifuged at 12,000 rpm for 10 min. After centrifugation, supernatant was collected and removed. Then, 700 µl Lysis Buffer SL1 was added to the tube in order to homogenize pellet and the homogenized solution was transferred to NucleoSpin® Bead Tube Type A containing ceramic beads. Following the homogenizing step, 150 µl Enhancer SX was pipetted into solution and a vortex adapter (Vortex-Genie) was utilized to destroy the cells, samples were vortexed at full speed and room temperature (18-25 °C) for 5 min. Then, samples were centrifuged at 12,000 rpm for 2 min to eliminate the foam caused by the detergent. After that, 150 µl Lysis Buffer SL3 was added and samples were left incubation for 5 min at 0-4 ºC. Then, samples were centrifuged for 1 min at 12,000 rpm. NucleoSpin® Inhibitor Removal Column was placed in a collection tube and 700 µl clear supernatant was loaded up onto the filter and the tubes were centrifuged for 1 min at 12,000 rpm. The process was completed according to the manufacturer's protocol. The quantity and quality of isolated DNA was determined by Qubit® 3 fluorometer (Thermo Fisher Scientific) by checking absorbance values of samples at 260 nm.

3.4.2. 16S Specific PCR Amplification

The primer pair to be used for the creation of the amplicon libraries targets a region of about 1400 bp covering the V1-V9 region of the 16S rRNA gene (Zeng et al., 2013; Klindworth et al., 2013). Oxford Nanopore Technologies barcode DNA sequences were added to the 5 'end of the target specific primer pairs. As it can be seen from the Table 3.3., the 3' flanking sequence of the

forward primer contains a wobble base (denoted by M; in the primer, the base is either an A or a C) in a variable region of the 16S gene.

Table 3.3. Primers used for 16S rRNA gene full reading.

Oligo name	5’ to 3’	Amplicon
16S-27F	ATCGCCTACCGTGAC - barcode - AGAGTTTGATCMTGGCTCAG	16S
16S-1492R	ATCGCCTACCGTGAC - barcode - CGGTTACCTTGTTACGACTT	16S

The PCR was performed using Proofreading DNA Polymerase 2x Reaction Mix and 200 nm from each primer. The following thermal cycle program was applied on the PCR device:” 1 minute at 95 °C; 35 cycles of 20 seconds at 95 °C, 30 seconds at 55 °C and 120 seconds at 65 °C; 5 minutes at 65 °C”. The PCR product was run on agarose gel to verify its size (~ 1450 bp) and purified using the PCR Product Purification Kit.

16S Barcoding Kit (SQK-RAB204; Oxford Nanopore Technologies) used to prepare the amplicon library and the amplicon library was loaded on the MinION™ (Oxford Nanopore Technologies) device for library preparation. It was performed according to manufacturer recommendation.

All barcoded 16S amplicons were quantified with standard technique and pooled in determined ratio. Finally, pooled sample was diluted to 50-100 ng with final volume of 10 µl. For the adapter ligation step, 1 µl of Rapid Adapter Mix was added into the pooled sample and it was incubated for 5 minutes at room temperature. The sequencing mix (11 µl DNA library) was mixed with Loading beads (LB) (25.5 µl), Nuclease-free water (4.5 µl) and the Sequencing Buffer mix (34 µl). The R9.4 flow cell to be used was made ready for loading by priming and the prepared sequencing mixture was transferred to the sample loading part of the flow cell.

A 48-hour (R9.4) sequencing protocol was performed using MinION™ control software, MinKNOW™ version 0.46.1.9 (R9.4). The reading data was obtained based on 1.2.2 rev 1.5 workflow and Metrichor™ agent (version 0.16.37960) software. Also, bioinformatics analysis of obtained results were in fast5 format and converted to fastq format using guppy v3.1.5 software (base-calling and de-multiplexing). Barcode and adapter sequences were cleaned with Porechop v0.2.3 software, and the universal primer was also removed from both ends of the sequences. After the sequences were cleaned, the readings of 1350-1450 bp length were filtered and the remaining readings were excluded from the analysis.

Cleaned readings were analyzed with customized workflow (Massive Bioinformatics, Turkey) using the mothur v.1.39.5 platform. Sequences were purified from chimeric structures, aligned, and operational taxonomic units (OTUs) were created by clustering readings that showed more than 99% similarity by measuring the distance between the similarity matrix. By comparing the created OTUs according to the RDP 16S rRNA database, taxonomic annotations were performed and the OTUs identified as the same genus were correlated.

3.5. Acidification Tests with High Methane Producing Sets of Substrates

At the end of BMP, the maximum biogas yield was observed with Turkish delight waste. Turkish delight and sunflower head waste, also Turkish delight, sunflower head, and tea waste mixtures have showed promising biogas yield as well. The acidogenesis stage was applied to these three substrates that generated the highest methane yield. The amounts of substrates were calculated, with an inoculum-to-substrate ratio of 2:1, eight experimental bottles (400 ml working volume-digesters) were set up in duplicate. Each reactor flushed with N_2 to provide anaerobic conditions. Substrates and 150 ml of seed sludge were added into all bottles and the bottles were filled up to 400 ml with tap water. The bottles were incubated at 37 ± 1.0 °C, under mesophilic conditions like BMP tests. Contrary to the BMP tests, the pH was initially adjusted to 5.5 ± 0.2 by adding HCl to improve the acidogenesis. The batch tests were monitored daily, and samples were taken from bottles during the 0^{th}, 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 7^{th}, 8^{th}, 10^{th}, 13^{th}, 15^{th}, 17^{th} experiment days for VFA analyses. Then the pH of each sample was measured**.** Sampling process from bottles is shown in the Figure 3.3.

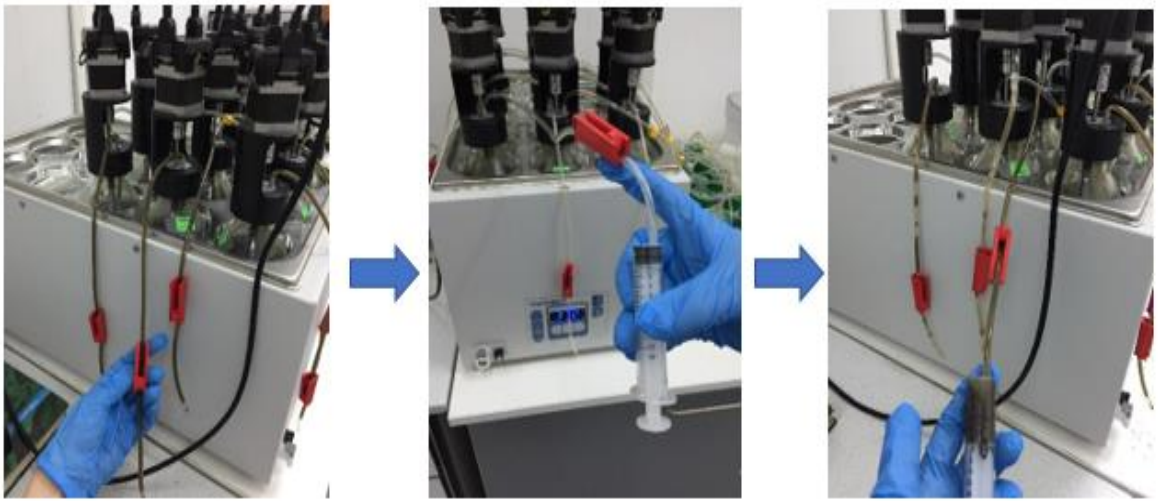


Figure 3.3. Samples from the digesters were collected daily for VFA analyses.

4. RESULTS AND DISCUSSION

In the first stage of research, Turkish delight waste (D), sunflower head waste (S), and tea waste (T) were used as substrates for mono digestion and co-digestion studies under mesophilic conditions to determine which substrates sets produce higher methane yields. D, DS and DST produced the highest methane yield in their groups. Bacterial characterization studies were implemented to D, DS, and DST on the 0^{th} and 30^{th} day and compared with each other using sequencing method. Then, bioinformatics analyses were carried out to realize and compare the bacterial diversity within digesters and between digesters. Acidification steps were also applied to the three sets D, DS, and DST that previously produced the highest methane yields. During the acidification steps only pH was set to 5.5 ± 0.2 to optimize VFA production. This pH range was predetermined due to previous studies of the R-D group. The results of methane production from mono and co-digestion sets and VFA productions from acidification studies were evaluated economically and ecologically.

4.1. Characteristics of Substrates and Seed Sludge

The characterizations of the seed sludge and raw substrates such as total solids (TS), total volatile solids (TVS), carbon/nitrogen ratio (C/N), pH, alkalinity, total chemical oxygen demand (TCOD), soluble chemical oxygen demand (sCOD) were measured using Standard Methods (APHA, 2005). Characterization results are shown in Table 4.1.

Table 4.1. The results of the characterization.

Samples	TS (% w/w)	TVS (% w/w)	TVS/TS (% w/w)	C/N	pH	Alkalinity (mg $CaCO_3$/L)	COD (mg/L)	sCOD (mg/L)
Turkish Delight Waste	43.5	34.7	80	253	4.1 ± 0.2	-	87120	49400
Sunflower Head Waste	71.3	63.4	89	23	7.8 ± 0.2	6500	34350	23400
Tea Waste	52.0	46.7	90	23	6.5 ± 0.2	2750	16225	5180
Seed Sludge	3.5	2.2	63	16	8.3 ± 0.2	7500	30100	740

4.2. Results of Mono and Co-digestion of Agricultural Wastes

After setting up AMPTS II system, several analyses were performed, and the values found were recorded. On the day AMPTS II was turned off, the same analyses were performed and noted. The values found are an indicator showing the efficiency of the digestion process.

4.2.1. TS/TVS Removal

TS, TVS of all digesters were measured on the 0^{th} and 30^{th}, days. The removal efficiency parameters were calculated and are shown in Table 4.2. The Turkish delight had the highest removal rate in terms of TS and TVS. Besides, Turkish delight provided better solids breakdown in co-digestion of DS, DT and DST when compared to mono-digestion of S, T, and co-digestion of ST, as reflected by the percent TS and TVS removal increased with containing Turkish delight in the digestion. Seed sludge had the lowest TS and TVS removal as expected, which is shown in Table 4.2.

Table 4.2. TS/TVS removal values in all digester, during the digestion time.

	TS Removal (%)	TVS Removal (%)
D	21	31
S	5	13
T	9	12
DS	7	14
DT	11	18
ST	11	17
DST	16	24
Seed Sludge (Control)	5	12

4.2.2. sCOD Removal and VFA Production/Removal in Anaerobic Digestion

On the 0^{th} and 30^{th}, days, sCOD concentrations of all digesters were measured and are shown in Figure 4.1 and Table 4.3. As shown in Figure 4.1, Turkish delight waste had higher sCOD values than the other substrates. Digesters without delight had lower sCOD values than digesters including delight. According to Table 4.3, Turkish delight waste had the highest rate of sCOD consumptions and digesters containing Turkish delight waste had higher rate of sCOD consumptions than the sCOD consumptions of digesters which did not contain. Although all digesters included same amount of volatile solids, the reason of the difference of sCOD consumptions in digesters was

related to the amount of Turkish delight. Furthermore, tea waste had the lowest rate of sCOD consumption; therefore, tea waste affected co-digestion sets and reduced their consumption rates.

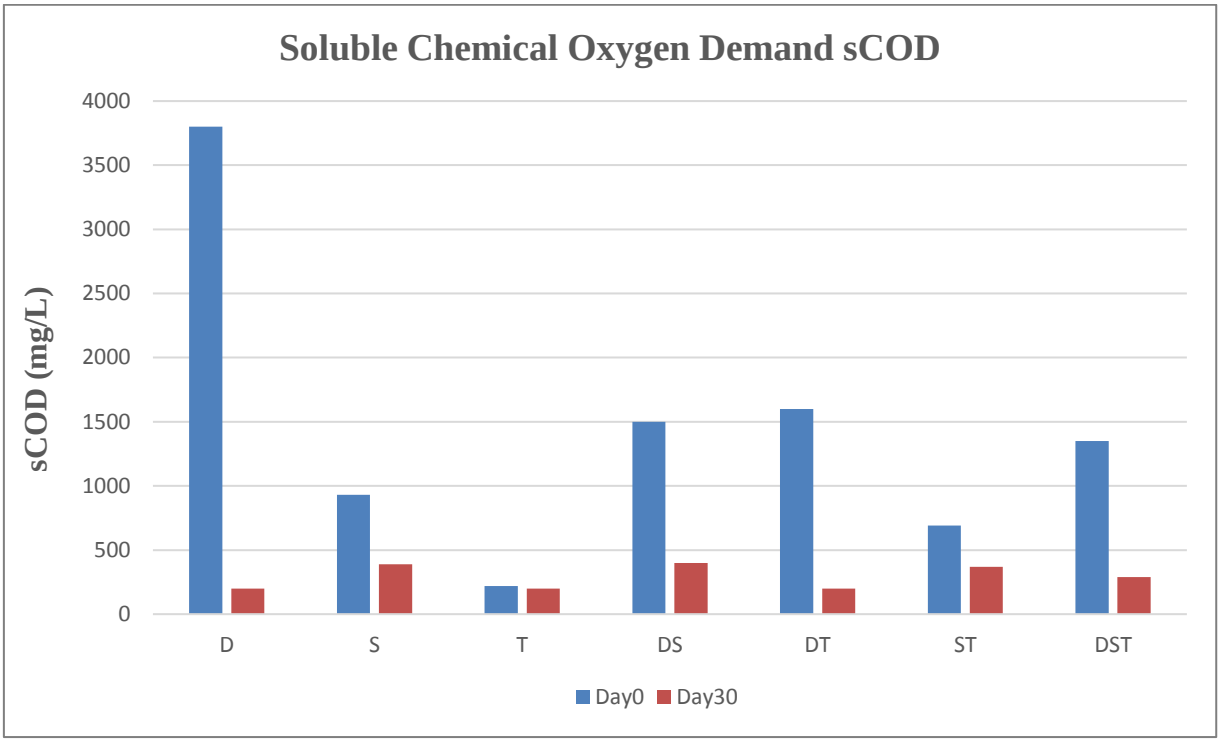


Figure 4.1. sCOD concentrations in all digesters with the digestion time.

Table 4.3. During digestion time, sCOD concentrations and consumptions in all digesters.

	sCOD (mg/L)		
Substrates	d0	d30	Consumption (%)
D	3800	200	95
S	930	390	58
T	220	200	8
DS	1600	200	88
DT	1500	400	73
ST	690	370	46
DST	1350	290	78
Seed Sludge	145	120	16

On the 0^{th} and 30^{th} days total VFA concentrations of the digesters were measured and calculated as total acetic acid (mg/L) which are shown in Figure 4.2 and given in Table 4.4. The common volatile fatty acids produced in all digesters during AD process was acetic, propionic and isobutyric acid along with smaller amounts of butyric, isovaleric and valeric acids. According to Figure 4.2 and Table 4.4, Turkish delight waste had the highest VFA production and VFA consumption. Besides, digesters that contained Turkish delight (D) which were DS, DT and DST had higher VFA productions and VFA consumptions than the digesters which did not contain D.

Tea waste had the lowest VFA production and VFA consumption; therefore, it affected the VFA production and consumption of the substrates with which it was co-digested. It was expected that Turkish delight would have the highest methane production and tea would have the lowest methane production, as high VFA consumptions contributed to increases in methane yields.

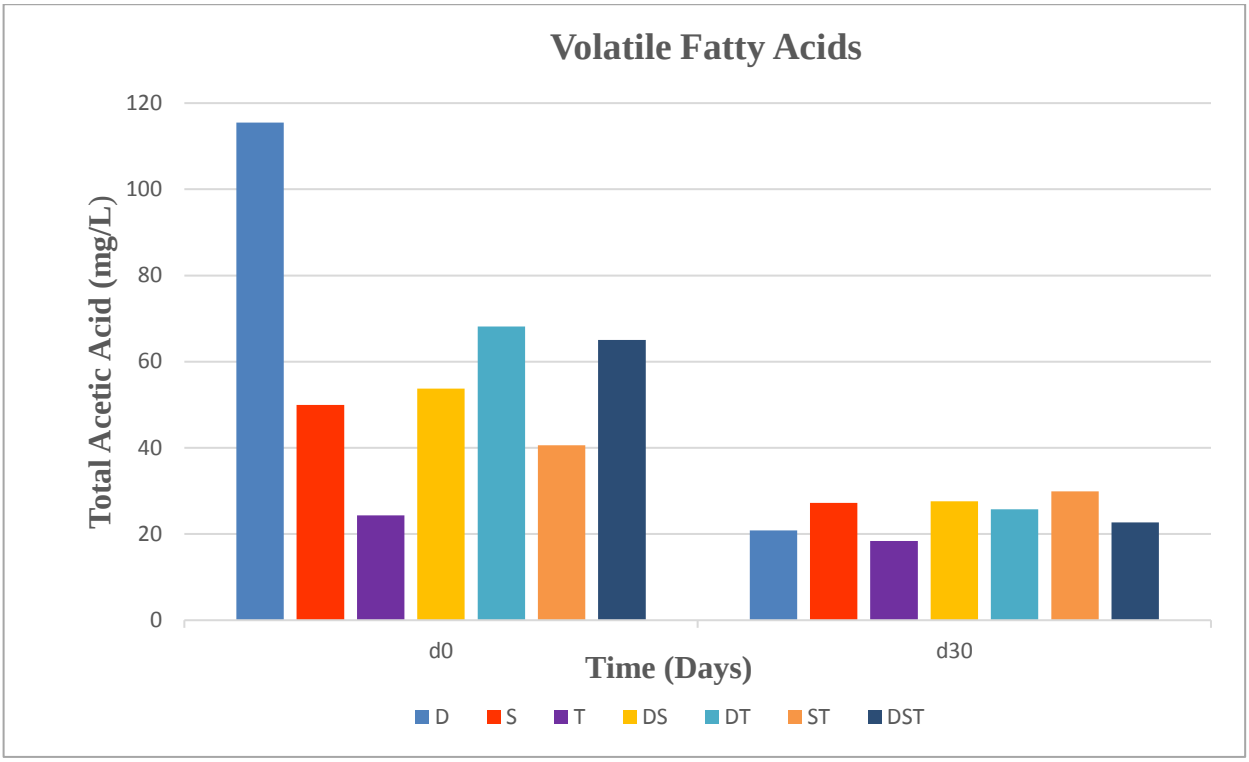


Figure 4.2. Results of VFA concentrations of the anaerobic digesters during digestion and co-digestion.

Table 4.4. VFA concentrations and consumption during digestion and co-digestion.

	Total Acetic Acid (mg/L)		
Substrates	d0	d30	Consumption (%)
D	116	21	82
S	50	27	46
T	24	18	23
DS	54	28	48
DT	68	26	62
ST	41	30	27
DST	65	23	65

The sensitivity of methanogens has been revealed by research. Although, it has no connection between low concentration short-chain fatty acids and the metabolic activity of methanogen, some volatile fatty acids like propionic and butyric acid have an impact on methanogens in anaerobic digestion process (Azman, 2016). In this case, accumulation of VFAs can lead to the inhibition. For

this reason, VFA concentration has been accepted as an important parameter to be followed in this study. Wang et al., (2009) observed that propionic acid concentration of 900 mg/L cause adverse consequences in anerobic digestion. As seen in Figure 4.2, the amount of total acetic acid (all acids were converted to acetic acid) did not exceed 116 mg/L in the beginning of AD process; therefore, it is not possible for butyric acid to exceed 900 mg/L in the following days in this system. Moreover, alkalinity concentrations in digesters might contribute to the stability of AD systems. As a result, there was not any inhibition in digesters in terms of accumulation of VFAs.

4.2.3. Methane Production

Methane measurements were recorded for 30 days. Methane production for all digester was stabilized approximately on the 16th day, as shown in Figure 4.3.

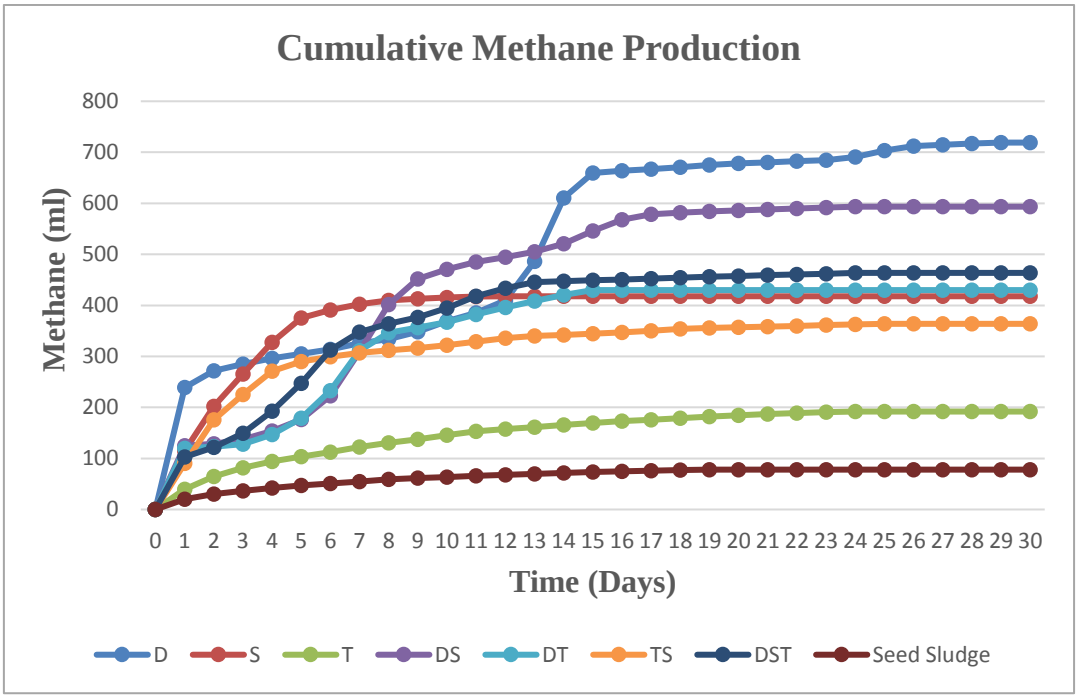


Figure 4.3. The cumulative methane productions during anaerobic digestion and co-digestion.

As shown in the Figure 4.3., the highest methane production was obtained from the mono digester set containing Turkish delight waste and the results indicated that the Turkish delight led to an increase in methane production because all digesters containing Turkish delight waste produced higher methane production than digesters without Turkish delight waste. The cumulative methane productions were recorded as D (719 ml), S (418 ml), T (192 ml), DS (593 ml), DT (430 ml), ST (364 ml), DST (464 ml) during 30 days anaerobic digestion.

Methane yield was obtained by dividing total produced methane, which the value was first subtracted from the produced methane amount 76 ml CH_4 obtained from seed sludge digester, to total added 1.65g volatile solids in the set-up of digesters (Bozan, 2018). Methane yield in terms of mL CH_4/g TVS_{used} is illustrated in Figure 4.4. The highest methane yield was obtained from the digester containing only Turkish delight waste; result was also related to the digester's VFA and sCOD consumption ratios.

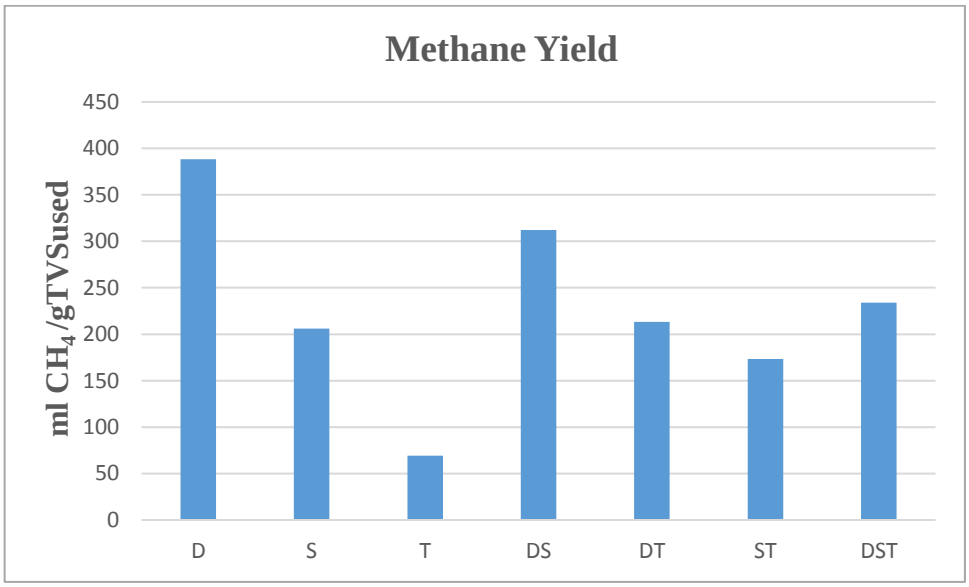


Figure 4.4. The calculated methane yields of the BMP digesters as per gram volatile solids added (CH_4/g TVS_{used}).

Methane yield for each digester was recorded as D (388 ml CH_4/g TVS_{used}), S (206 ml CH_4/g TVS_{used}), T (69 ml CH_4/g TVS_{used}), DS (312 ml CH_4/g TVS_{used}), DT (213 ml CH_4/g TVS_{used}), ST (173 ml CH_4/g TVS_{used}), and DST (234 ml CH_4/g TVS_{used}). In this study, methane production yield recorded from the digestion of untreated sunflower head was (206 mL CH4 g^{-1} TVS), when compared to other studies; Monlau et al. (2013), obtained higher methane potential from sunflower stalks (259 ± 6 mL CH4 g^{-1} raw TVS) after pretreatment at 55 °C with 4% NaOH for 24 h and in another study, Hesami et al. (2015), achieved much higher values, BMPs from sunflower stalks after hydrothermal (180 °C, 60 min) and isopropanol-based organosolv pretreatment (160 °C, 30 min, 1% H_2SO_4), of 234 and 278 mL CH4 g^{-1} TVS, respectively. Since pre-treatment was not performed in this study, these values were expected. Besides, results could be affected by the sunflower variety and geographic location (Amon et al., 2007).

In this study, methane production yield recorded from the digestion of tea waste was (69 mL CH4 g^{-1} VS). Higher data were found in spent tea than raw tea waste which is used for this

research. For instance, Ozbayram, (2020) operated BMP with the inoculum/substrate ratio of 2 with various substrates. At the end of the operation period, the lowest production was observed as 149 mL CH_4 g^{-1} VS in the mono-digesters operated with only spent tea waste. Although this result was lower than expected, it was found that spent tea had a higher methane yield than raw tea used in this study. In addition, the presence of high lignin content of tea waste could affect its gas production. In another study, Khayum et al. (2018), found that co-digestion of spent tea waste and cow manure increased the biogas potential by 170% compared to the mono digestion of cow manure. In this case, spent tea can be considered as a more suitable substrate than raw tea due to heat treatment.

As it can be seen in Figure 4.4, methane yield was higher in digesters containing Turkish delight waste. On the other hand, the methane yield did not show high results for co-digestion as was achieved in Turkish delight waste digestion, because mono Turkish delight digester produced and consumed more sCOD and VFA than its co-digestion. According to the results, tea and sunflower head wastes when mixed with Turkish delight reduced the yield of methane. For this reason, appropriate substrates should be defined to be co-digested with Turkish delight in order to get more methane yields. Although sunflower head waste is better alternative than tea waste, results obtained show that sunflower head and tea waste were not the best option to be co-digested with Turkish delight. Some pre-treatment processes which cause better hydrolysis of lignocellulosic contents may be required for tea and sunflower head waste to produce higher methane yield because of their higher lignocellulosic contents.

4.3. Bacterial Community Analyses of Mono and Co-digested Sludges

In this study, bacterial communities found in digesters were evaluated and applied NGS methodology for the samples taken from the 0^{th} and 30^{th} day of digesters. Bacterial characterization studies were implemented to D, DS, and DST that produced the highest methane yield in their groups. Digester containing only seed sludge was a control reactor for all reactors. Bacterial community profiling was observed by sampling from each reactors containing seed sludge from day 0 and 30, Turkish delight waste from day 0 and 30, Turkish delight and sunflower head waste from day 0 and 30, Turkish delight, sunflower head and tea waste from day 0 and 30; were labeled as BC01, BC02, BC03, BC04, BC05, BC06, BC07, BC08, respectively.

The bacterial community composition on the 0^{th} and 30^{th} day of the seed sludge, which was taken from a municipal wastewater treatment plant, was presented at the phylum level in Figure 4.5 and Figure 4.6, respectively. Hydrolytic microorganisms were used as seed sludge to initiate the

digestion process. *Proteobacteria, Firmicutes, Bacteroidetes,* are some of the hydrolytic bacterial phylum. As it can be clearly seen in Figure 4.5, the three most abundant bacterial phyla are *Proteobacteria* (75%), *Bacteroidetes* (9%) and *Firmicutes* (7%) on the 0th day of sample gathered from the seed sludge digester. These results are consistent with previous studies, which examined bacterial community structure for a wastewater treatment plant (Numberger et al., 2019).

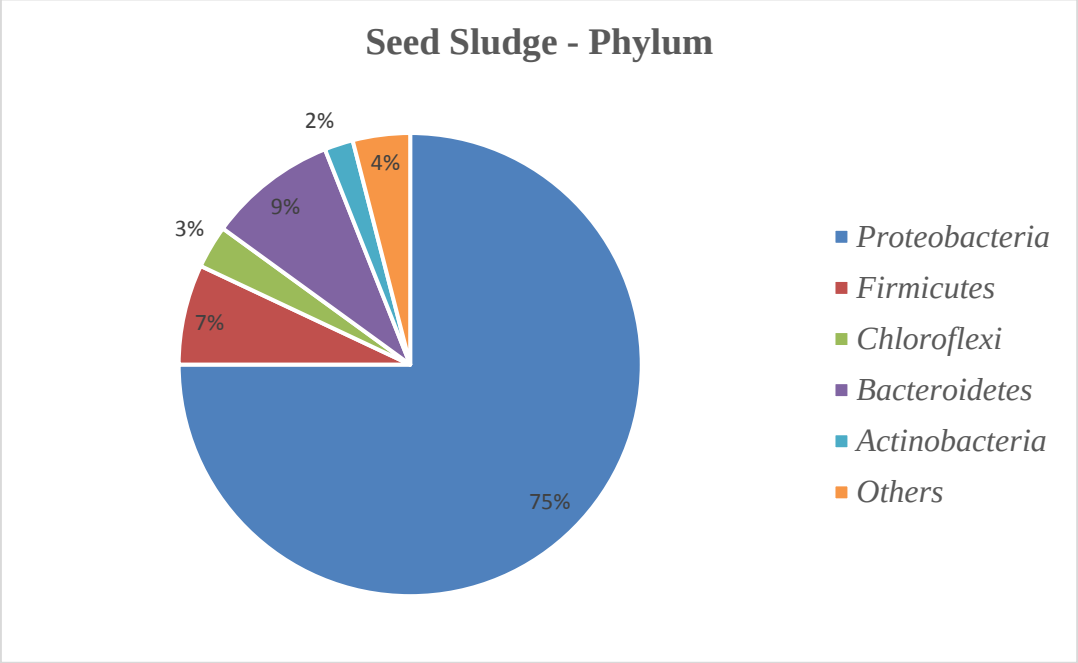


Figure 4.5. Bacterial communities in the seed sludge displaying phylum on the 0th day of AD.

Bacterial phyla of seed sludge on the 30th day of anaerobic digestion is indicated in Figure 4.6, to compare with other digesters. As it can be seen in Figure 4.6, *Proteobacteria* was the most abundant phylum (71%) followed by *Firmicutes* (11%) and *Bacteroidetes* (7%) on the 30th day of sample gathered from the seed sludge digester. Although, *Firmicutes* were not the most abundant phylum found in the seed sludge used in this research, in previous studies *Firmicutes, Bacteroidetes,* and *Proteobacteria* were found to be the most abundant phyla found in anaerobic digesters (Treu et al., 2016, Krause et al., 2008). *Chloroflexi* and *Actinobacteria* were also observed but in less amounts (Treu et al., 2016). *Chloroflexi* and *Actinobacteria* lower than the *Proteobacteria, Firmicutes,* and *Bacteroidetes*.

Proteobacteria are one of the most important bacteria in AD process. *Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria* which are class of Proteobacteria are known as the glucose, propionate, butyrate, and acetate-utilizing microorganisms. (Guo et al., 2015). *Bacteroidetes* can degrade complex molecules and they convert amino acids into acetate, and they are mainly responsible for

the digestion of starch and starch is digested faster and produces more VFA. *Bacteroidetes* are capable of metabolizing carbohydrates to produce VFAs and *Firmicutes* are described as fermenting bacteria by degrading VFA such as butyrate; therefore, VFA, proteins, and carbohydrate degradation were mostly controlled by *Firmicutes* and *Bacteroidetes* in anaerobic digesters (Walter et al., 2018). When the 0^{th} and 30^{th} days of seed sludge samples were compared, *Firmicutes* phyla increased, while that of *Proteobacteria* and *Bacteroidetes* decreased. VFA production may have affected between the 0^{th} and 30^{th} day by these changes.

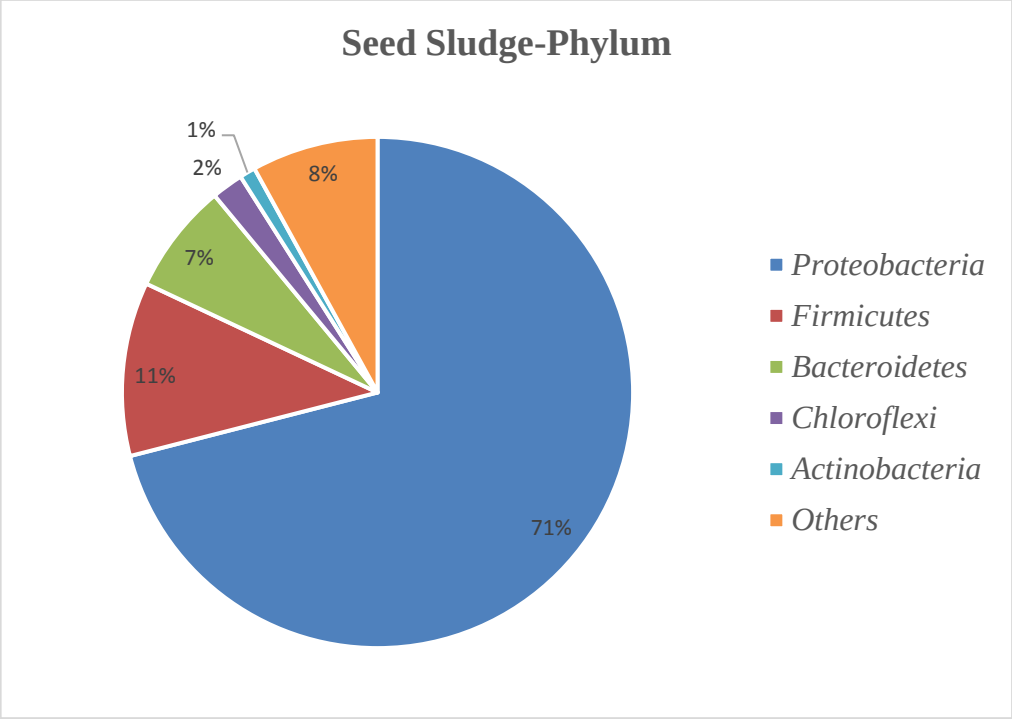


Figure 4.6. Bacterial communities in the seed sludge displaying phylum on the 30^{th} day of AD.

Bacterial diversity of seed sludge on the 0^{th} and 30^{th} day of anaerobic digestion is indicated in Figure 4.7 and Figure 4.8., respectively.

Although *Xanthomonadaceae* (phylum: *Proteobacteria*) genus were not detected in the seed sludge on the 0^{th} day sample, the majority of the total sequences were represented by *Xanthomonadaceae* (24%) at the family level in all systems which can be seen in Figure 4.7. The family *Xanthomonadaceae* belongs to the *Gammaproteobacteria* which have 250 genera with a diverse range of aerobicity, and of temperature adaptation (Williams et al., 2010). Other genera, shown in Figure 4.7. were *Frateuria* (21%), *Pseudoxanthomonas* (5%), *Prolixibacteracae* (4%), *Sedimentibacter* (2%), *Smithella* (2%), and so on.

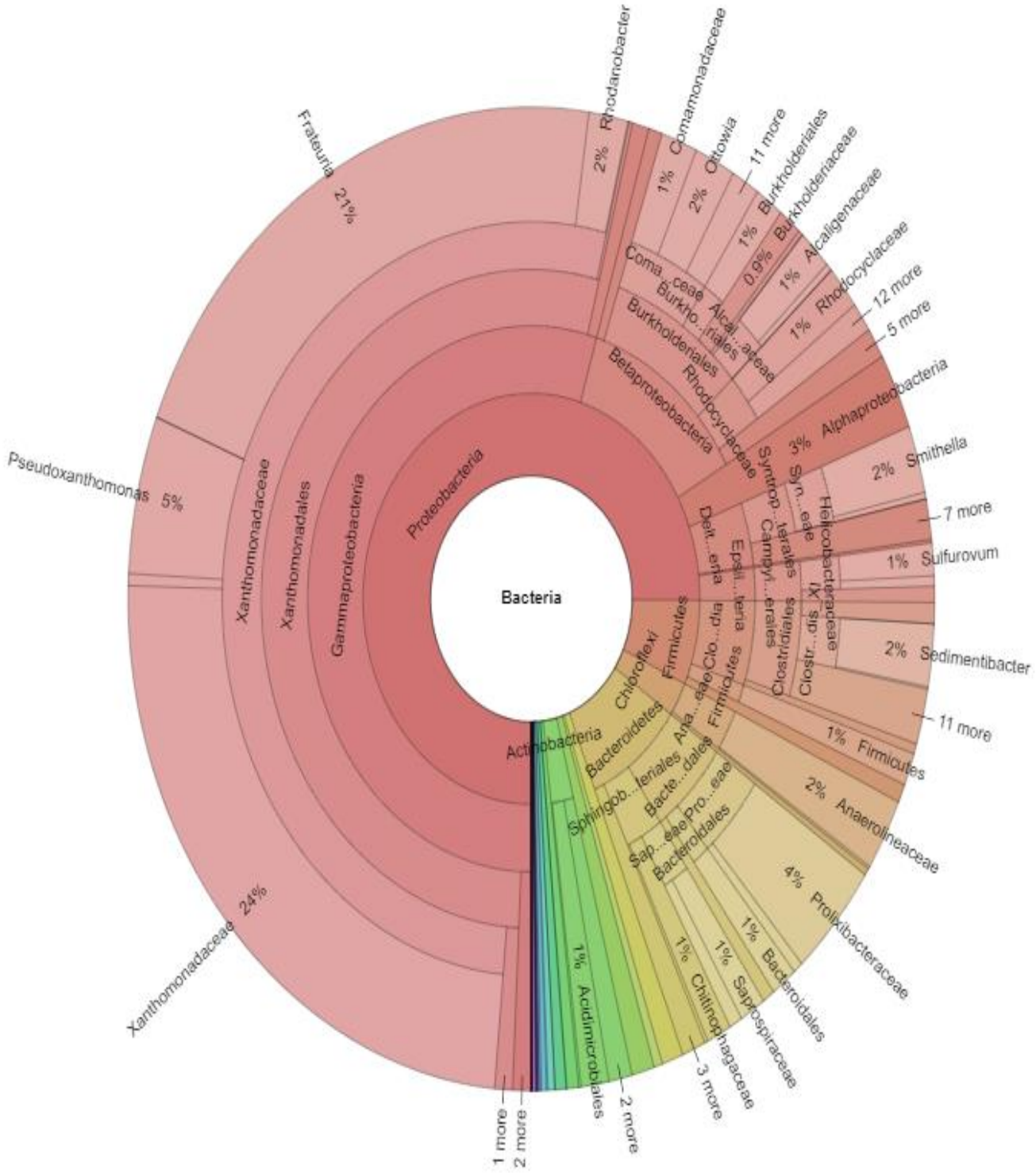


Figure 4.7. Bacterial communities in the seed sludge showing detailed species diversity on the 0th day of AD.

Most abundant bacterial genus in the seed sludge on the 30th day sample was *Smithella* (16%). Other genera, as can be seen clearly from Figure 4.7, were *Sulfurovum* (12%), *Prolixibacteracae* (4%), *Comamonadacaea* (4%), *Ottowia* (4%), and so on. When the 0th and 30th days of seed sludge

samples were compared, *Smithella* which are syntrophic propionate-oxidizing bacteria and *Sulfurovum* which are sulfide oxidizing bacteria increased, while that of *Xanthomonadaceae* decreased (Haosagul et al., 2019).

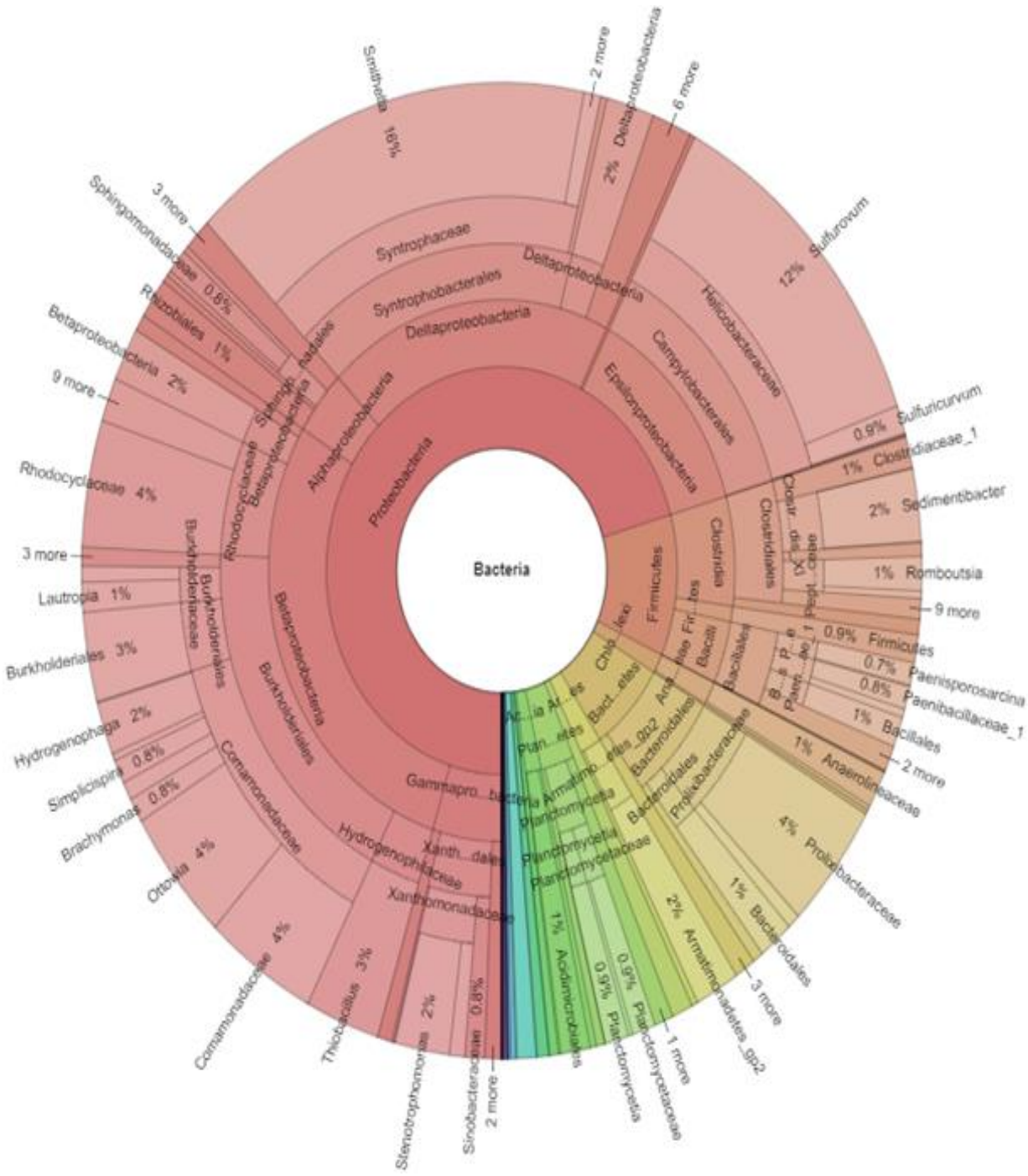


Figure 4.8. Bacterial communities in the seed sludge showing detailed species diversity on the 30th day of AD.

Bacterial community patterns at the genus level are indicated in Figure 4.9 and Table 4.5. The figure and table were formed with genera with more than 1% in the sample.

The family that dominated the sample from the digester of "BC01-Seed Sludge-0th day" was *Xanthomonadaceae* (24%). Besides, the most dominant genus was *Frateuria* (21%). Comparing the digester of "BC01-Seed Sludge-0th day" and "BC02-Seed Sludge-30th day", *Smithella* and sulfur-oxidizing bacteria *Sulfurovum* increased, while *Xanthomonadaceae* decreased. The genus *Smithella* (belonging to the phylum of Proteobacteria) is strictly anaerobic, syntrophic, VFA oxidizing bacteria. They grow syntrophically on propionate oxidation that acetate and small amounts of butyrate are formed as end products with methanogenic bacteria and utilize H_2 (Liu et al., 1999). *Xanthomonadaceae* are described as obligate aerobes, therefore decline was expected.

Turkish delight produced the highest methane yield in its groups. The bacterial community in the "BC03-Turkish Delight-0th day" reactor was dominated by sulfur-oxidizing bacteria *Sulfurovum* (34%) and *Sulfuricurvum* (12%). Comparing the digester of "BC03-Turkish Delight-0th day" and "BC04-Turkish Delight-30th day", *Sulfurovum* and *Sulfuricurvum* disappeared, while *Pseudomonadaceae*, a family of Gram-negative *Gammaproteobacteria* and *Thiobacillus*, a family of *Hydrogenophilaceae* increased. Although *Pseudomonadaceae* (phylum: *Proteobacteria*) species were not detected in the sample, it could be *Pseudomonas spp.* because *Pseudomonas spp*. are key players in agricultural biogas substrate degradation. Buettner and his colleagues (2019) observed that *Pseudomonas spp*. have a highly conserved carbohydrate metabolism. *Thiobacillus* is the most common H_2S-oxidizing bacteria. Considering the biogas flow rates that need to be treated at high H_2S concentrations, the amount of nitrate required can be substantial; *Thiobacillus* can reduce nitrate to nitrogen for complete denitrification. In this case, H_2S would not exceed the limit value (Dumont, 2015).

The most dominant genus of the bacterial community in the sample from digester of "BC05-Turkish Delight and Sunflower Head-0th day" was *Sulfurovum* with an abundance of 55%. Other genera shown in Figure 4.9, were *Arcobacter* (6.45%), *Trichococcus* (4.45%), *Sulfuricurvum* (3.17%) and so on. Comparing the digester of "BC05-Turkish Delight and Sunflower Head-0th day" and "BC06-Turkish Delight and Sunflower Head-30th day", *Sulfurovum* dramatically decreased, *Arcobacter* and *Trichococcus* disappeared, and while *Sulfuricurvum* increased. At the phylum level, *Proteobacteria, Firmicutes, Bacteroidetes,* and *Chloroflexi* were identified after anaerobic digestion. Although in previous studies, there was no Turkish delight and sunflower head waste co-

digestion, if the results evaluated as the phylum level, they are consistent with a previous study, which revealed similar bacterial phyla for a sunflower oil cake process (Rincón et al., 2011).

The bacterial community in the "BC07-Turkish Delight, Sunflower Head and Tea-0th day" reactor was dominated by sulfur-oxidizing bacteria *Sulfuricurvum* (26.2%), *Sulfurovum* (11.8%) and *Arcobacter* (11.3%). Comparing the digester of "BC07-Turkish Delight, Sunflower Head and Tea-30th day" and "BC08-Turkish Delight, Sunflower Head and Tea-30th day", *Sulfuricurvum* (4.3%) and *Sulfurovum* (3.6%) decreased, while *Arcobacter* disappeared. On the other hand, the bacterial members of *Gammaproteobacteria* class increased noticeably in number from 0% to 10.1%. Surprisingly, *Gammaproteobacteria* reads could not be assigned to any known bacterial genus in the present study.

Significant differences in bacterial communities were determined between the 0th and 30th day of digesters. Substrates increased bacterial diversity inside of anaerobic digesters according to bioinformatics analysis. The family *Pseudomonadaceae* were found more in the Turkish delight sample that producing the highest methane. Most digesters contain sulfur-oxidizing bacteria *Sulfurovum* and *Sulfuricurvum.* On day 0th, *Sulfurovum* dominates Turkish delight and Turkish delight and sunflower head digesters which are the two digesters that produce the highest methane. On day 30th, after anaerobic digestion, the percentage of *Sulfurovum* was either significantly reduced or completely zeroed.

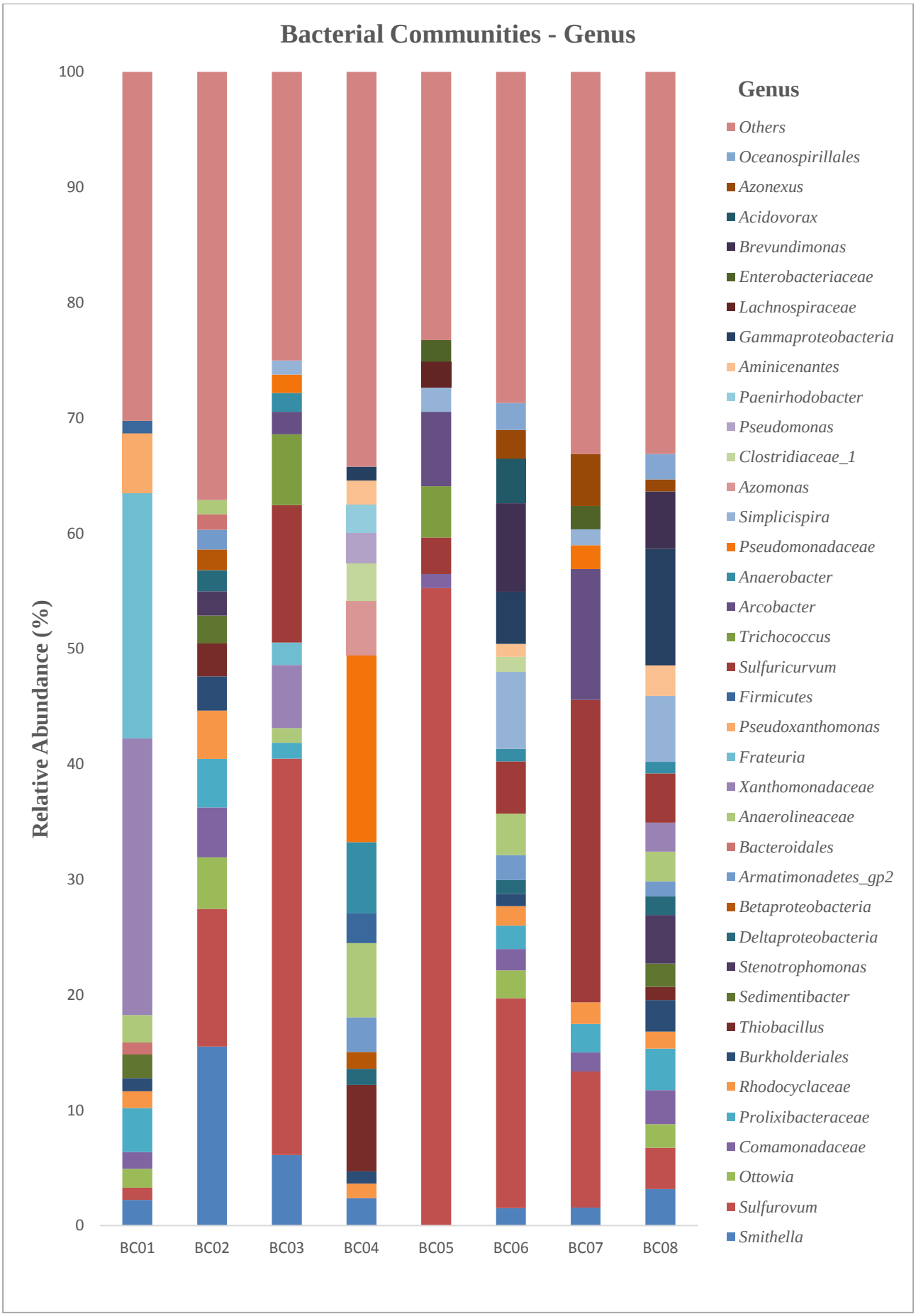


Figure 4.9. Most abundant bacteria genera found in the mesophilic digesters.

Table 4.5. The genus-level classification of bacteria found in digesters.

Genera	BC01	BC02	BC03	BC04	BC05	BC06	BC07	BC08
Smithella	2.22	15.52	6.11	2.38	0.00	1.52	1.55	3.17
Sulfurovum	1.06	11.93	34.36	0.00	55.27	18.19	11.80	3.59
Ottowia	1.64	4.46	0.00	0.00	0.00	2.40	0.00	2.04
Comamonadaceae	1.45	4.33	0.00	0.00	1.21	1.86	1.66	2.94
Prolixibacteraceae	3.82	4.21	1.38	0.00	0.00	2.01	2.46	3.61
Rhodocyclaceae	1.45	4.20	0.00	1.25	0.00	1.70	1.88	1.47
Burkholderiales	1.14	2.96	0.00	1.08	0.00	1.07	0.00	2.74
Thiobacillus	0.00	2.86	0.00	7.47	0.00	0.00	0.00	1.13
Sedimentibacter	2.06	2.41	0.00	0.00	0.00	0.00	0.00	2.04
Stenotrophomonas	0.00	2.10	0.00	0.00	0.00	0.00	0.00	4.21
Deltaproteobacteria	0.00	1.83	0.00	1.40	0.00	1.19	0.00	1.61
Betaproteobacteria	0.00	1.78	0.00	1.46	0.00	0.00	0.00	0.00
Armatimonadetes_gp2	0.00	1.71	0.00	3.00	0.00	2.16	0.00	1.30
Bacteroidales	1.04	1.35	0.00	0.00	0.00	0.00	0.00	0.00
Anaerolineaceae	2.38	1.23	1.28	6.45	0.00	3.60	0.00	2.58
Xanthomonadaceae	23.98	0.00	5.45	0.00	0.00	0.00	0.00	2.51
Frateuria	21.25	0.00	1.96	0.00	0.00	0.00	0.00	0.00
Pseudoxanthomonas	5.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	1.07	0.00	0.00	2.57	0.00	0.00	0.00	0.00
Sulfuricurvum	0.00	0.00	11.91	0.00	3.17	4.52	26.21	4.27
Trichococcus	0.00	0.00	6.13	0.00	4.45	0.00	0.00	0.00
Arcobacter	0.00	0.00	1.96	0.00	6.45	0.00	11.34	0.00
Anaerobacter	0.00	0.00	1.63	6.17	0.00	1.09	0.00	1.01
Pseudomonadaceae	0.00	0.00	1.59	16.24	0.00	0.00	2.08	0.00
Simplicispira	0.00	0.00	1.23	0.00	2.07	6.69	1.38	5.72
Azomonas	0.00	0.00	0.00	4.68	0.00	0.00	0.00	0.00
Clostridiaceae_1	0.00	0.00	0.00	3.27	0.00	1.30	0.00	0.00
Pseudomonas	0.00	0.00	0.00	2.67	0.00	0.00	0.00	0.00
Paenirhodobacter	0.00	0.00	0.00	2.42	0.00	0.00	0.00	0.00
Aminicenantes	0.00	0.00	0.00	2.08	0.00	1.10	0.00	2.64
Gammaproteobacteria	0.00	0.00	0.00	1.21	0.00	4.53	0.00	10.10
Lachnospiraceae	0.00	0.00	0.00	0.00	2.27	0.00	0.00	0.00
Enterobacteriaceae	0.00	0.00	0.00	0.00	1.86	0.00	2.01	0.00
Brevundimonas	0.00	0.00	0.00	0.00	0.00	7.68	0.00	4.97
Acidovorax	0.00	0.00	0.00	0.00	0.00	3.85	0.00	0.00
Azonexus	0.00	0.00	0.00	0.00	0.00	2.49	4.49	1.05
Oceanospirillales	0.00	0.00	0.00	0.00	0.00	2.35	0.00	2.21
Others	30.26	37.12	25.01	34.21	23.26	28.69	33.13	33.11

4.4. Results of VFA Production of High Methane Producing Sets of Substrates

In anaerobic digestion process, the cumulative methane productions were recorded as D (719 ml), S (418 ml), T (192 ml), DS (593 ml), DT (430 ml), ST (364 ml), DST (464 ml) on 30th day. The best three digesters in terms of methane production which were D, DS and DST were selected for the acidification stage, and they were conducted for 17 days at pH 5.5 ± 0.2.

The experiment was conducted for 17 days at pH 5.5 ±0.2, to observe the VFA production in the acidification. Although acetic and butyric acid were the dominant species, the various components of VFA observed, such as propionic acid, butyric acid and valeric acid. Since propionic, butyric and valeric acid were comparatively much lower than the acetic and butyric acid, they were neglected. Acetic and butyric acid concentrations obtained from D, DS, and DST are shown in Table 4.6. Also, the result of acetic and butyric acid concentrations is shown as graphical forms in Figure 4.10, Figure 4.11, and Figure 4.12, respectively. According to Table 4.6, total VFAs production reached the highest value on the 7th day.

Table 4.6. Acetic and butyric acid concentrations of the Turkish delight (D); Turkish delight and sunflower head (DS); and Turkish delight, sunflower head and tea waste (DST) during the acidification.

	D		DS		DST	
Day	Acetic Acid (mg/L)	Butyric Acid (mg/L)	Acetic Acid (mg/L)	Butyric Acid (mg/L)	Acetic Acid (mg/L)	Butyric Acid (mg/L)
0	47	8	47	7	32	7
1	257	1245	357	607	286	302
2	475	1190	564	575	482	347
3	600	1250	682	654	433	350
4	560	1120	705	570	466	312
7	810	1120	934	485	487	252
8	860	1054	662	480	132	277
10	874	1046	440	472	180	237
13	656	978	110	28	110	30
15	420	542	57	25	45	27
17	115	247	30	23	26	22

Turkish delight waste had a very high butyric acid concentration on day 1st, and then it tends to decrease slowly as can be seen in Figure 4.10. Acetic and butyric acid concentrations were very low on the 17th day.

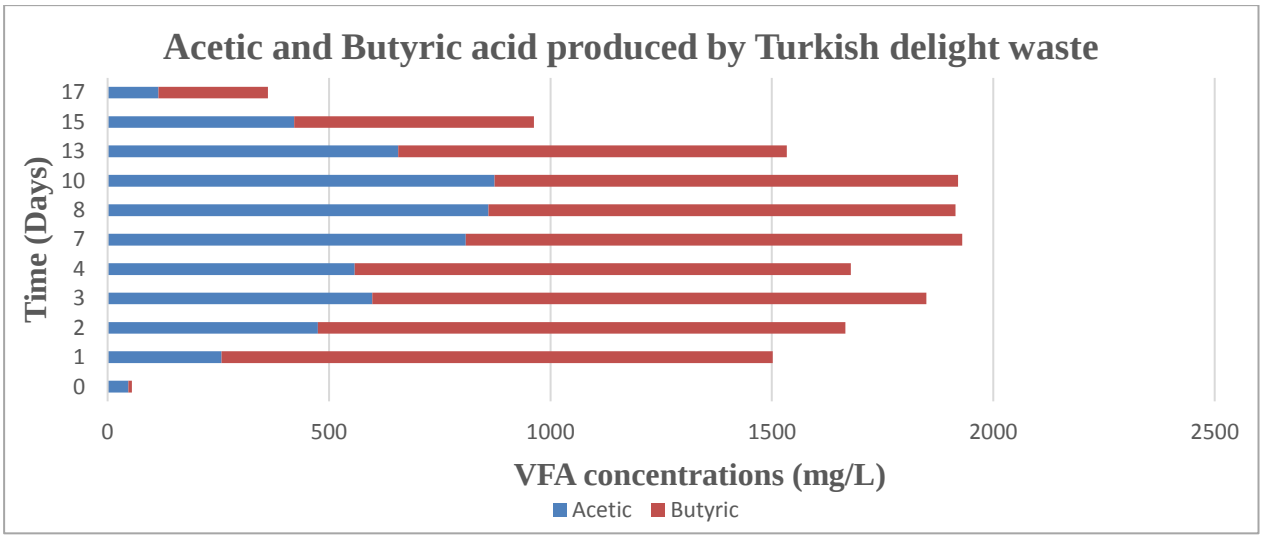


Figure 4.10. Results of acetic and butyric acid concentrations of the Turkish delight waste during the acidification.

As it can be clearly seen in Figure 4.11, VFAs concentrations in the digesters containing Turkish delight and sunflower head wastes were at their highest level on the 7th day.

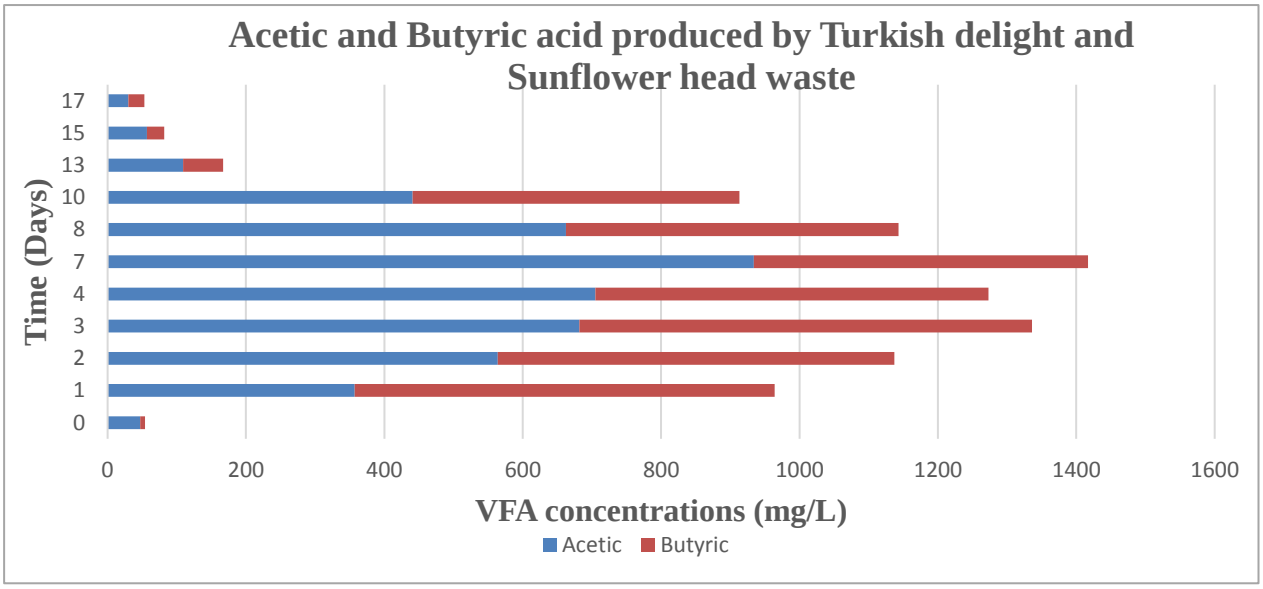


Figure 4.11. Results of acetic and butyric acid concentrations of the Turkish delight and sunflower head waste during the acidification.

The mixture of Turkish delight, sunflower head, and tea waste had a very high VFAs concentration on day 2nd, according to Figure 4.12. For this reason, it can be said that DST started acidification before D and DS. However, DST had the lowest total concentration in terms of VFAs comparing to D and DS. The low amount of Turkish delight waste may have caused it to produce a low concentration of VFAs.

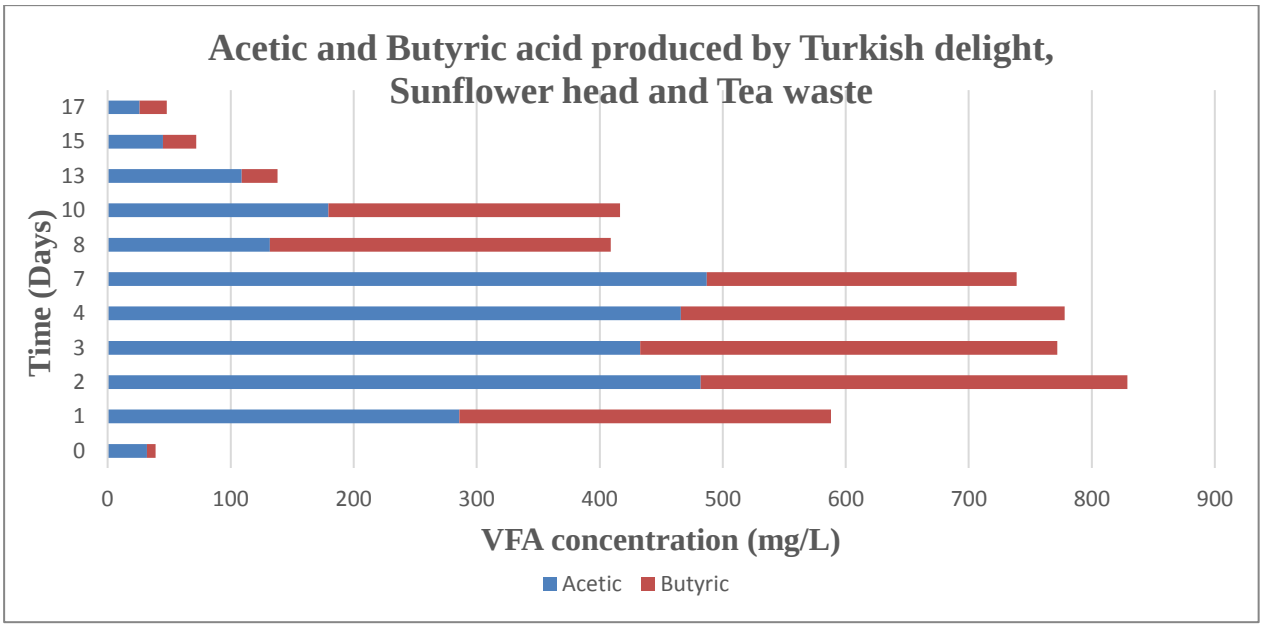


Figure 4.12. Results of acetic and butyric acid concentrations of the Turkish delight, sunflower head and tea waste during the acidification.

4.5. Economic Evaluation

4.5.1. Valorization of agro-industrial wastes for methane production

The prices of biogas are valued at USD 150/ton (Calt, 2015). Biogas is mainly composed of methane (CH_4) at a concentration of 50-70% and carbon dioxide (CO_2) at a concentration of 30-50% (Angelidaki et al., 2018). Since the AMPTS II device gives the result of only methane gas, the calculation was made based on 70% CH_4 concentration and USD 214/ton was used as the methane price. As a result, Turkish delight was calculated as USD 20/ton, Turkish delight, and sunflower head waste mixture as USD 22/ton, Turkish delight, sunflower head, and tea waste mixture as USD 17/ton.

According to Figure 4.4, although the Turkish delight had the highest methane yield, DS was higher than D in terms of valorization. The reason for this result was D had low TVS content. In other words, since digesters were set up by creating the same TVS content, more grams of Turkish delight waste were used into digester which contained only Turkish delight waste than digester containing the mixture of Turkish delight and sunflower head. The reason for DST was the lowest in terms of valorization was that it produced the least methane yield when compared to D and DS, due to the tea waste content.

4.5.2. Valorization of agro-industrial wastes for volatile fatty acids production

The prices of VFAs can vary between USD 600 to 3,815 /ton according to the number of carbon atoms in the molecular structure (Calt, 2015). When acetic acid prices range from USD 600 /ton and butyric acid prices range from USD 2,163 /ton, the results are given in Table 4.7. According to Table 4.7, Figure 4.10, and Figure 4.11 VFAs production reached the highest value on the 7th day for D and DS, while the highest value was seen on the 3rd day in table 4.7 where the economic evaluation was calculated. This was because butyric acid concentrations were higher on the 3rd day for D and DS, and also the butyric acid value was higher than the acetic acid one in terms of USD/ton. The highest value and concentration were on the 2nd day for DST.

Table 4.7. Daily total valorization coming from acetic and butyric acid production (USD/ton).

	Daily total valorization from VFA (as acetic and butyric acid) production. (USD/ton)										
Days	0	1	2	3	4	7	8	10	13	15	17
D	4	240	241	260	232	245	235	235	210	120	50
DS	5	166	172	200	180	174	156	140	14	10	7
DST	4	90	115	110	105	92	75	68	14	9	7

4.5.3. Economic comparison of VFAs and biogas production

As stated in Table 4.8, comparing the biogas studies and acid fermentation of D, DS, and DST reactors, the acidification stage looks more profitable.

Table 4.8. The value of methane and VFAs per ton of waste (USD/ton) in the acidification stage.

	The value of methane per ton of waste (USD/ton)	On the 3rd day the value of VFAs per ton of waste (USD/ton)
D	20	260
DS	22	200
DST	17	110

It can be seen in Table 4.8, the value of VFAs produced out of 1 ton of waste was higher than the methane gas produced from the same amount of waste. It has been determined that the valorization results are 6 to 13 times higher in acidification process depending on the substrate type. This is because the market value of VFA is much higher than biogas.

According to Perimenis et al. (2018) which compared between the acidogenic and methanogenic potentials of six different agricultural industry residues, it was found that VFA production from residues could have economic value three times higher than methane production. In another research, an economic comparison of VFAs and biogas production was made using sewage sludge by anaerobic digestion. As a result of the full-scale study, VFA had a net profit of 9.12 USD/m^3, while biogas had a net value of only 3.71 USD/m^3 (Liu et al., 2018).

5. CONCLUSIONS AND RECOMMENDATIONS

Regarding the mono-co digestion studies, methane yields for each digester were recorded as D (388 ml CH_4/g TVSused), S (206 ml CH_4/g TVSused), T (69 ml CH_4/g TVSused), DS (312 ml CH_4/g TVSused), DT (213 ml CH_4/g TVSused), ST (173 ml CH_4/g TVSused), and DST (234 ml CH_4/g TVSused) after 30-days in anaerobic digestion. The highest methane yield was obtained from the Turkish delight waste. Besides, digesters containing Turkish delight waste; DS, DT, DST had higher methane yield than digesters without delight waste. Turkish delight together with sunflower head both can be considered as moderately efficient in terms of methane yields obtained. Bio-methane production ceased approximately on the 16^{th} day in all digesters.

Regarding the bacterial characterization studies, substrates increased the bacterial diversity in the anaerobic digesters according to bioinformatics analyses. The family *Pseudomonadaceae* was observed more numerous in digester D that produced the highest methane than the digesters of DS and DST. Especially, *Sulfurovum* were clearly observed higher ratio in digesters compared to the control digesters, the main reason behind this should be investigated in future research. Both bacterial and archaeal characterization studies should be adopted in future studies.

Regarding the acidification studies, acetic and butyric acids were the dominant species. The total VFAs production reached the highest value on the 7^{th} day, while the highest economical value was observed on the 3^{rd} day due to the highest butyric acid production. According to the economic evaluations, total valorization potentials (coming from acetic and butyric acid) were 6-13 times higher in acidification studies than bio methane from the digestion studies.

Agro-wastes should be considered as a valuable source of energy and resources, not wastes. Besides, the transition of anaerobic digestion to carboxylates production has shown to provide better valorization. Similar studies at larger scale and with wider range of organic substrates (wastes) must be tested and system hydraulics, optimum solids loading, VFA and methane production, etc., should thoroughly be considered.

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