ENHANCED ANAEROBIC MONO-DIGESTION AND CO-DIGESTION OF CROP RESIDUES BY NAOH ALKALI PRE-TREATMENT

by

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B.Sc. in Chemical Engineering, Suez University, 2016

Submitted to the Institute of Environmental Sciences
in partial fulfillment of the requirements for the degree of
Master of Science
in
Environmental Technology

Boğaziçi University

2021

Dedicated to my dear mom,
Huveyda Ibrahim

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to Prof. Dr. Bahar İnce for her invaluable advice, continuous support, and patience during my MSc research. Her guidance and suggestions paved the way for me to accomplish my research. She always encouraged me and gave me the freedom in all the time of my research.

I would like to extend my gratitude to Prof. Gözde Özbayram, whose immense knowledge and plentiful experience have helped me solve many problems and learn new things during my research work. I would like to thank her for the unwavering support and help she provided.

I would like to express my appreciation to the jury members Prof. Dr. Ferhan Çeçen, Prof Dr. Emine Ubay Çokgör, Prof. Dr. Bülent Mertoğlu, and Prof. Dr. Burak Demirel for their valuable suggestions and supportive critics.

My gratitude, love, and appreciation are extended to my real brother Sina Maghami Nick who was always helpful. Throughout this master's journey, I learned many things from him that I would never forget. His precious support and help made everything easier to complete my study.

I would like to thank Prof. Dr. Nadim Copty. He was always helpful and supportive throughout my MSc study. I also appreciate all the support and help I received from my colleagues and friends. Special thanks go to my brother Şeref Mert who believed in me and never left me alone with no help, and my friends Ömer Uzun, Aslınur Çalışıyor, Ibrahim Cem, and Merve Oter for their valuable friendship and support throughout this research. I would also like to thank Filiz Ayılmaz for her help during my lab work.

Last but not least, I really appreciate all the support and love I received from my family, especially my parents Huveyda Ibrahim and Mansur Abu-Bakir, and my lovely sister Aya Mansur. Without their tremendous understanding and encouragement in the past few years, it would be impossible for me to complete my study.

ABSTRACT

ENHANCED ANAEROBIC MONO-DIGESTION AND CO-DIGESTION OF CROP RESIDUES BY NAOH ALKALI PRE-TREATMENT

The main objective of the study was to determine the effect of different dosages of NaOH alkali pre-treatment on the lignocellulosic structures and the methane production of crops' residues. In this regard, NaOH pre-treatment was implemented with different dosages on three different crops' residues: wheat, rye, and rice straws. Substrates were soaked into NaOH solutions at different dosages with solid to liquid ratios of 100 and 200 gTS/L to investigate both conditions. Anaerobic digestion process was conducted using AMPTS II under mesophilic conditions.

By 8 % NaOH pre-treatment dosage, the reduction of hemicellulose and lignin was 81 % and 62 % for rice straw, 86 % and 69 % for wheat straw, 80 % and 74 % for rye straw, respectively. The concentrations of CODs in the liquid fraction after pre-treatment were regarded as 3 times higher than untreated samples. Pre-treatment with 8 % NaOH dosage increased the methane yield of rice, wheat, and rye straws by 55 %, 40 %, and 46 % respectively. During the co-digestion test, an increase in VFA production was regarded by (rice/wheat) and (rice/wheat/rye) sets by values of 1930 and 1710 mg/L total acetic acid on the 5^{th} day.

Samples from digesters that produced the highest methane yield were chosen for further molecular analysis. Proteobacteria was found the most abundant bacterial phylum in the inoculum used, while *Methanothermobacter* and *Methanoculleus* were the major archaeal communities found. Compared to untreated digesters, 16S rRNA gene amplicon sequencing revealed more diverse microbial communities in NaOH digesters.

ÖZET

NAOH ALKALI ÖN İŞLEMİYLE BİTKİ ARTIKLARININ GELİŞTİRİLMİŞ ANAEROBİK MONO-SINDIRIMI

Çalışmanın temel amacı, farklı dozlarda NaOH alkali ön işleminin lignoselülozik yapılar ve bitki artıklarının metan üretimi üzerindeki etkisini belirlemektir. Bu bağlamda, buğday, çavdar ve pirinç samanı olmak üzere üç farklı ürün kalıntısına farklı dozlarda NaOH ön işlemi uygulanmıştır. Substratlar, her iki koşulu da araştırmak için 100 ve 200 GTS/L katı/sıvı oranlarında farklı dozajlarda NaOH çözeltilerine batırıldı. Anaerobik çürütme işlemi, AMPTS II kullanılarak mezofilik koşullar altında gerçekleştirilmiştir.

%8'lik NaOH ön-muamele dozu ile, hemiselüloz ve lignin azaltımı sırasıyla pirinç samanı için %81 ve %62, buğday samanı için %86 ve %69, çavdar samanı için %80 ve %74 olmuştur. Ön işlemden sonra sıvı fraksiyondaki KOİ konsantrasyonları, işlenmemiş numunelerden 3 kat daha yüksek olarak kabul edildi. %8 NaOH dozajı ile ön işlem pirinç, buğday ve çavdar samanlarının metan verimini sırasıyla %55, %40 ve %46 artırmıştır. Birlikte sindirme testi sırasında VFA üretiminde (pirinç/buğday) ve (pirinç/buğday/çavdar) setlerinde 1930 ve 1710 mg/L toplam asetik asit değerleri ile 5. günde artış olduğu düşünüldü.

Daha fazla moleküler analiz için en yüksek metan verimini üreten çürütücülerden numuneler seçildi. Proteobacteria, kullanılan inokulumda en bol bulunan bakteri filumu bulunurken, Methanothermobacter ve Methanoculleus bulunan başlıca arke topluluklarıydı. İşlenmemiş sindiricilerle karşılaştırıldığında, 16S rRNA gen amplikon dizilimi, NaOH sindiricilerde daha çeşitli mikrobiyal topluluklar ortaya çıkardı.

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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	gNaOH/gTS
NH_3	Ammonia	
NO_3^-	Nitrate	
N_2	Nitrogen	
SO_4^{-2}	Sulphate	
µl	Microliter	

Abbreviation	Explanation
AMPTS II	Automated Methane Potential Test System II
BMP	Biomethane Potential
AD	Anaerobic Digestion
cDNA	Complementary DNA
C:N	Carbon:Nitrogen
COD	Chemical Oxygen Demand (mg/L)
dH2O	Distilled Water
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
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
sCOD	Soluble Chemical Oxygen Demand (mg/L)
TS	Total Solids (mg/L)
TVS	Total Volatile Solids (mg/L)
VFA	Volatile Fatty Acid (mg/L)
0 % Rice Day 30	Untreated Rice Straw on the 30^{th} Day
8 % Rice Day 30	8 % gNaOH/gTS Pre-treated Rice Straw on the 30^{th} Day
8 % Rice Day 0	8 % gNaOH/gTS Pre-treated Rice Straw on the 0^{th} Day

1. INTRODUCTION

Over the past 2 centuries and since the beginning of the industrial revolution, energy demand has rapidly increased all around the world. Being available and easy to access, the world started to depend mainly on fossil fuels to meet their energy need. This has led to the depletion of the available fossil fuel reservoirs along with rising prices (Sari & Budiyono, 2014).

Dependence on fossil fuels hasn't caused only economic problems but also environmental. Burning oil, coal, and natural considerably contributes to global climate change due to the emission of greenhouse gases (GHGs) coupled with degradation of the environment and human health problems. Therefore, the energy supply for the future has become one of the most important global issues (Vats, et al., 2020).

To address the growing energy demand and avoid the negative effects of fossil fuels, introducing alternative sources of energy has become crucial. Bioenergy production from renewable sources is considered to be a great solution to overcome those limitations. Among all biofuel technologies, biogas production has shown promising results in many aspects. It is economical and environmentally friendly technology. In terms of energy input and output, the technology is so efficient compared to other technologies of energy production (Chandra, et al., 2012). All these advantages have encouraged many countries to consider this technology, and nowadays according to The World Biogas Association there are around 50 million micro-digesters, 132,000 small, medium, and large-scale digesters, and 700 upgrading plants operating globally (Global Potential of Biogas 2019).

One of the most used feedstocks for biogas production processes is energy crops. Due to their high energy potential to produce biogas, energy crops form a high percentage of the feeding mixture in many agricultural biogas plants. However, they count for approximately 30-35 % of the total biogas production cost which makes them unfeasible without high benefits (Schievano, et al., 2015). As a substitution for energy crops, agriculture wastes are considered a promising solution to overcome the high production cost and maintain the biogas production potential. Moreover, agriculture residues don't compete with food production like energy crops. Being available in high quantities and at a low cost, lignocellulosic wastes have been one of the most attractive alternatives for biogas production. Every year, the world produces massive quantities, around 8.2 billion tonnes, of agricultural lignocellulosic residues (e.g. straw, corn stover), which makes them the most abundant source for the production of biogas through anaerobic digestion (Croce, et al., 2016; Dahmen, et al., 2019).

Approximately 90 % of the lignocellulosic biomass is formed of a complex structure of lignin, cellulose, and hemicelluloses. Among these constituents, lignin is highly resistant to chemical and biological (microbial and enzymatic) degradation. As a result, the digestibility of lignocellulosic biomass for biogas production has become a challenging process. To increase the digestibility, a pre-treatment step must be carried out to change the compact structure so the microbes and enzymes can reach cellulose and hemicelluloses easily to break them down and to convert them into fermentable sugars (Ahmad, et al., 2018).

The main goal of the pre-treatment step is to reduce structural and compositional impediments of lignocellulosic biomass and to increase the accessibility of cellulose and hemicellulose to a microbial breakdown which will lead to an increase in biomass degradation rate and biogas yield. To reach this goal, several pre-treatment strategies have been investigated recently including biological, chemical, physical processes, or a combination of them (Zheng, et al., 2014).

Alkaline pre-treatment is one of the current most promising pre-treatment techniques as it has shown many advantages such as the solubilization of lignin and neutralization of various acidic products degraded from the lignocellulosic complex. Moreover, the trace amount of alkali left in the treated solids may help to avoid the drop of pH during the acidogenesis process. Therefore, alkaline pre-treatment is considered to be more effective and compatible when compared to other techniques of pre-treatment. NaOH was found to be the most effective alkaline chemical for lignin removal and biogas production among three kinds of chemicals (NaOH, KOH, lime) tested for pre-treatment (Zhu, et al., 2010).

Cereal residues, including both on-site residues and processing residues, represent a renewable and abundant source for lignocellulosic biomass. After harvesting cereal crops, a huge amount of agricultural residues is produced such as s rice straw, wheat straw, corn stover, and sugarcane bagasse. With a great availability of biomass and high energy potential, cereal crops are considered one of the best options for biogas production (Paudel, et al., 2017).

This study focuses on bioenergy production from three of the most abundant agricultural residues: wheat straw, rice straw, and rye straw. Three of them represent the largest fraction of agriculture wastes in many countries including Turkey. The main concern of this study is to analyze the effect of the NaOH alkali pre-treatment technique on each of them and make a clear comparison between the behavior of the untreated and substrates for effective biogas production through anaerobic digestion. In addition, a co-digestion test will be conducted using the three substrates to

decide on the set with the best results of methane production. In the end, bacterial characterization studies will be implemented to digesters with the highest methane production to get an idea of the microbial communities responsible for increasing the methane yield.

2. LITERATURE REVIEW

2.1. Anaerobic Digestion Process

The use of conventional ways of fuels and energy has created several economic and environmental problems, such as air pollution, water pollution, and global warming. This has motivated the researchers to search for alternatives with a focus on renewable and sustainable sources of energy (Widiasa & Johari, 2010). Anaerobic digestion is a biochemical process in which complex organic matter is decomposed and biodegraded in the absence of oxygen by various anaerobic microorganisms. The process of anaerobic digestion can be applied to treat a wide range of substrates like wastewater and organic solid wastes. The biogas produced from the process is considered to be a promising alternative to conventional resources of energy since it's economic and environment friendly (Adekunle & Okolie, 2015). Biogas consists mainly of methane, carbon dioxide along with a small amount of other gases and trace elements. It's a combustible gas that can be used as a substitution of fossil fuel sources such as firewood, coal, and natural gas. Currently, many countries use biogas as vehicle fuels or in fuel cells. It's used for combined heat and power generation (CHP) or it is upgraded and fed into natural gas grids as well (Jingura & Kamusoko, 2017).

Anaerobic digestion has been introduced as a waste-to-energy technology to treat wastes sustainably. It's been fully accepted and proven technology for biodegradation of organic matter to produce a renewable source of energy (De Bere, 2000). Biogas produced from anaerobic digestion offers high advantages over the other methods of bioenergy production. Limitation of carbon dioxide and other gases emissions, reduction of air and water pollution, and energy production from biomass are making anaerobic digestion a more promising and attractive method for waste management. Lignocellulosic biomass is considered to be a great source of substrates to be treated by anaerobic digestion for biogas production, since these materials are rich in carbohydrates. However, the recalcitrance nature of these materials makes them very difficult to be biodegraded, as their structure hinders the microbial hydrolysis step in the process which makes the usage of lignocellulosic materials in biogas production is so limited today (Teghammar, 2013).

The interest in the anaerobic digestion of organic wastes is increasing day by day due to the wide range of advantages offered by the process regarding the reduction of organic waste, production of sustainable energy sources, and decreasing the environmental impact caused by fossil fuels. Although many other technologies have been offered for waste treatment (gasification, pyrolysis, plasma, etc.),

these technologies haven't been applied and are widespread the same way as anaerobic digestion. According to the European Biogas Association, the European biogas market reached around 17,783 biogas plants with an electricity production capacity of 65,179 GWh in 2017 (EBA, 2019).

2.2. Biochemistry of Anaerobic Digestion Process

Anaerobic digestion is a biochemical process during which complex organic compounds are biodegraded and decomposed by an assortment of microbes under oxygen-free conditions converting them into a methane and carbon dioxide mixture (about 50-75 % CH_4 and 25-50 % CO_2) (Frigon & Guiot, 2010). The digestion itself is considered to be a complex process consisting of several biochemical reactions taking place under anaerobic conditions. The formation of biogas in anaerobic digestion involves four different steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis as shown in Figure 2.1.

The rate-limiting step in anaerobic digestion can be defined as the step in which the whole process fails under imposed kinetic stress (Aslanzadeh, 2014). For complex organic compounds, researchers report that the rate-limiting step is the hydrolysis step due to the recalcitrance nature of these materials and the formation of toxic and non-desired byproducts formed during hydrolysis. However, methanogenesis is thought to be the rate-limiting step for easy biodegradable compounds (Lu, et al., 2008)

The microorganisms responsible for the degradation reactions in each step differ widely according to physiology, nutritional needs, growth kinetics, and sensitivity to the environment. The two major groups of microorganisms in the process are: acid-forming and methane forming microorganisms. Despite the presence of fungi and protozoa in anaerobic reactors, bacteria and archaea are the dominant groups in biodegradation (Demirel & Yenigün, 2002).

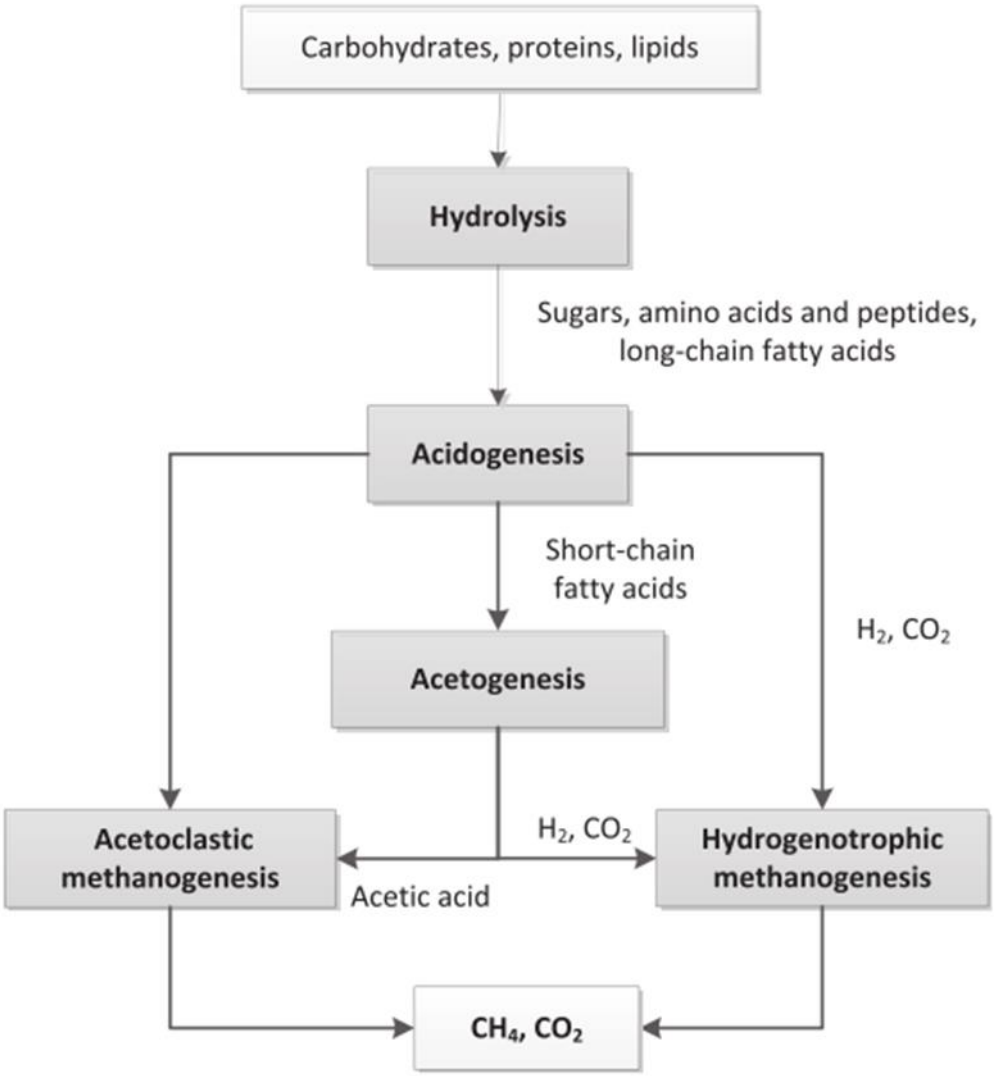

Figure 2.1. Process flow during anaerobic digestion (Zheng, et al., 2014).

2.2.1. Hydrolysis Stage

Hydrolysis is the first step in anaerobic digestion, and it includes the transformation of insoluble organic materials and higher molecular mass compounds such as lipids, polysaccharides, proteins, fats, nucleic acid, etc. into soluble organic materials i.e. to compounds that can be metabolized to provide energy or can be used as starting material for the synthesis of other required compounds such as monosaccharides, amino acids, and other simple organic compounds.

Hydrolysis is carried out by strict anaerobic microorganisms such as Bacteroides, Clostridia (phylum: Firmicutes), and facultative bacteria such as Streptococci, etc. (Christy, et al., 2014). Since large organic compounds and polymers are too large to be absorbed and digested by microorganisms as a substrate/food source, this step is considered to be very critical. To reach biodegradation, certain

microorganisms produce different types of enzymes, called extracellular enzymes, which “break down” the larger molecules up into smaller pieces which the microorganism can then absorb and use as a source of energy and nutrition. Some microorganisms produce several different enzymes, which allow them to break down different types of organic materials. Other microorganisms are specialized.

Table 2.1 indicates examples of some different groups of extracellular enzymes. Each group involves several enzymes that are specialized in various substrates. The rate of decomposition during the hydrolysis stage depends greatly on the nature of the substrate (Adekunle & Okolie, 2015).

Table 2.1. Some important groups of hydrolytic enzymes and their functions (Adekunle & Okolie, 2015).

Enzymes	Substrates	Breakdown products
Proteinase	Proteins	Amino acids
Cellulase	Cellulose	Cellobiose and glucose
Hemicellulase	Hemicellulose	Sugars, such as glucose, xylose, mannose and arabinose
Amylase	Starch	Glucose
Lipase	Fats	Fatty acids and glycerol
Pectinase	Pectin	Sugars, such as galactose, arabinose and polygalacticuronic acid

2.2.2. Acidogenesis Stage

Acidogenesis is the second step of anaerobic digestion in which fermentative microorganisms are involved in the biodegradation of amino acids, sugars, and some fatty acids which are produced in the hydrolytic phase. They are degraded further into short-chain organic acids, acetic acid, valeric acid, acetate, propionate, butyrate, carbon dioxide, and hydrogen by the activity of Clostridia, Mycoplasmas, and Streptococci (Table 2.2). The products formed vary with the type of bacteria as well as with temperature, pH, and nature of the substrate. In general, during this stage, simple sugars, fatty acids, and amino acids are converted into organic acids and alcohols (Gerardi, 2003).

Table 2.2. Major acids and alcohols produced through fermentation processes in anaerobic digestion (Gerardi, 2003).

Substrate	Chemical formula
Acetate	CH_3COOH
Butanol	$CH_3 (CH_2)_2 CH_2OH$
Butyrate	$CH_3 CH_3 CH_2COOH$
Caproic acid	$CH_3 (CH_2)_4 COOH$
Formate	$HCOOH$
Ethanol	CH_3CH_2OH
Lactate	$CH_3 CHOHCOOH$
Methanol	CH_3OH
Propanol	$CH_3 CH_2 CH_2OH$
Propionate	CH_3CH_2COOH
Succinate	$HOOCCH_2CH_2COOH$

2.2.3. Acetogenesis Stage

The acids and alcohols produced in the acidogenic phase are further consumed as substrates for the other microorganisms in the third phase of anaerobic digestion. In the acetogenesis phase, products that cannot be directly converted to methane by methanogenic bacteria are converted into methanogenic substrates. Volatile fatty acids and alcohols (VFA) are oxidized into methanogenic substrates like acetate, hydrogen, and carbon dioxide, VFA with carbon chains longer than one unit are oxidized into acetate and hydrogen.

So we get plenty of organic acids, alcohols, and organic-nitrogen compounds, some can be used directly as a substrate by methane-forming bacteria (Table 2.3) and some that can be used indirectly (Table 2.4) by being degraded to acetate by fermentative bacteria, then to methane by methane-forming bacteria. If the methane-forming bacteria do not degrade the products of the second stage, the products will accumulate and produce an acid medium.

Table 2.3. Alcohols, organic-nitrogen compounds, and organic acids used as substrates by methane-forming bacteria (Gerardi, 2003).

Substrate	Chemical formula
Acetate	CH_3COOH
Formate	HCOOH
Methanol	CH_3OH
Methylamine	CH_3NH_2

Table 2.4. Alcohol and organic acids used indirectly as substrates by methane-forming bacteria (Gerardi, 2003).

Substrate	Chemical formula
Ethanol	CH_3CH_2OH
Butyrate	CH_3 CH_3 CH_2COOH
Propionate	$CH3CH_2COOH$

2.2.4. Methanogenesis Stage

In the methanogenic phase, methane is formed mostly from acetate, carbon dioxide, and hydrogen gas by methanogenic bacterial under strictly anaerobic conditions. Methane is also formed from other organic compounds other than acetate (Table 2.4). So, all fermentative products must be converted to compounds that can be used directly or indirectly by methane-forming bacteria (Aslanzadeh, 2014).

As long as the compounds can be converted to substrates that are capable of being degraded by methane-forming bacteria, the decomposition of complex organic compounds will proceed relatively fast. Therefore, methanogenesis is commonly considered to be the rate-limiting step or “bottleneck” in the final degradation of complex organic compounds. However, for organic compounds that are hardly biodegradable such as lignocellulosic biomass, the hydrolysis stage may become the rate-limiting step (Gerardi, 2003).

2.3. Environmental and Nutritional Requirements

2.3.1. Appropriate Environment

2.3.1.1. Temperature

Temperature is considered as one of the most important parameters in the process due to its significant influence on the microorganisms' activity within the reactor. There are two common ranges of temperature used in anaerobic digestion: one at 30-40 °C (optimum 37 °C) for mesophilic microorganisms, and the other at 45-60 °C (optimum 55 °C) for thermophilic microorganisms. The temperature must be adjusted very well according to the microorganisms used in the process. Increasing or decreasing the temperature may have both advantages and disadvantages affecting the whole process (Kanokwan, 2006).

The increasing temperature might have a positive effect on the process such as increasing the solubility of organic compounds, increasing the biological and chemical reaction rates, and pathogen deaths. On the other hand, high temperatures increase the fraction of free ammonia and this causes inhibition for the microorganisms. Therefore, the thermophilic process is more susceptible to inhibition (Kanokwan, 2006). However, the mesophilic process provides a slower reaction rate and lower methane production but it's less expensive than the thermophilic process (Meegoda, et al., 2018).

2.3.1.2. pH, Alkalinity and Volatile Fatty Acids

Each group of microorganisms is considered to have a specific pH range. Although methanogenic bacteria which is responsible for methane production are very sensitive to pH with an optimum range between 6.5 and 7.2, fermentative bacteria can adapt to a wider pH range between 4.0 and 8.5 (Appels, et al., 2008).

VFAs are also affected by pH values. At low pH ranges, products are mainly acetic and butyric acid, and at high pH values acetic and propionic acid are more produced.

The values of pH decrease as VFAs are continuously 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 since the system's pH value is controlled by CO_2 concentration in the gas phase, and HCO_3 concentration in the liquid phase. If CO_2 levels stay constant, the possible addition of HCO_3 alkalinity may increase the pH of the digester (Appels, et al., 2008).

2.3.2. Nutrients

2.3.2.1. Macronutrients: Nitrogen, Phosphorus, and Sulfur

The organics in the feedstock represent the food for most of the anaerobic system organisms. Since the systems are considered heterotrophs, except for the autotrophic methanogens converting hydrogen to methane, the carbon source needed comes from the organics in the feedstock. However, a specific amount of inorganic nutrients is necessary for the growth of the bacteria's cells. Therefore, the system must be supplied with those nutrients to assure high removal efficiency (Gerardi, 2003).

Macronutrients are defined as nutrients that are needed for the cell growth of bacteria such as nitrogen and phosphorous together with carbon which is the biodegradable part of organics. In the case of anaerobic digestion of complex wastes, there is a ration of macronutrients that is known to be giving satisfactory results. This ration is C/N/P: 250(400-200) / 5 / 1.

Nitrogen is regarded as the most important macronutrient required in the highest concentration for bacterial growth. During waste degradation, Ammonia (NH_3) and the portion of organic nitrogen are released and directly unutilized by microorganisms. Therefore, they are considered the major nitrogen sources in the anaerobic system. Nitrate (NO_3 -) and nitrite (NO_2-) nitrogen are mostly lost as they are reduced to nitrogen gas, so they are not available for growth under anaerobic conditions (Stronach, et al., 2012).

Phosphorous is just as important as nitrogen for microbial growth. It was reported that the microbial uptake of phosphorus in anaerobic digestion is approximately one-fifth to one-seventh of that for nitrogen. Other studies show that it is 2 percent of the biological solid waste. Most guidelines in researches refer to a ratio on a COD-basis of COD: N:P / 1000:7:1, for low loaded processes (<0.5 kg. COD/kg VSS/day).

Besides nitrogen and phosphorous, some sulfide sources must be added, commonly in the sulfate form. The reason behind that is most methanogens utilize sulfide as a sulfur source which is required for the synthesis of proteins

2.3.2.2. Micronutrients: Trace Metals and Organic growth factors

Along with macronutrients (nitrogen, phosphorous, carbon) some other nutrients have to be shown in the anaerobic system as they are responsible for about 4 percent of the dry weight of the cell. These compounds are referred to as micronutrients. The most common micronutrients are trace metals and organic growth factors.

The actual concentration of trace metals to exist in the anaerobic system is difficult to be calculated as the sulfide needed by methanogens precipitates these elements. Therefore, leaving low concentrations of trace metals within the system at equilibrium is suggested. Those metals are Na, K, Mg, Ca, Fe, S, Ni, Co, Mo.

The addition of nickel, iron, and cobalt has been reported to help achieve high volatile solids concentration. Some metals like iron, cobalt, and nickel show a very important role in converting acetate to methane by methanogens. Molybdenum, selenium, and tungsten are considered mandatory as well for methanogens.

Organic growth factors are considered very important for the stimulation of methanogenic activity. They include the coenzyme-M, the factor F420, acetate, 2-methyl butyric acid, vitamins, N-acetyl glucosamine, riboflavin, B12, and some other compounds (Nizami, 2012).

2.3.3. Electron donor and acceptor

The component that plays the role of electron donor which provides energy for the biomass activity in the system is the biodegradable COD. The electron acceptor differs according to the system type. In aerobic systems, the electron acceptor is oxygen which is reduced to water. However, anoxic systems reduce nitrate/nitrite to nitrogen gas. In anaerobic systems, CO_2 or sulfate are operated as electron acceptors. CO_2 is reduced to methane gas, while sulfate is reduced to H_2S (Gerardi, 2003).

2.3.4. Mixing

Proper mixing is playing an important role in increasing the digestion rate by facilitating the way of nutrients and food to reach the cells and by removing wastes from them. Mixing has mainly 2 functions. First, the food, which is the substrates, easily reaches the microorganism cell wall from the

bulk solution. Second, the waste products from the cell wall are transferred to the bulk solution. Mixing can be done in two ways; gas recirculation and mechanical mixing. Gas recirculation is accomplished by means of reinjecting compressed digester gas. Mechanical mixing can be conducted by mechanical pumping or "jettling" of sludge (Stronach, et al., 2012).

2.3.5. Toxicity

Free oxygen elements and compounds containing oxygen like NO3-, H2O2, and SO4-2 are considered to be toxic and undesirable to the process as they can negatively affect the microorganisms' activity, so they should be carefully controlled and monitored during the process.

The substrates that contain high ranges of Sulfate compounds can cause the growth of sulfate-reducing bacteria and the production of H_2S, so they are undesirable.

Ammonia in high concentration is also thought to be very toxic to the system. Increasing the free ammonia nitrogen concentration above the threshold level has a highly negative effect on the anaerobic digestion process. Free ammonia nitrogen, produced from proteins and urea during biological hydrolysis of substrates, is an inhibitor for the anaerobic digestion process therefore it should be managed carefully throughout the process (Chen, et al., 2008).

2.4. Anaerobic Reactor Types

Over a long time, anaerobic digesters have evolved greatly from that of a simple chemostat to the modern high-rate anaerobic processes that permit operation at very low HRTs. Anaerobic digesters are designed to treat insoluble wastes and soluble wastewaters. Soluble wastewaters are treated by high-rate anaerobic digesters. The reason behind this is that wastewaters do not need hydrolysis or solubilization of wastes, so the treatment is much faster. However, high-strength wastes are usually treated by suspended growth digesters. Fixed-film digesters are usually used for the treatment of soluble wastewaters. There are several anaerobic systems available for the treatment of both soluble wastewaters and insoluble wastes as shown in Table 2.5. Each digester affects hydraulic retention time (HRT) and solids retention time (SRT) (Gerardi, 2003).

Table 2.5. Typical operating conditions of various anaerobic digesters

Reactor type	Load (kg COD/m^3day)	HRT (hour)	COD removal (%)
Conventional anaerobic digester	1-5	240-360	60-80
Anaerobic contact reactor	1-6	24-120	60-80
Anaerobic sequencing batch reactor	1-10	6-24	75-90
Anaerobic filter	2-15	10-85	80-95
Fluidized bed	2-50	1-24	80-90
Upflow anaerobic sludge blanket (UASB)	2-30	2-72	80-95
Anaerobic baffled reactor	3-35	9-32	75-95
Two-stage anaerobic digestion	5-30	20-150	70-85

2.5. Substrates for Anaerobic Digestion Process

A variety of biomass types can be used as substrates (feedstock) in the anaerobic digestion process for biogas production. However, the substrates should meet specific characteristics and nutritional requirements needed for microorganisms' growth. The substrate should also include some components like trace elements and vitamins needed for the activity of microbial enzymes systems. The substrate composition is very important as well in the degradation process. The substrate composition significantly affects the whole process regarding the quality of digestate produced, nutrition content, and process contamination potential (metals, organic compounds, disease-causing organisms, etc.) (Aslanzadeh, 2014).

The composition of a substrate can affect the microorganism's activity which in turn can affect process stability and gas production. During the anaerobic digestion process, the ratio of carbon to

nitrogen (C/N ratio) shows great importance for process stability, so the performance of the whole process can be enhanced by a different substrate from a different source with the right amount to control the ratio. Researches show higher results for co-digestion of substrates from different sources regarding gas production than that expected from single digestion of one substrate (Adekunle & Okolie, 2015).

It's also more advantageous to use a substrate that is not too diluted which will contain too much water compared to an organic fraction. If the substrate is too diluted with a high water content that will result in microorganisms wash out in a continuous process because their growth rate will be very low. That's why a highly diluted substrate is treated with different ways to retain the microorganisms like using a carrier material or adding back biomass (Adekunle & Okolie, 2015).

2.6. Biogas Production from Lignocellulosic Biomass

2.6.1. Biomass Supply

Biomass is a renewable source of organic material that comes from plants and animals. It used to be the most important and abundant source of energy until the mid-1800s. Biomass contains stored chemical energy from the sun produced by plants through photosynthesis. Biomass can be burned directly for heat or converted to renewable liquid and gaseous fuels through different processes. (EIA, 2020).

In 2018, the domestic supply of biomass was 55.6 EJ globally accounting for 12 % of the world's energy supply. In 2019, biomass provided nearly 5 quadrillion British thermal units (Btu) and about 5 % of total primary energy use in the United States. In 2018, 637 TWh of electricity was generated from biomass globally. 66 % of all biopower generated was from solid biomass sources (WBA, 2020).

The most common biomass used in European Biogas plant production are listed below:

- Animal manure and slurry.
- Agricultural residues and digestible organic wastes.
- Organic fraction of municipal waste and from catering.
- Sewage sludge.
- Dedicated energy crops (e.g. maize, miscanthus, sorghum).

2.6.2. Lignocellulosic Biomass

Lignocellulosic biomass is an abundantly available source of biomass with an annual global yield of over 200 billion dry metric tons per year. Agri- and forest residues, and dedicated energy crops are the common examples of these renewable resources. The basic structure of lignocellulosic biomass mainly consists of cellulose (35-50 %), hemicellulose (20-35 %), and lignin (10-25 %), along with smaller quantities of other organic and non-organic compounds like proteins, lipids, and other extractives (Sawatdeenarunat, et al., 2015). Table 1 summarizes the typical composition of some commonly used lignocellulosic feedstocks.

Table 2.6. Percent composition of cellulose, hemicelluloses, and lignin in agricultural residues and wastes (Ahmad, et al., 2018).

Lignocellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40–55	24–40	18–25
Softwood stems	45–50	25–35	25–35
Nut shells	25–30	25–30	30–40
Corn cobs	45	35	15
Grasses	25–45	35–50	10–30
Paper	85–99	0	0–15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15–20	85–85	0
Cottonseed hairs	80–95	5–20	0
Newspaper	40–55	25–40	18–30
Wastepaper	60–70	10–20	5–10
Swine waste	60	28	NA
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Coastal Bermuda grass	25	35.7	6.4
Switchgrass	45	31.4	12.0
sunflower stalk	34–42	19–21	12–30
Corn stover	37–39	23–31	14–18
Eucalyptus	34–44	18–19	19–30
Maize stems	36–38	10–30	3.5–10.5
Poplar	40–43	12–26	18–25
Rice straw	27–44	14–34	13–26

2.6.3. Properties of Lignocellulosic Biomass

Large quantities of lignocellulosic biomass are accumulated by agricultural, municipal and forestry, and other activities, that’s why it’s considered as one of the most abundant sources of

biomass. Cellulose, hemicellulose, and lignin are the main three polymers forming lignocellulosic biomass. The carbohydrate part (cellulose and hemicellulose) are fermentable and can be biodegraded after hydrolysis, which makes lignocellulosic biomass a promising substrate for bioenergy production. However, there are some characteristics of lignocellulosic biomass which resist biodegradation by enzymes and microbes such as structural and chemical properties (Zheng, et al., 2014).

2.6.4. Structure of Lignocellulosic Biomass

The amounts of the three main components of lignocellulosic biomass (cellulose, hemicellulose, and lignin) vary between different species. They can also vary due to maturation and growth conditions in the same species. Cellulose is the main component of almost all plant cell walls, so it's considered to be one of the most abundant renewable polymers on Earth. At the molecular level, cellulose $(C_6H_{10}O_5)$ n is a linear (unbranched) homopolysaccharide polymer consisting of 10,000 to 15,000 D-glucose units linked by β(1→4) glycosidic linkages (Sawatdeenarunat, et al., 2015).

Cellulose chains are linked together by hydrogen bonds and van der Waals forces, resulting in microfibrils with high tensile strength. Cellulose molecules vary greatly throughout the structure, this creates a different level of crystallinity. Because of that, cellulose consists mainly of 2 different regions: amorphous (low crystallinity) and crystalline (high crystallinity) regions. Crystallinity index can be used to characterize the crystallinity level of cellulose. So, the higher the crystallinity index, the more difficult for cellulose to become biodegraded. Furthermore, cellulose microfibrils are linked together by hemicellulose and/or pectin and covered by lignin as shown in Figure 2.2. Due to such a complicated structure, the biological degradation of cellulose became a difficult process (Zheng, et al., 2014).

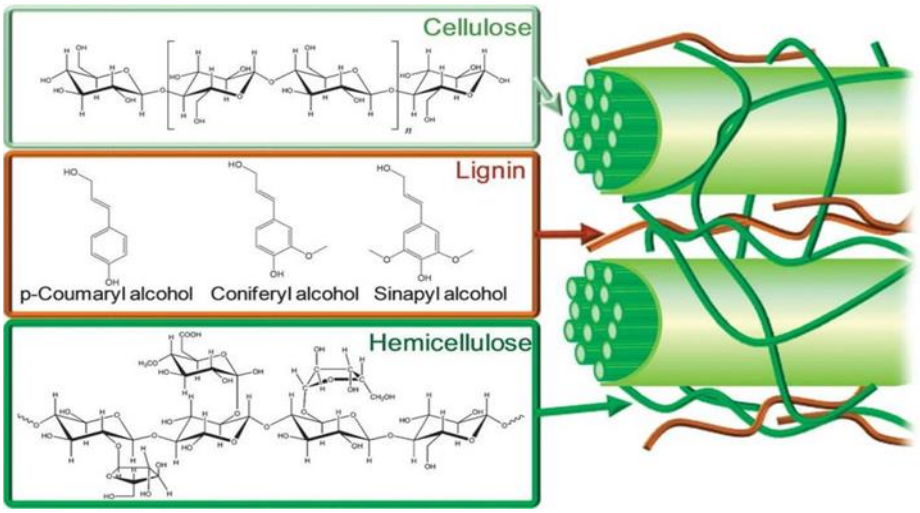

Figure 2.2. Structure of lignocellulosic biomass with cellulose, hemicellulose and lignin represented (Alonso, et al., 2012).

Opposite to cellulose, hemicellulose has a lower crystallinity level so it's more likely to be amorphous. Moreover, hemicellulose is branched heterogenic polysaccharides consisting of various pentoses (xylose and arabinose), acids (glucuronic acid, methyl glucuronic acid, and galacturonic acid), and hexoses (glucose, galactose, mannose, and/or rhamnose). Being amorphous and branched, hemicellulose is highly affected by the biological, thermal, and chemical hydrolysis of their monomer compounds. However, the short-branched chains of hemicellulose polymers interact with cellulose microfibrils and lignin forming a matrix that is extremely rigid and resistant to biodegradation (Zheng, et al., 2014).

The third component of lignocellulosic biomass is lignin. Lignin is the second most abundant organic compound in nature after cellulose. It is a large and complex aromatic and hydrophobic amorphous heteropolymer. Lignin consists of phenylpropane units such as coniferyl alcohol and sinapyl alcohol with hydroxyl, methoxyl, and carbonyl functional groups. It acts as a cement in linking cellulose and hemicellulose to form a very rigid matrix of the cell wall structure. Lignin is water-insoluble and optically inert. These lignin properties make the most resistant component of the plant cell wall to degradation. So, the higher the lignin content, the greater the resistance of the biomass to chemical and biological degradation. Therefore, lignin is the main physical barrier to the utilization of lignocellulosic biomass in biodegradation processes (Grabber, 2005).

2.6.5. Resistance of Lignocellulose to Anaerobic Digestion

As mentioned before, lignocellulosic biomass is recently viewed as a promising source of renewable energy due to its abundance and availability as a fuel source. However, the physical and chemical characteristics of lignocellulosic biomass mass make it resistant to bioconversion. The major problem that makes the conversion process hard is the complexity and variability of biomass chemical structure. Biomass chemical structure has a great impact on the biodegradability process including cellulose crystallinity, presence of lignin and hemicellulose, accessible surface area, degree of cellulose polymerization, and degree of hemicellulose acetylation (Kim & Holtzapple, 2006).

As a result, hydrolysis of the lignocellulosic biomass is the key rate-limiting step in the whole anaerobic digestion process. The rigid structure of lignocellulose resists any enzymes and microbes attack which in turn reduces their accessibility to cellulose and hemicellulose compounds.

To overcome this obstacle and improve biogas production a pre-treatment process is necessary to free the carbohydrate from lignin compounds and facilitate the accessibility of enzymes and microbes to cellulose and hemicellulose. Because of that, pre-treatment is a crucial step in breaking the physical and chemical barriers and increasing the biodegradability of carbohydrates and lignocellulose (Paudel, et al., 2017).

2.6.6. Pre-Treatment Methods to Improve the Digestibility of Lignocellulosic Biomass

The main goal of the pre-treatment process is to break the naturally recalcitrant carbohydrate-lignin shields that decrease the accessibility of enzymes and microbes to cellulose and hemicellulose. Pre-treatment also helps to reduce the structural crystallinity and increase the porosity of the lignocellulosic biomass, which in turn improves biodegradability and biogas production (Ahmad, et al., 2018).

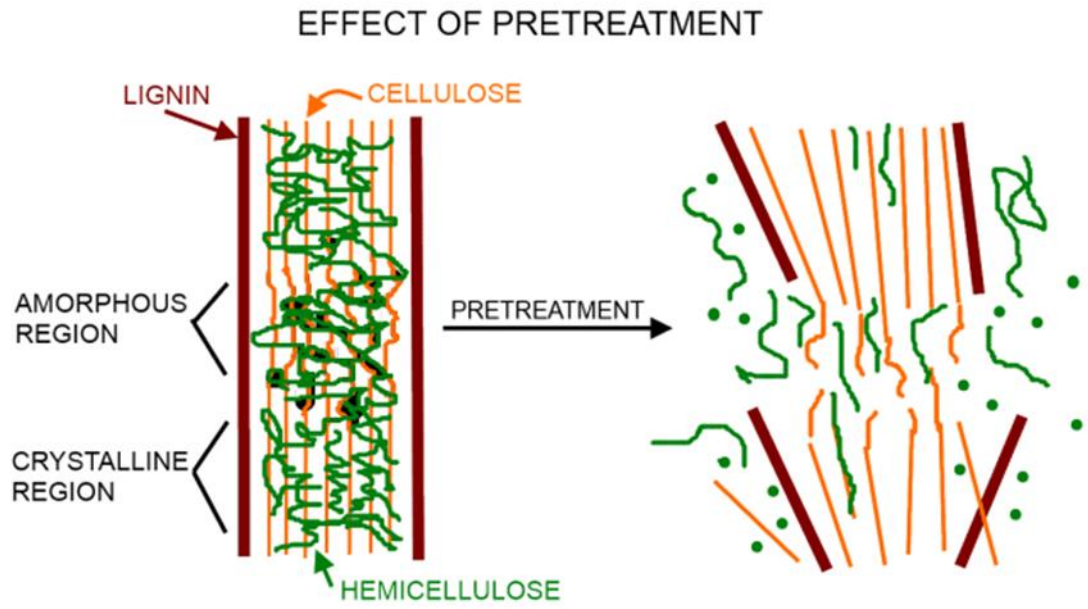

Figure 2.3. Schematic of pre-treatment of lignocellulosic biomass (Muley & Boldor, 2017).

The advantages of the pre-treatment process have been recognized for a long time, but several factors must be considered during the choice of the pre-treatment technologies, e.g. the nature of lignocellulosic biomass and the type of biological conversion processes. Table 2.7 summarizes the effects of different pre-treatment methods on the chemical composition and physical characteristics of lignocellulosic biomass. Pre-treatment techniques are reviewed under three categories: physical, chemical, and biological (Zheng2014).

Table 2.7. The effect of pre-treatment on the compositional and structural alteration of lignocellulosic biomass (Zheng, et al., 2014).

Pretreatment	Increase of accessible surface area	Decrystallization of cellulose	Solubilization of hemicellulose	Solubilization of lignin	Alteration of lignin structure	Formation of furfural/ hydroxymethylfurfural (HMF)
Mechanical	●	●				
Irradiation	●	○	○			○
Steam-explosion	●		●	○	●	●
Liquid hot water	●	ND	●	○	○	○
Catalyzed steam-explosion	●		●	●/○	●/○	●
Acid	●		●	○	●	●
Alkaline	●		○	●/○	●	○
Oxidative	●	ND		●/○	●	○
Ionic liquids	●	●	○			
Thermal acid	●	ND	●			●
Thermal alkaline	●	ND	○	●/○	●	○
Thermal oxidative	●	ND	○	●/○	●	○
Ammonia fiber explosion	●	●	○	●	●	○
Biological pretreatment	●	ND	●	●	●	

[a] ● = major effect, ○ = minor effect, ND = not determined, and blank = no effect.

2.6.6.1. Physical Pre-treatment

As mentioned before, the pre-treatment step can highly improve the bio-digestibility of the lignocellulosic biomass. Physical pre-treatment refers to processes that don't include any chemicals or microorganisms in the pre-treatment process. By increasing the accessible surface area for enzymes and microbes, and reducing crystallinity and polymerization of cellulose and hemicellulose, the bio-degradation process will be easier and faster, which in turn will enhance the biogas production. This can be done by several mechanical processes such as chopping milling (e.g. hammer milling, ball milling, two-roll milling, and colloid milling) and grinding (Zheng, et al., 2014).

2.6.6.2. Mechanical Pre-treatment

Large sizes of lignocellulosic biomass hinder the anaerobic process and are not effective for biogas production. Microorganisms' activity is greatly affected as it becomes harder for them to handle large sizes of lignocellulosic biomass, and this makes it difficult for them to carry out the bioconversion process. Smaller sizes provide a more accessible surface area for microorganisms and reduce the crystallinity and the resistance to biodegradation. Mechanical pre-treatment involves techniques for size reduction such as chipping with reduces the particle size of biomass to range from 10 to 30 mm. Further size reduction can be done by grinding and milling which can reduce the size particles to ranges from 0.2 to 2 mm. The relation between particle size and biogas production is shown in Figure 2.4 (Ahmad, et al., 2018).

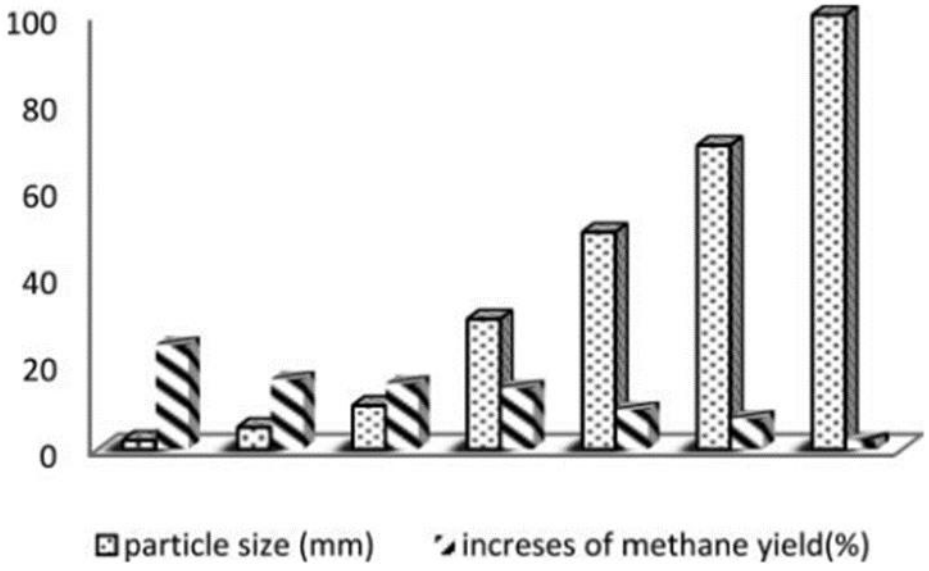

Figure 2.4. Relation between particle size and biogas production (Ahmad, et al., 2018).

2.6.6.3. Thermal Pre-treatment

Thermal pre-treatment involves the treatment of lignocellulosic biomass in a wide range of temperatures (50 ~ 250 ℃), which enhances the bioconversion and the digestibility of biomass. Along with enhancing the digestibility, the thermal pre-treatment also eliminates the pathogens from waste material. That is why this method has been applied so far on a massive scale for the pre-treatment of sewage sludge, agricultural biomass, and organic fraction of municipal solid wastes. There are three common types of thermal pre-treatment applied to enhance the biodegradability of lignocellulosic biomass; hydrothermal, steam explosion, and microwave heating (Cesaro & Belgiorno, 2014).

The thermal pre-treatment process has many advantages such as enhancing the digestibility of biomass and removal of pathogens. However, high-temperature pre-treatment can unexpectedly arouse reactions that form complex recalcitrant or inhibitory substrates. This will negatively affect the biogas production and the efficiency of the whole process. So, a deep analysis study is required for substrates, the temperature needed, and the time required to guarantee good results of pre-treatment (Paudel, et al., 2017).

2.6.6.4. Hydrothermal Pre-treatment

In the hydrothermal pre-treatment process, the lignocellulosic biomass is subjected to high pressure to maintain the water in the liquid phase in high temperatures. Biomass is subjected to high-temperature cooking in water under high pressure. During the process, water can go through the cell structure of biomass hydrating cellulose, solubilizing hemicellulose, and slightly removing lignin. There are three methods of liquid hot water (hydrothermal pre-treatment) are used: (1) co-current, (2) counter-current, and (3) flow-through (Mosier, et al., 2005).

Hydrothermal pre-treatment has many advantages including increasing the accessible and susceptible surface area of cellulose, decreasing crystallinity of cellulose, and improving the digestibility of biomass. As a result, it is considered one of the most effective ways to treat lignocellulosic biomass and enhance methane yield (Zheng, et al., 2014).

2.6.6.5. Steam-explosion

Steam explosion pre-treatment technique has been used for the treatment of lignocellulosic biomass for enhancement of methane production, including wheat straw, corn stalks, hardwoods, and food processing wastes. It was proved to be effective for enhancing methane yield over many substrates (Zheng, et al., 2014).

In this process, the biomass is subjected to high-pressure saturated steam for a short time then the pressure is quickly reduced to stop the reactions. Therefore, the biomass particles undergo explosive decompression. The temperature and pressure fall with the ranges of 160-260^{o} C, 0.69-4.83MPa, respectively. The time of the process is ranged from several seconds to a few minutes.

The process is considered to be an autohydrolysis process for lignocellulosic linkage where hemicellulose is hydrolyzed into its component sugars and lignin is transformed to a certain degree, therefore the pre-treated biomass becomes more biodegradable. Hydrothermal pre-treatment is regarded as one of the most effective pre-treatment processes for lignocellulosic biomass. It has many advantages including small energy demand, no recycling costs for waste stream, and low pollution levels.

2.6.6.6. Chemical Pre-treatment

Chemical pre-treatment is the usage of a variety of chemicals such as acids, bases, or oxidants to break down the linkage between compounds in the lignocellulosic biomass. So, the main function of chemical pre-treatment is to make the biomass more biodegradable by destructing the internal structure of biomass. This happens by penetrating the lignin-carbohydrate link and cellulose crystalline matrix, or the hydrolysis of hemicellulose. Table 2.5. shows mechanisms and performance of chemical pre-treatment methods used for AD of agricultural biomass.

Alkali Pre-treatment

In alkali pre-treatment, the biomass is applied to a dilute base such as sodium hydroxide (NaOH), potassium hydroxide (KOH), calcium hydroxide (Ca $(OH)_2$), and ammonia (NH_3) for some time before anaerobic digestion to remove lignin, hemicellulose, and/or cellulose. Therefore, the lignocellulosic biomass becomes more degradable to microbes and enzymes. The base helps to

decrease the degree of polymerization and crystallinity of biomass by the saponification and penetration process of lignin-carbohydrate linkages.

This leads to the disruption of the lignin structure, increasing the accessible surface area and porosity of biomass (see Figure 2.5). This causes the biomass to be more digestible to microbes and enzymes which in turn improves methane production. Being one of the strongest bases, NaOH has gained massive interest in studies related to its effect on enhancing the degradability of lignocellulosic biomass and improving methane production. NaOH has the ability to weaken the linkage between lignin and hemicelluloses and break through bonds of the lignin–carbohydrate complexes.

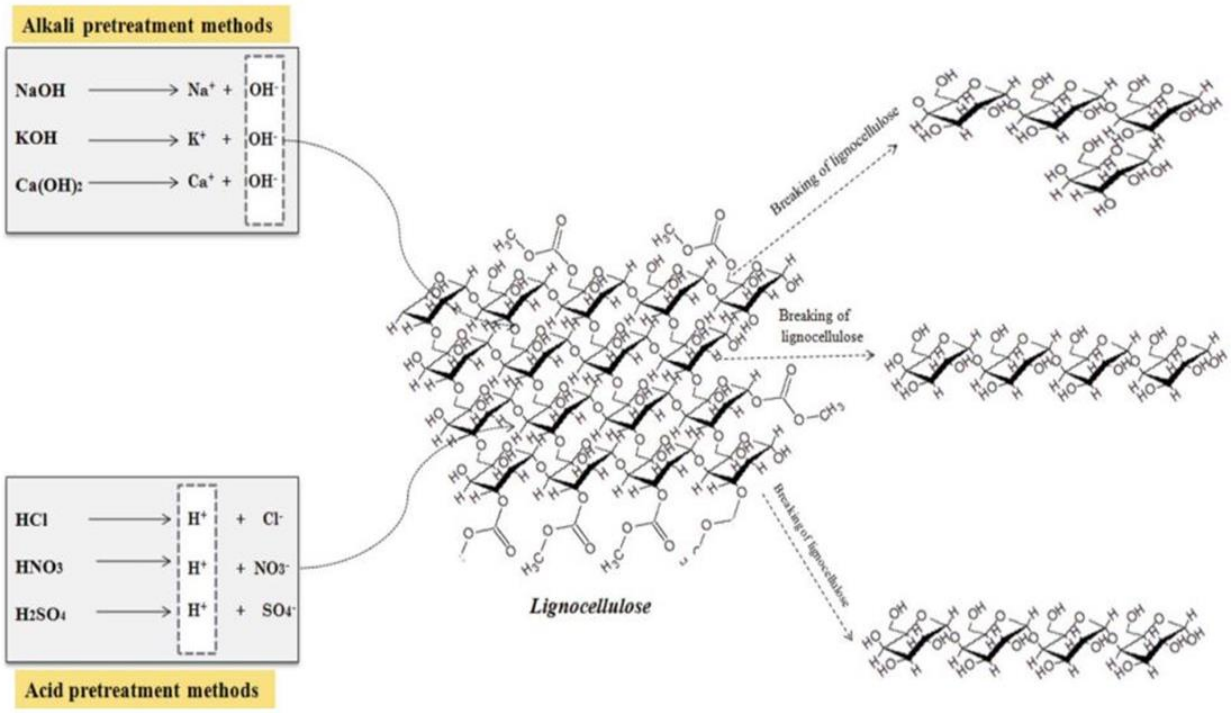

Figure 2.5. Mechanistic route for acid and alkali pre-treatment methods (Ahmad, et al., 2018).

Table 2.8. Mechanisms and performance of various pre-treatment methods used for AD of agricultural biomass (Paudel, et al., 2017).

Methods	Mechanism	Feedstock	Pre-treatment Condition	Effect of pre-treatment	CH_4 yield (mL/g VS) and enhancement
Chemical pre-treatment					
Acid	Enlarged surface area; Solubilization of HC*; Alteration of Lg* structure	Sunflower stalks Dairy cow manure	4% HCl; 170 °C 2% HCl; 37 °C; 72 h	12% HC removal -	233 mL/g VS; 21% 400 mL/g VS; 20.6%
Alkali	Alteration of Lg* structure Enlarged surface area; Solubilization of Lg; Alteration of Lg structure	Corn stover Corn stover Sorghum forage Grass silage Ensiled Napier grass	2% NaOH; 20 °C; 72 h 6% NaOH; 35 °C, 10% NaOH; 40 °C; 1 h 7.5% NaOH; 100 °C; 48 h 1% NaOH; 24h	34.6% digestion time shortened 56.3% VS reduction 31% Ce & 44% Lg removal 21.2% Ce removal -	220 mL/g VS; 73.4% 466 mL/g VS; 48.5% 346 mL/g VS; 29% 452.5 mL/g VS; 28% 110.4 mL/g VS
H_2O_2	Enlarged surface area; Solubilization of Lg; Alteration of Lg structure	Sunflower stalks	4% H_2O_2; 55 °C; 24 h	35% Lg removal	225 mL/g VS; 33%
Thermal pre-treatment					
Hydro-thermal	Enlarged surface area; Solubilization of HC	Wheat straw Rice straw Fruits & vegeTables Ensiled Napier grass	200 °C; 1.55 MPa; 10 min 200 °C; 5% NaOH; 10 min 170 °C; 1 h 100 °C; 1 h	- - - -	94.1 mL/g VS; 20% 132.7 mL/g VS 326 mL/g VS; 16.1% 99 mL/g VS
Steam explosion	Enlarged surface area; Solubilization of HC; Alteration of Lg structure	Bamboo Rica straw Fruits & vegeTables Harvested hay	243 °C; 3.5 MPa; 5 min 120 °C; 2 min 120 °C; 15 min 175 °C; 10 min	- 67% biodegradation rate increase	215 mL/g TS; 80% 328.7 mL/g TS; 51% 930 mL/g VS; 43% 281 mL/ g VS, 16%
Mechanical pre-treatment					
Grinding/milling/chipping	Enlarged surface area;	Horse manure	40 °C	-	272 mL/g VS; 26.5%
	Decrystallization of Ce*	Rice Straw Wheat Straw Harvested meadow grass	Size 0.3–0.75 mm; 37 °C Mesh grating plate & chopping: size < 1.5 cm	24.4% biodegradation enhanced 31.5% biodegradation enhanced	65.7 mL/g VS; 13% 93.1 mL/kg VS; 38.7% 359 mL/g VS; 22%

Table 2.8. Continued.

Biological pre-treatment					
Fungal pre-treatment	Enlarged surface area; Solubilization of Hc & Lg; Alteration of Lg structure	Yard trimmings Rice straw	28–37 °C; 12 d – 8 weeks incubation 20 g solid; 3 weeks incubation	20.9% Lg removal 47.51% Lg removal	44.6 mL/g VS; 15% 479.4 mL/g VS; 46.2%
Microbial consortium		Napier grass	3 g solid; 3 weeks incubation; 30 °C	35% Lg, 22% Ce, 40% HC removal	279 mL/g VS; 49.2%

**Cellulose (Ce); Hemicellulose (Hc); Lignin (Lg*

Acid pre-treatment

In the acid pre-treatment process, lignocellulosic biomass is subject to either concentrated acid (e.g. 30-70 %) and low temperature (e.g. 40^{o} C) or dilute acid (e.g. 0.1 %) and high temperature (e.g. 230^{o} C). Organic and inorganic acids can be used for dilute acid pre-treatment such as sulfuric acid(H_2SO_4), hydrochloric acid (HCl), nitric acid (HNO_3), phosphoric acid (H_3PO_4), and acetic acid. Although concentrated acids are very efficient on cellulose and hemicellulose hydrolysis (see Figure 2.5), it's not preferred in the pre-treatment process, as it has many negative effects on the whole process such as it is extremely toxic, it needs special material to handle and it is highly corrosive.

As a result, dilute acid pre-treatment is preferred over concentrated one for lignocellulosic biomass pre-treatment. Dilute acids can hydrolyze up to 100 % of hemicellulose into its sugar components (e.g.xylose, arabinose, and galactose). It can disrupt lignin to a high level. As a result, the biodegradability of cellulose and hemicellulose by enzymes and microbes increases (Zheng, et al., 2014).

2.6.6.7. Biological Pre-treatment

Unlike most physical, chemical, and thermal pre-treatment methods, biological pre-treatment doesn't require high energy or chemicals input. Therefore, it doesn't create any bad effects on the process such as severe temperature, pH, and inhibitory byproducts, which can negatively affect methane production. As it doesn't require high energy or chemical expenses, biological pre-treatment is regarded to be more economical compared to other methods. Moreover, it doesn't create any inhibitory byproducts which are considered to be safer than other pre-treatment techniques (Paudel, et al., 2017).

Industrial enzymes, lignolytic enzymes, and cellulase can be effective in this process to pretreat the biomass and degrade the lignocellulosic components. Due to the high unit prices of these enzymes and the requirement of large doses of them, external addition of these enzymes is not feasible in this process therefore indirect addition of these enzymes by a biological route is more applicable. For example, the usage of rot-fungi is able to provide such extra-cellular enzymes.

Compared to other pre-treatment methods, biological pre-treatment requires a relatively long time which has limited the application of this method on a commercial scale. Moreover, there is a competition for carbohydrates between pre-treatment and biogas production, because microbes and

enzymes during biological pre-treatment require certain levels of carbohydrates to degrade the lignocellulosic compounds. In contrast to this, biogas production is improved by increasing the accessibility of cellulose (See Figure 2.6) (Zheng, et al., 2014).

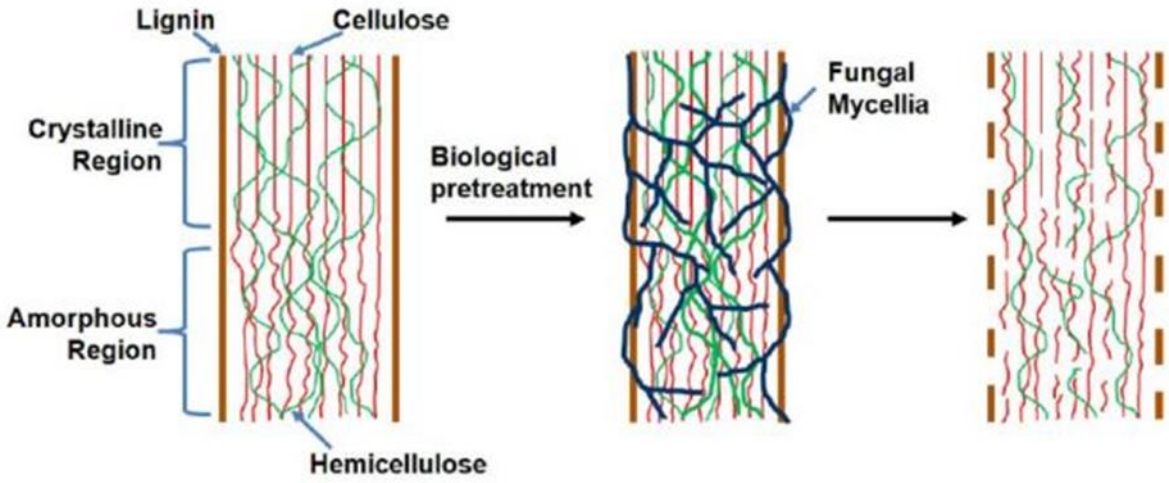

Figure 2.6. Effect of biological pre-treatment on lignocellulosic structure.

2.7. Alkali Pre-treatment of Cereal Straws Using Sodium Hydroxide

Since it's available in many places with high quantities and at low cost, lignocellulosic biomass is regarded to be one of the most attractive alternatives for bioenergy production among other renewable sources. Agriculture residues like wheat and rice straw present a very important source of lignocellulosic biomass and therefore, they have a great potential for unutilized energy. Three types of bioenergy can be produced from lignocellulosic biomass by thermochemical or biochemical processing: liquid fuels such as bioethanol, gaseous fuels such as biogas, and electricity by combustion.

However, methane production from biomass is always more efficient than bioethanol production. Biomass consists mainly of (carbohydrates, fats, and proteins). In the anaerobic digestion process, and with the help of different types of anaerobic micro-organisms, most biomass contents are converted into biogas (CH_4+CO_2). On the other hand, when it comes to the alcoholic fermentation process, only the carbohydrate part is converted into simple sugar, which finally turns into ethanol. Therefore, the methane recovery yield is richer than the ethanol recovery yield (Chandra, et al., 2012).

Biogas is a clean and renewable source of energy that can be produced through the biochemical processing of lignocellulosic biomass. It is composed mainly of methane and carbon dioxide with different percentages. It can be produced in small-scale implements like a small farm and a massive scale like a biogas facility. Biogas can be produced by the anaerobic digestion process of biomass. Since its high energy recovery and environmental benefits, anaerobic digestion is considered one of the most efficient technologies for energy recovery (Bolado-Rodríguez, et al., 2016).

Table 2.9. Methane yields (in terms of TS) are associated with various agricultural biomasses (Mussoline, et al., 2013).

Agricultural biomass	Methane yield (L/kg TS)
Triticale chopped/squashed	310/320
Rape chopped/squashed	300/350
Oat chopped/squashed	240/280
Jerusalem artichoke squashed	240
Sunflower squashed	200
Wheat squashed	290
Rye squashed	290
Maize chopped/squashed/ripped	300/330/300
Maize drying up residues	378
Tomato skin and seeds	227
Barely straw	239
Grape stalks	290
Grape marc	171
Rice straw	202
Rice straw	240

However, the digestibility of lignocellulosic biomass is limited by its cell wall structure. Therefore, an efficient pre-treatment step is a great solution to accelerate hydrolysis and improve methane production. Depending on the characteristics and nature of lignocellulosic biomass, different pre-treatment techniques have been studied such as biological, chemical, physical processes, or a combination of them (Sambusiti, et al., 2013).

Currently, alkaline pre-treatment is regarded as one of the most efficient pre-treatment methods. It has shown many positive effects and advantages over other pre-treatment techniques, including the high ability of lignin solubilization and neutralization of various acidic products degraded from the lignocellulosic complex. Moreover, the presence of small excess of residual alkali in the treated substrates may help prevent the drop of pH during the acidogenesis process. In addition, the alkaline pre-treatment decreases the possibility of inhibition in methane fermentation and provides a lower cost of production (Bolado-Rodríguez, et al., 2016).

Alkaline pre-treatment is commonly used for treating lignocellulosic biomass with high lignin content, such as wheat and rice straws. Alkaline pre-treatment becomes very effective if it's performed using strong bases such as sodium, potassium, calcium, and ammonium hydroxides. Strong bases are able to modify the structure and solubilize the lignin which in turn improves the biogas production of the anaerobic digestion process. Among all kinds of alkali tested for pre-treatment of rice straw, NaOH has shown to be the most effective in lignin removal and biogas production (Zhu, et al., 2010).

2.7.1. Rice Straw

Rice is one of the most grown agricultural crops in the world. It is ranked at third place among all the major agricultural crops with a total cultivated area of 161.42 million hectares with a gross grain yield production of 503.17 million metric tons according to the United States Department of Agriculture (USDA) (Anon, 2021). The estimated dry lignocellulosic biomass resulting from rice cultivation is around 905 million tons per annum. This massive amount of lignocellulosic biomass can play an important role in meeting the increasing energy demand in the world by converting them to a renewable source of energy (Sari & Budiyono, 2014).

Rice straw presents the main crop residue from rice cultivation. For every ton of rice harvested, approximately 1.35 tons of rice straw remain in the field with high energy potential. It is fibrous biomass consisting mainly of cellulose, hemicellulose, and lignin. Straws appear like flat fibers with approximate dimensions of 0.5 cm in width and 20–60 cm in length (See Figure 2.7).

Figure 2.7. Rice straw left after grain processing and harvesting.

Traditionally, people reused rice straw in different ways such as animal feeding, fuels for cooking by direct burning, house heating, and paper industry. However, a massive amount of rice straw is left in the field unused and burned in open areas to get rid of them, causing serious environmental and health problems, such as air pollution, fire disaster, and respiratory diseases. On the other side, rice straw is a source of biomass (organic material) that can be used to produce a renewable source of energy. Through biological or thermochemical processes, Rice straw can be converted to biogas and bioethanol respectively. However, straw contains lignocellulosic compounds which limit the biodegradation process, making it hard and challenging for biogas production (Croce, et al., 2016).

Alkali pre-treatment with NaOH solution is an effective step to solubilize the lignin content of straw and increase the accessibility of cellulose and hemicellulose by enzymes and microbes to enhance digestibility. Many studies have worked on this technique and got positive effects during NaOH pre-treatment related to the changes in biodegradability and biogas production.

Compared to untreated rice straw, around a 3.2 %-58.1 % increase in biogas yields were obtained with 4 %-10 % NaOH-treated rice straws. These results proved the effectiveness of NaOH pre-treatment in improving biodegradability and enhancing the biogas production of rice straw. Changes in composition have also been recorded. Hemicellulose, cellulose, and lignin were decomposed by 35.2 %-54.2 %, 14.2 %-16.4 %, and 8.0 %-44.5 %, respectively, for 4 %, 6 %, 8 %, and 10 % NaOH-treated rice straws (He, et al., 2009).

According to Chandra et al. (2012), an increase of 87.5 % in methane yield was obtained by using alkali pre-treatment with 40 % NaOH. In another study, results showed that an increase of 27.3-64.5 % in methane yield was obtained by 6 % NaOH-treated rice straw. This increase was achieved by an improvement of straw biodegradability by changes of straw composition as degradation of 16.4 % cellulose, 36.8 % hemicellulose, and 28.4 % lignin was observed (He, et al., 2008)

Table 2.10. Rice straw composition (Mussoline, et al., 2013).

Feedstock component	Dry wt. %
Glucan	38.9
Mannan	0.0
Galactan	0.5
Xylan	20.4
Arabinan	3.4
Lignin	13.5
Extratives	5.3
Ash	18.0
Total	100

2.7.2. Wheat Straw

Wheat is the most grown agricultural crop worldwide. In the marketing year of 2019/2020 and according to the United States Department of Agriculture (USDA), the global production volume of wheat reached 765 million metric tons. Wheat straw is an agricultural residue resulting from wheat cultivation. In 2009, the global annual production of wheat straw was estimated to be 681.92 million tons from 225.437million hectares of total cultivated area. This massive amount of straw would cause many environmental problems if it wasn't reused properly. A major part of wheat straw is utilized for animal feeding, heating, and cooking by direct burning. The rest remains unutilized, so it's dumped in an open area or burned in the field which is an unsustainable practice causing many bad effects on the environment (Chandra, et al., 2012).

Kaparaju et al. (2009) showed that the use of wheat straw for biogas production via anaerobic digestion is energetically more efficient than utilizing wheat straw for bioethanol production. It is also reported that the production of biogas can be a more economical process for the utilization of biomass in wheat straw. Because of the lignocellulosic composition of wheat straw, biodigestibility becomes a challenging process. The pre-treatment step of wheat straw offers an enhancement of the biodegradation process and improvement for biogas production. Alkali pre-treatment of wheat straw gives a great result in reducing polarization and crystallinity of straw structure and increasing the accessible surface area for enzymes and microbes (Sambusiti, et al., 2012).

Sambusiti et al. (2012) reported an increase of 17 to 47 % in biogas production at 1 and 10 % NaOH pre-treated dosages of wheat straw, respectively. The concentration of soluble chemical oxygen demand (CODs) in the liquid phase after the pre-treatment was also improved up to 24 % for the pre-treated wheat straw. The total sugars content was also increased up to five times at 10 %

gNaOH/gTS, showing that pre-treatment has improved the hydrolysis of cellulose and hemicelluloses.

In another study, an improvement of 87.5 % higher biogas production and 111.6 % higher methane production was reported by 4 % NaOH dry basis of wheat straw for 120 h. the untreated wheat straw substrate had resulted in 78.4 L/kg VS methane and 188.4 L/kg VS biogas, while 165.9 L/kg VS methane and 353.2L/kgVSa biogas for 4 % NaOH pre-treated wheat straw (Chandra, et al., 2012).

2.7.3.Rye Straw

Rye is one of the most grown cereal crops in the world. In 2018, the global production of rye reached 10.5 million metric tons with an annual increase of 2.2 %. Rye is regarded as the most important crop in many European countries especially Northern Europe. Only Germany accounted for 24 % of the global production of rye in 2016 (USDA, 2021).

Rye straw is an abundant agricultural waste used traditionally for animal feeding, cooking, and heating. As a rich biomass source, rye straw has gained great interest as raw material for biorefineries. However, rye straw contains lignocellulosic compounds which limit the hydrolysis step and as a result, the biodegradation process becomes more challenging. Mostly, rye straw has been used for bioethanol production by following some pre-treatment steps to accelerate and enhance digestibility. Squashed rye was used for biomethane production through the anaerobic digestion process. BMP test resulted in the production of 290 (L/kg TS) methane yield according to Mussoline et al. (2013).

Table 2.11. Rye straw composition (Petersson, et al., 2007).

Feedstock Component	Dry wt. %
Glucan	40.8
Xylan	22.3
Galactan	1.2
Mannan	Nd
Arabinan	2.6
Klason	16.1
Ash	5.1
Extractives	7.1
Residual	4.9

2.8. Molecular Methods Applied for Microbial Analyses in Anaerobic Digesters

There are diverse amounts of enzymes and microbes responsible for the biodegradation of lignocellulosic biomass and the production of biogas. These microorganisms work in a balanced way and have a balanced relationship with each other. Furthermore, if a sufficient balance is achieved between all microbial populations, this will guarantee a better performance of the whole anaerobic digestion process. There are some methods to determine the microbial community of anaerobic digesters: real-time polymerase chain reaction (Q-PCR), denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH), metagenomics, and genomic sequencing (Bozan, 2018).

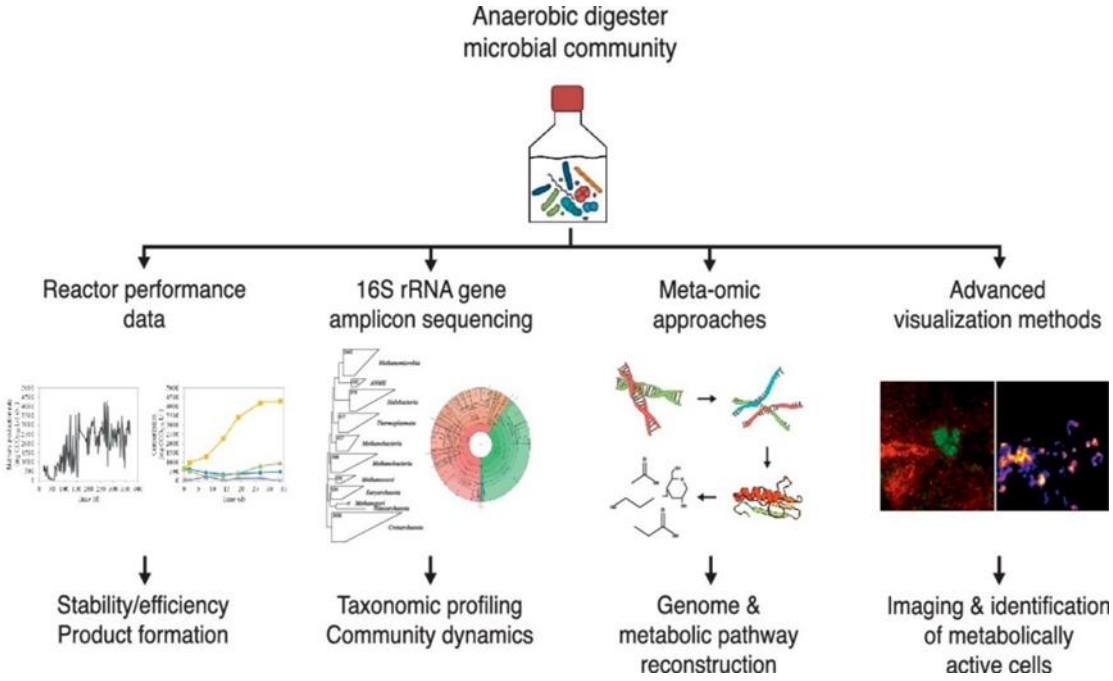

Figure 2.8. Applications of some special methods to determine the microbial ecology of digesters (Vanwonterghem, et al., 2014).

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

Quantitative polymerase chain reaction (Q-PCR) is a technique that can be used to determine the amount of PCR product. It is also a useful method for enhancing functional genomics, and highly effective for analyzing gene expression. There are different kinds of Q-PCR. The basic Q-PCR is useful for detecting the gene without quantifying its expression. To monitor the amount of PCR product over time, the data generated during the reaction is used, and by the effect of parameters such as melting temperature, the PCR product can be determined. Reverse transcriptase PCR is commonly

used to determine the expression. To do that, the RNA is extracted and converted into cDNA before the Q-PCR reaction (Maddocks & Jenkins, 2016).

2.8.2. Fluorescence in situ Hybridization (FISH)

Through Fluorescence in situ hybridization (FISH) method we can determine specific groups of microorganisms and it is also can be used to provide information about both the culturable and the unculturable microorganisms. They are many ways to define the microorganisms in the sample such as domain, family, genera, and species. Moreover, Fluorescence in situ hybridization can be used to get some ideas of the function and structure of the complex microbial community. However, it is hard to observe under fluorescence microscopy, because of the special characteristics of its cell wall (Dinova, et al., 2018).

2.8.3. Metagenomics

Metagenomics technique is commonly known to be used for complex microbial communities' analysis. For high throughput data, Amplicon based method and whole metagenomic shotgun sequencing are the two most used methods to reach this. Shotgun metagenomic analysis can be used to identify most of the organisms in the environmental sample and it can be divided into two types: sequence-based screens and functional screens. The amplicon-based method includes 16S ribosomal RNA for bacteria, internal transcribed spacer, and 18S region for fungi and eukaryotes, respectively (Ghosh, et al., 2019).

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. Next-generation sequencing (NGS) technologies have changed genomic research, parallel sequencing was massively increased in the second generation. Third-generation sequencing technologies enable longer read sequencing than second-generation which allows direct sequencing of single DNA molecules (Heather & Chain, 2016).

The promise of nanopore sequencing is the most recent expected technology in the area of genomic sequencing. This new technology can be the spark of using nanopores for the detection and quantification of all manner of biological and chemical molecules. The first company to offer nanopore sequencers is Oxford Nanopore Technologies (ONT). It has generated a great deal of

excitement over their nanopore platforms GridION and MinION. 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, the MinION device is very usable (Heather & Chain, 2016).

In 2014, Oxford Nanopore Technologies (ONT) released the MinION which is the first commercial sequencer using nanopore technology. To understand how the MinION works, we need to look at the changes in the electrical conductivity generated as DNA strands pass through a biological pore. These changes are measured by the MinION and then it identifies the DNA bases. It's portable, affordable, and fast in data production which makes it suitable for real-time applications. By the release of the long-read sequencer MinION, much excitement and interest were generated in the genomics community especially in pathogen surveillance and clinical diagnostic applications (Lu, et al., 2016).

3. AIM OF THE STUDY

The main objective of this MSc study is to improve the biodegradability of lignocellulosic biomass by implementing an alkali pre-treatment technique using NaOH on three different cereal crops' residues: wheat straw, rye straw, and rice straw, which in turn will enhance the biogas production through the anaerobic digestion process. In this regard, NaOH alkali pre-treatment was implemented with different dosages. (0 %, 4 %, 6 % 8 % and 10 % (w/w)) were tested for each substrate to set on the best pre-treatment concentration causing the fastest biomass degradation and the highest biogas production. Furthermore, an anaerobic co-digestion step was implemented between the three different substrates double and tribble sets, using the best NaOH pre-treatment concentration to Figure out how results change by combining more substrates in the digester. In each step, microbial diversity was analyzed by 16S rRNA gene amplicon sequencing.

The specific objectives of the study can be summarized as follows;

- Determination of the effect of different dosages of NaOH alkali pre-treatment on the chemical composition and the anaerobic biodegradability of cereal crops and their harvesting residuals.
- Enhancement of hydrolysis and acidification rates by the application of NaOH alkali pre-treatment during AD of lignocellulosic biomass.
- Increasing the understanding of using NaOH pre-treatment for improved biogas production in AD processes
- Determination of most promising NaOH concentration and crop materials with respect to different dosages of NaOH alkali pre-treatment methods
- Assessment of the biochemical methane potentials (BMP) and biogas microbiome during the AD of cereal crops' residues.
- Explanation of the relation between alkali pre-treatment approaches and selected lignocellulolytic enzymes expression levels together with microbial community dynamics in AD tests.

4. MATERIALS AND METHODS

4.1. Substrates and Inoculum Characterization

4.1.1. Substrates

This study depends on 3 main cereal crops' residues considered among the most agriculture residues produced in Turkey and worldwide. Wheat and rye straw was collected from Konya, Turkey. The harvest time was August 2020. The third substrate used in the study was rice straw, which was collected from Catalca, Istanbul, Turkey. The harvest time was between September-October of 2020.

After samples were collected and sent to the Institute of Environmental Science, Bogazici University, Biomass, and Microbial Ecology Lab, they were oven-dried at 105 ^{o}C for one day till a moisture content less than 10 %. Thereafter, samples were grounded by a kitchen blender into 5-10-mm particles as shown in Figure 4.1. Eventually, samples were kept in air-tight bags at 4 ^{o}C until the following procedures.

Figure 4.1. The substrates after being ground to 5-10-mm particles; rice straw and wheat straw.

4.1.2. Anaerobic Seed Sludge

The activated sludge used as inoculum for BMP tests was taken from the effluent of a mesophilic anaerobic digester processing mixed sludge from Hurma Municipal Wastewater Treatment Plant in Antalya, Turkey. The inoculum was kept in a container under anaerobic conditions at 4 ^{o}C before use.

4.1.3. Analytical Determinations

Total solids (TS), volatile solids (VS), soluble Chemical Oxygen Demand (sCOD), alkalinity, and Total Kjeldahl Nitrogen (TKN) were measured according to Standard Methods (Federation & Association, 2005). The carbon to nitrogen (C: N) ratio was determined using an automated elemental analyzer (ECS 4010 CHNS-O Analyzer, COSTECH Analytical Technologies, INC., USA) with dried samples. pH was measured by Hach, Pocket Pro+ pH meter.

Alkalinity was determined by titration method with 0.1N H_2SO_4, amount of consumed sulfuric acid used for alkalinity calculation. Cellulose, hemicellulose, and lignin contents of crop residues were analyzed according to Standard Forage Analysis (Goering & Van Soest, 1970). Soluble Chemical Oxygen Demand (sCOD) of samples prior to and during anaerobic digestion were measured for chemical analysis. Untreated and pre-treated straw samples were collected after 24h and filtered.

The liquid part left after filtration of each sample was taken for sCOD analysis. For samples collected from digesters, they were centrifuged at 14,000 rpm at 4°C for 30 min and supernatants were collected in a beaker. Later, the supernatants were filtered through filters with 0.45-µm pore sizes prior to sCOD analysis.

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 a 1 µL gas sample was injected by 0.5 mL syringe. Prior to VFA analysis, 10N phosphoric acid was added into the final filtrates as 10 % (v/v) to fix all biological activity.

Table 4.1. Initial characterization of straws and seed sludge.

Samples	pH	TS (%)	VS (%)	VS/TS (%)	Alkalinity (mg $CaCO_3$/L)	sCOD (mg/L)	TKN (mg/L)	C:N	Cellulose (% TS)	Hemicellulose (% TS)	Lignin (%TS)
Rice straw	6.8	92.8	76.2	82	1710	5490	454	88:1	33.5	28.2	7.4
Wheat straw	6.6	96.4	87.9	92	1050	6280	182	81:1	40.8	22.5	11.6
Rye straw	6.7	94.7	85.8	91	1125	7210	565	85:1	33.4	28.4	14.1
Seed sludge	8.3	3.1	2	63	7500	31,875	1797	16:1	-	-	-

4.1.4. Microbial Characterization of the Anaerobic Seed Sludge

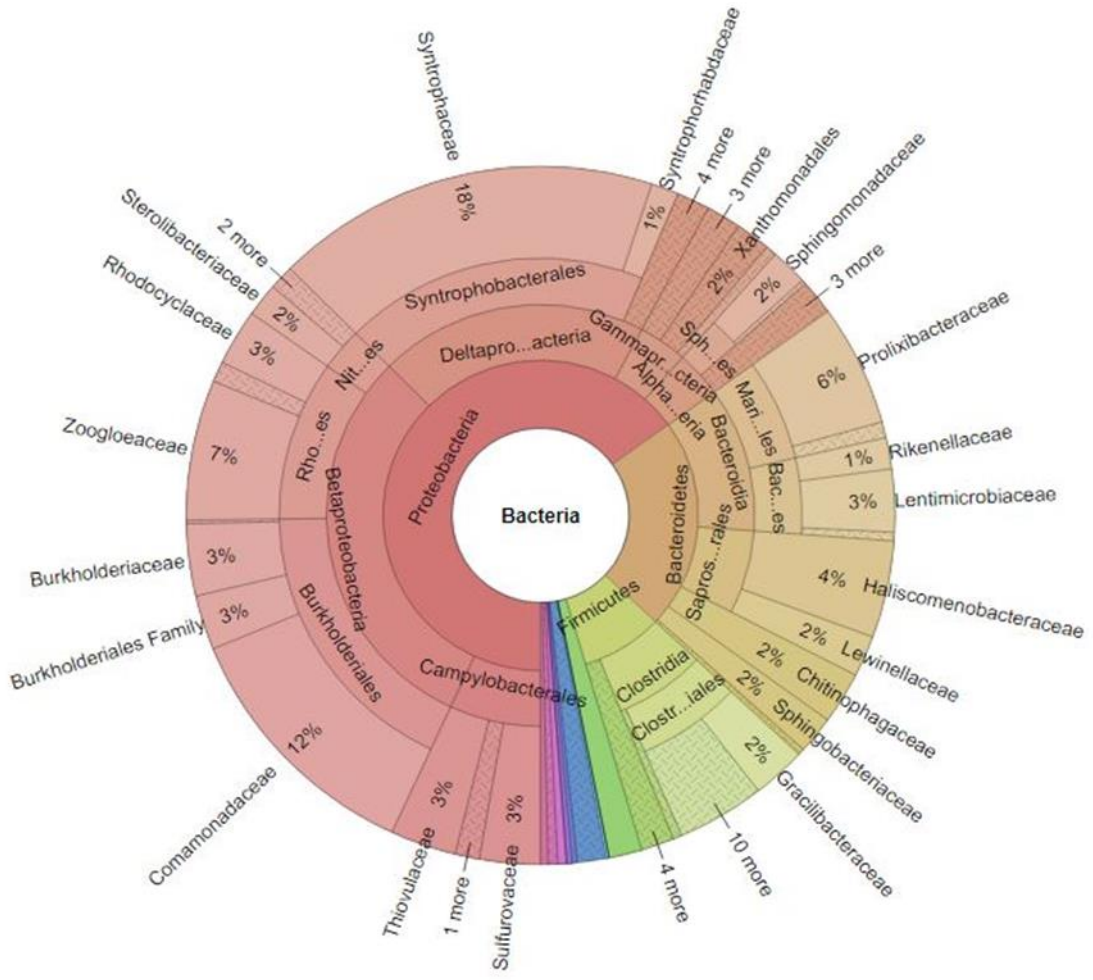

Figure 4.2. The bacterial communities in phylum, class, order, and family levels of the seed sludge.

4.2. Experimental Set-up

4.2.1. Alkaline Pre-treatment

In this research work, sodium hydroxide was used for the pre-treatment of substrates before anaerobic digestion. Rice, wheat, and rye straws were pre-treated by NaOH with different doses. Four NaOH doses of 4 %, 6 %, 8 %, and 10 %, based on the dry matter of substrates were used.

First, solutions of different doses (4 %, 6 %, 8 %, and 10 % gNaOH/gTS) were prepared with a solid to liquid ratio of 100 gTS/L to maintain 10.0 % total solids concentration as recommended by Chandra et al. (2012). In this study, another solid to liquid ratio of 200 gTS/L (20.0 % total solids concentration) was also used for pre-treatment. Therefore, the pre-treatment experiment was classified into 2 groups for each sample; A group where solutions of four different NaOH doses were prepared with a solid to liquid ratio of 100 gTS/L, and another group with a solid to liquid ratio of 200 gTS/L.

Then, the calculated amounts of each sample were soaked into the solutions and kept in closed bottles at room temperature for 24 h, without stirring as shown in Figure 4.3. The pre-treatment room temperature and contact time (24 h) were chosen according to some previous studies with the best pre-treatment results (Sambusiti, et al., 2013; Zhu, et al., 2010).

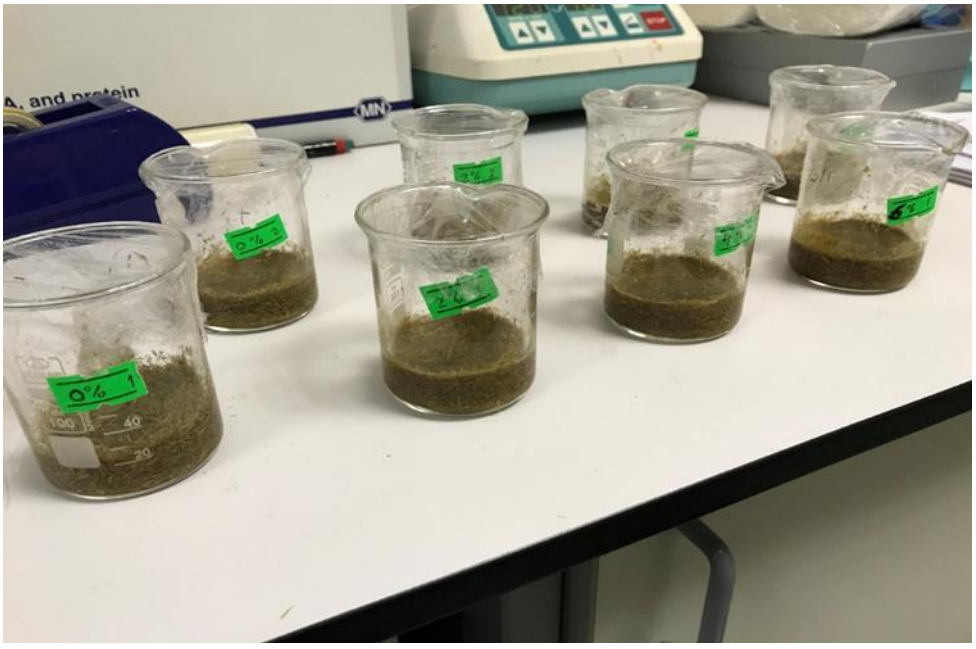

Figure 4.3. Alkali pre-treatment conducted in closed bottle for 24h without stirring.

Some samples with 0 % NaOH (only soaked into tap water) were used as control samples. After 24h, pre-treatment waste was completed, and samples were filtered through a sieve of 0.20mm of pore size. The sieve was used to separate solids and the liquid fractions were taken for compositional analyses (see Figures 4.4 and 4.5).

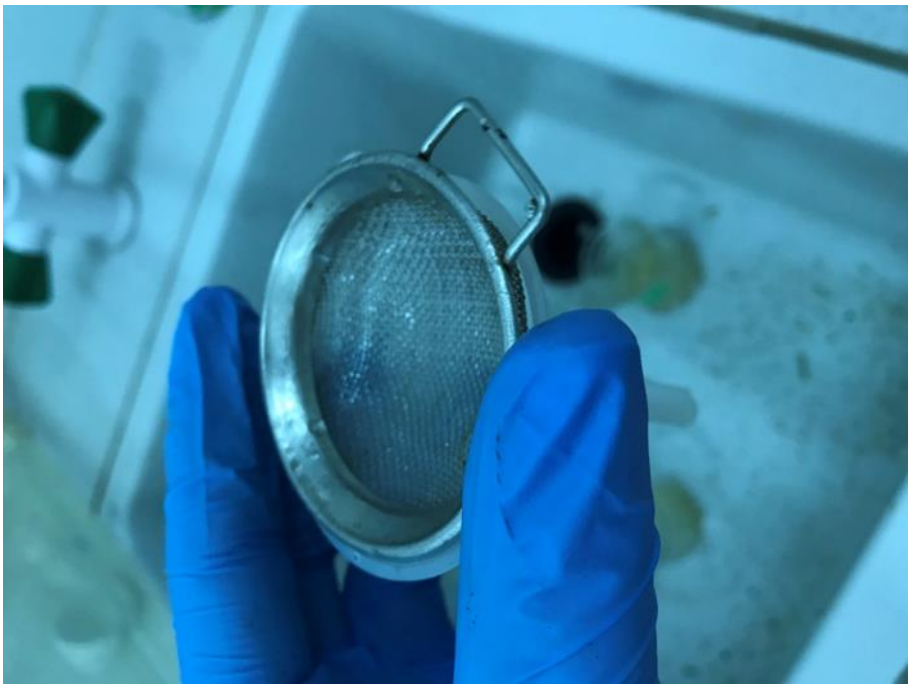

Figure 4.4. The 0.20mm-sieve used for solid separation after pre-treatment.

Figure 4.5. The liquid fractions left after solid separation of the samples.

4.2.2.Biochemical Methane Potential Tests

The anaerobic digestion process was the next step after pre-treatment. Biochemical Methane Production (BMP) tests were conducted to measure the possible amount of biogas that can be produced out of each sample by anaerobic digestion. (BMP) tests were performed in duplicate, by using a volumetric gas production method (AMTPS II, Bioprocess Control Sweden AB, 2014).

Automated Methane Potential Test System (AMPTS II.) is a commercial laboratory instrument used for methane yield determination for different substrates by the anaerobic digestion process. The system is shown in Figure 4.6.

Figure 4.6. Automated Methane Potential Test System II (AMPTS II).

As shown in Figure 4.6, AMPTS II is a volumetric device consisting of 15 gas-tight glass bottles (500mL of working volume) each of which represents a separate digester. All bottles are placed in a water bath at 35 ± 0.5 °C to hold the digesters' temperature in the mesophilic range. Each bottle has a rotary stirrer (112 revolutions per min; for 1 min after a 1 min pause; repeat) that continuously mixes all the digester's content. When the system is on, biogas starts to be produced from each digester. Biogas produced is a mixture of methane and carbon dioxide. For CO2 separation, the biogas produced from each bottle passes through a NaOH solution (3M) that absorbs most of the CO_2 content.

Methane flows through a liquid-displacement automated measuring unit with a resolution of 11–13 mL. A data acquisition system allows flow rate data to be recorded continuously.

4.2.3. Setup of AMPTS II System

After the pre-treatment of substrates and the separation of liquid fractions, substrates were placed into the digesters (gas-tight glass bottles) and mixed with inoculum. In each digester, an amount of 3.3 gVS of seed sludge (corresponding to a volume of 165mL), was mixed with 1.65 gVS of each sample (raw and pre-treated before sieve-separation) resulting in a inoculum/substrate ratio around 2 gVS/gVS, as suggested by Raposo et al. (2009), Labatut et al. (2011) and Akyol et al. (2016). The calculations of the amount of seed sludge and each substrate sample in every digester are shown in Table 4.2.

Then, all bottles were filled up to 450 ml with tap water. The pH value in each digester was set to 7.5 ± 0.2. An amount of 50 mL was taken from each bottle for compositional analysis, so the final volume in each of them was 400 mL. A mixture of inoculum, and tap water, without the addition of substrate, was prepared as a blank sample. To supply anaerobic conditions to microorganisms, all bottles were flushed with N_2 gas flow for 120 seconds before running the test. After replacing all batch digesters in the thermostatic water bath and connecting all wires and cables, the system was on and the motor started running.

AMPTS II system needed to be checked periodically to make sure whether the whole system was working properly or facing any problem. The water level in the thermostatic water bath also needed to be checked and refilled with deionized water as needed. The BMP test lasted for 30 days and the temperature was set to $35^{o} \pm 1^{o}C$.

Table 4.2. Amounts of substrates, NaOH, water, and inoculum needed for pre-treatment and BMP tests.

Substrate	NaOH Pre-treatment	Amount of substrate (g) equivalent to 1.65 gVS	Amount of NaOH (mg)	Water (mL) added to reach solid to liquid ratio of 100 gTS/L and 200 gTS/L		Amount of inoculum (mL) equivalent to 3.3 gVS
Rice Straw	0 %	2.2	0.0	20.2	10.1	165
	4 %		87			
	6 %		130			
	8 %		173			
	10 %		217			
Wheat Straw	0 %	1.9	0.0	18.8	9.4	
	4 %		75			
	6 %		113			
	8 %		150			
	10 %		186			
Rye Straw	0 %	1.9	0.0	19.23	9.60	
	4 %		77			
	6 %		115			
	8 %		154			
	10 %		192			

4.2.4. Sampling and Analytical Methods

The cumulative methane production during anaerobic digestion was measured daily by AMTPS II. For each sample, the BMP test lasted for 30 days. During the test, Samples were taken from each digester during the 0th, 10^{th}, and 30^{th} experiment days for further compositional analysis. Samples taken during the test were analyzed for TS and VS, according to the APHA standard methods. soluble Chemical Oxygen Demand (sCOD), Total Kjeldahl Nitrogen (TKN), and alkalinity were measured according to Standard Methods (APHA/AWWA/WEF, 2012). pH was measured by Hach, Pocket Pro+ pH meter. Finally, the volatile fatty acids (VFA) test was determined for each sample by a gas chromatograph (GC-2025, Shimadzu Co., Japan) equipped with an auto-injector (AOC-20i, Shimadzu Co., Japan).

4.2.5. Anaerobic Co-digestion Test with 8 % NaOH Pre-treatment

At the end of BMP tests for all samples with different NaOH pre-treatment concentrations, it was noticed that the best performance for anaerobic digestion regarding the highest methane yield was observed with 8 % g NaOH/g TS pre-treatment concentration. Therefore, an additional co-

digestion BMP test was performed using combinations of the three different straws with 8 % gNaOH/gTS pre-treatment concentration to decide on the best set with the maximum biogas yield. Table 4.3 shows the combinations of substrates and their amounts used in the co-digestion test.

Besides this, VFA tests were conducted for all sets during the 0^{th}, 3^{rd}, 5^{th}, and 30^{th} experiment days to observe the VFA production in the acidogenesis phase. The co-digestion test was conducted for 30 days as well. The same compositional analyses were performed on samples from each set, and the methane yield was recorded using AMPTS II system.

Table 4.3. Combinations and amounts of substrates, NaOH, water, and inoculum used for pre-treatment and co-digestion BMP test.

Substrate	NaOH Pre-treatment	Amount of substrates (mg) equivalent to 1.65 gVS	Amount of NaOH (mg)	Water (mL) added to reach solid to liquid ratio of 100 and 200 gTS/L		Amount of inoculum equivalent to 3.3 gVS
Rice/Wheat	8 %	1118 / 940	165	20.6	10.3	165 mL
Rice/Rye		1118 /962	166	20.8	10.4	
Wheat/Rye		940/962	152	19.0	9.5	
Rice/Wheat/Rye		720/630 /640	169	20.0	10.0	

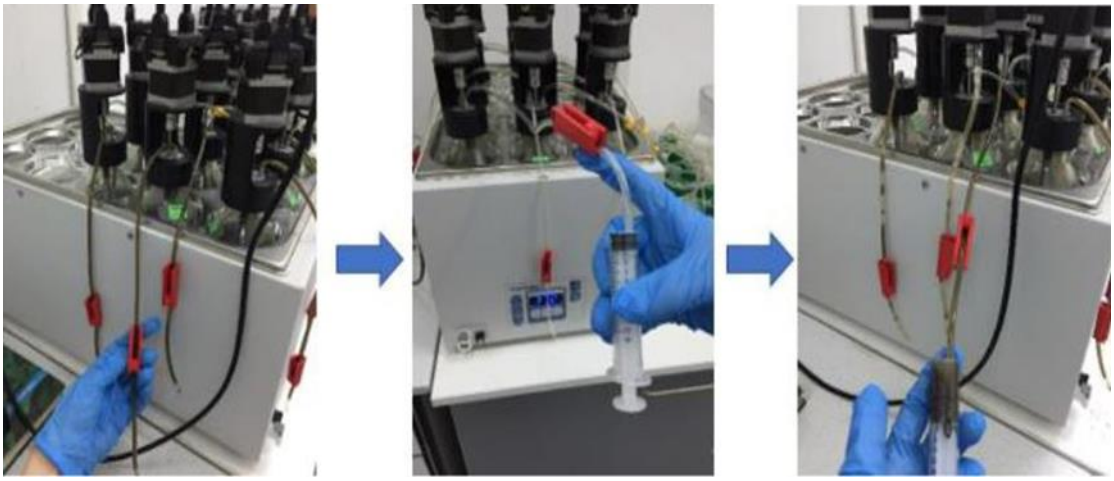

Figure 4.7. Samples collected from digesters on days 0, 3, 5, and 30 to observe the VFA production and the depletion in the acidogenesis phase.

4.3. Metagenomic Analysis

4.3.1. DNA Extraction

Samples from digesters that produced the highest methane yield were chosen for further molecular analysis. Genomic DNA was isolated from samples taken on days 0 and 30 of those samples during anaerobic digestion with the Soil Extraction Kit (Machenery-Nagel, Germany) according to the manufacturer's protocol. First, a sample of 5 mL was taken from each digester and then centrifuged at 12,000 rpm for 10 min. supernatant produced after centrifugation was collected and removed. In order to homogenize the pellet, 700 µl Lysis Buffer SL1 was added to the tube and the homogenized solution was transferred to NucleoSpin® Bead Tube Type A that contains ceramic beads. Just after the homogenizing step, 150 µl Enhancer SX was pipetted into solution, and to destroy the cells, a vortex adapter (Vortex-Genie) was utilized, 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 for incubation at 0-4 ºC for around 5 min. 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.

According to the manufacturer's protocol, the DNA extraction process was completed once it came to that last step. Qubit® 3 fluorometer (Thermo Fisher Scientific) was utilized to check the quantity and quality of the isolated DNA by determining the absorbance values of samples at 260 nm. The results of the Qubit® 3 fluorometer are shown in Table 4.4.

Table 4.4. The absorbance values of samples at 260 nm by Qubit® 3 fluorometer.

Samples	Qubit® 3 fluorometer
Rice 8 % Day 0	43
Rice 8 % Day 30	53.6
Rice 0 % Day 30	18.9
Wheat 0 % Day 30	29
Rye 0 % Day 30	12.2
Seed Sludge	12.7
(Rice/Wheat) 8 % Day 5	50.8
(Rice/Wheat/Rye) 8 % Day 5	16.7
(Rice/Wheat) 8 % Day 30	37.4
(Rice/Wheat/Rye) 8 % Day 30	44.8

4.3.2. 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 Table 4.5, 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 4.5. 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. 16S Barcoding Kit (SQK-RAB204; Oxford Nanopore Technologies) was used to prepare the amplicon library and the amplicon library was loaded on the MinIONTM (Oxford Nanopore Technologies) device for library preparation. It was performed according to manufacturer recommendations.

4.3.3. Sequencing

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 was in fast 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.

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.

5. RESULTS AND DISCUSSION

The main objective of this study is to investigate the effect of NaOH alkali pre-treatment on the chemical composition and the methane production of agriculture wastes. Being three of the most abundant lignocellulosic biomasses among agricultural residues in the world, wheat, rice, and rye straws were chosen as substrates for this study. The straws were first pre-treated by NaOH for 24 h and then anaerobically digested at mesophilic temperature.

Alkali pre-treatment was chosen for this study due to the advantages it has over the other pre-treatment techniques, including the high ability of lignin solubilization and neutralization of various acidic products degraded from the lignocellulosic complex. Furthermore, it decreases the possibility of inhibition during methane fermentation and provides a lower cost of production (Bolado-Rodriguez 2016). Strong bases such as sodium, potassium, calcium, and ammonium hydroxides give high performance in terms of lignin solubilization. Specifically, NaOH is the most effective base in lignin removal and biogas production out of rice straw (Zhu2010).

The pre-treatment step was conducted in closed bottles at room temperature. The samples were soaked in NaOH solutions at different dosages (0 %, 4 %, 6 %, 8 %, and 10 % gNaOH/gTS), with a solid to liquid ratio of 100gTS/L and 200gTS/L. After 24 h of pre-treatment, samples with a solid to liquid ratio of 100gTS/L were filtered and proceeded to the anaerobic digestion stage. The others, which were pre-treated with a 200gTS/L solid to liquid ratio, were proceeded directly to the BMP test without any filtration.

BMP tests were conducted using AMTPS II and lasted for 30 days under mesophilic conditions. In the end, the best NaOH pre-treatment dosage was defined and used for further anaerobic co-digestion tests using all substrates to determine which substrate sets produce the highest methane yield. During BMP, samples were taken on different days for compositional analysis

To define the best pre-treatment NaOH dose, a comprehensive comparison was conducted using all samples with different pre-treatment dosages and solid to liquid ratios. The comparison was based on some factors such as the effect of pre-treatment on the chemical composition of substrates (reduction of cellulose, hemicelluloses, and lignin contents due to pre-treatment), soluble chemical oxygen demand removal (sCOD), methane production, TS/TVS removal and volatile fatty acid (VFA) production for all substrates with different pre-treatment dosages.

Bacterial and archaeal characterization studies were conducted on the inoculum used and the sets with the highest methane yield on different days of the BMP test. Then, the sequencing method was used to reveal the diversity of microbial communities within each digester. Afterward, bioinformatics analyses were carried out to realize and compare the bacterial diversity within digesters and between the chosen digesters.

5.1. Effect of Pre-treatment on the Chemical Composition of Substrates

According to BMP test results, pre-treatment with 8 % gNaOH/gTS dose showed the best results in terms of higher methane production, that's why this pre-treatment dose was chosen for fibrous composition analysis. Two samples of untreated and 8 % NaOH of each straw type were analyzed to determine the change in cellulose, hemicellulose, and lignin contents before and after the pre-treatment. The comparison was made between 0 % and 8 % pre-treated straws and the reduction in cellulose, hemicelluloses, and lignin contents were regarded. Figure 5.1 shows samples after and without pre-treatment. Cellulose, hemicellulose, and lignin contents of straw samples were analyzed according to Standard Forage Analysis (Goering and Van Soest, 1970).

Figure 5.1. Pre-reated and untreated wheat straw samples ready for fibrous composition analysis.

Rice, wheat, and rye straws consist mainly of lignin, cellulose, and hemicellulose which are considered the main carbon source provided for microorganisms to perform the anaerobic digestion process. As mentioned before, the lignocellulosic structure of straw is highly resistant to microorganisms' attacks and the removal of lignin and hemicellulose parts increases the accessibility of cellulose which in turn increases the biodegradability of straw.

After applying the NaOH pre-treatment step, alkali chemical reactions take place between NaOH and the chemical structure of straws resulting in the change of chemical composition and physical properties. The chemical composition of straws was analyzed before and after pre-treatment (as given in Table 5.1) to determine the effect of pre-treatment on the digestibility of straws and the biogas production.

Table 5.1. Fibrous composition of untreated and NaOH rice, wheat, and rye straws.

Sample	Cellulose (% TS)	Hemicellulose (% TS)	Lignin (% TS)
Untreated rice straw	33.50	28.20	7.40
8 % rice straw	4.70	5.30	2.80
Untreated wheat straw	40.80	22.50	11.60
8 % wheat straw	8.70	3.10	3.60
Untreated rye straw	33.40	28.40	14.10
8 % rye straw	1.50	5.60	3.70

5.1.1. Lignin

The application of NaOH alkali pre-treatment had a great effect on the lignin content of straws. The chemical reaction that occurred between NaOH and lignin caused the breakdown of the linkages of lignin units and the functional groups. It can be referred to as depolymerization or partial degradation process of lignin into simpler substances which leads to the reduction of lignin content in the sample. Liu et al. (2015) regarded a decrease of 54.7 % in lignin content of wheat straw by pre-treatment with 50 % KOH solution. Another study showed a reduction of 80 % in the lignin content of sugarcane bagasse after being using NaOH 1M (Rabelo, et al., 2011). In the case of rice straw in this study, the lignin removal was around 62 % of total lignin content after pre-treatment with 8 % NaOH dosages. A lignin removal of 69 % and 74 % was observed after the pre-treatment of wheat and rye, respectively.

5.1.2. Hemicellulose

The hemicellulose part of straw consists of a limited number of sugar units connected by relatively strong linkages. During NaOH pre-treatment, NaOH reacts with both the sugar units and the linkages. This reaction results in the breakage of the bands and the change of hemicellulose functional sugar units. Therefore, hemicellulose is degraded to simpler substances which increase the digestibility of straw. Sambusiti et al. (2013) observed a reduction of 60 % and 45 % in the hemicellulose contents of sorghum and wheat straw, respectively by pre-treatment with 10 % g NaOH/g TS for 24 h. in this study, after pre-treatment of samples with 8 % NaOH doses, hemicellulose fraction was reduced by approximately 81 %, 86 % and 80 % of the total hemicellulose content of rice, wheat and rye straw, respectively.

5.1.3. Cellulose

A relatively high decrease of cellulose content was observed in each sample after being pre-treated with 8 % NaOH dose. The chemical reaction of NaOH with cellulose led to the change of the functional groups of cellulose and the breakage of the linkages between them which in turn resulted in the reduction of cellulose content. This made cellulose more biodegradable and easier to be attacked by anaerobic microorganisms.

After pre-treatment with 8 % NaOH dose, cellulose content was reduced by 85 %, 79 %, and 95 % for rice, wheat, and rye straws, respectively. The swelling and hydrolysis of hemicelluloses and cellulose during the pre-treatment process resulted in partial degradation of those compounds and a release of sugars which increased the sugars content of the pre-treated samples compared to the untreated ones. This explains the high cellulose reduction in each sample after being alkali pre-treated (Bolado-Rodríguez, et al., 2016)

5.2. Effect of Pre-treatment on Soluble COD of Samples

During the pre-treatment process, samples were soaked in NaOH solutions for 24 hours. Two different solid to liquid ratios were used for solutions preparation; 100 and 200 gTS/L. Samples pre-treated with 200 gTS/L ratio were almost completely saturated with the solution and had no liquid part left, so these samples were sent directly to the BMP test without being filtered (See Figure 5.2). On the other hand, samples with 100 gTS/L NaOH solution ratio were fully saturated and had some

liquid solution left. Therefore, these samples were filtered before the BMP test and the filtrates (the liquid left after pre-treatment) were sent for compositional analysis.

Figure 5.2. Rice straw sample with 200 gTS/L NaOH solution ratio

Due to swelling and hydrolysis of hemicelluloses and cellulose during pre-treatment, the release of CODs (in the form of sugars and other organic compounds) increased in filtrates which was observed clearly in the filtrates' color resulted from treated and untreated samples. The color differences can be seen in Figure 5.3. To measure the difference between CODs released from treated and untreated samples, soluble COD tests were conducted on the filtrates produced after 24 h of pre-treatment. A control sample was used with 0 % NaOH solution (only water was used). To run the test, filtrates were diluted with deionized water to a ratio of 1:10.

Figure 5.3. Filtrates released after 24 h pre-treatment of rice straw by 0 %, 4 %, 6 %, and 8 % NaOH dosages, respectively.

Differences in color after dilution are shown in Figure 5.4. By checking the diluted samples, we can see that the color of each sample is getting darker while moving from untreated towards higher-dose treated ones. This shows that the released CODs in the filtrates increases as the pre-treatment NaOH dosage increases. It gives proof of better pre-treatment results which means more lignin, hemicellulose, cellulose removal, and more sugar release.

Figure 5.4. Differences in color of diluted filtrates of pre-treated rye straw samples with 0 %, 4 %, 6 %, 8 %, and 10 % NaOH dosages, respectively.

As sCOD analysis was a good way to Figure out how efficient pre-treatment was by measuring the amounts of CODs released in filtrates produced from treated and untreated straw samples, a chart was created using these sCOD values to compare the effect of pre-treatment with different NaOH dosages on each straw sample. By having a look at the chart Figure 5.5, we can notice that the sCOD value for each straw increases, as pre-treatment NaOH dosage increases. It starts at its lowest value for untreated samples, and it goes higher by increasing NaOH dosages for ones. This can be explained by the fact of the chemical reactions occur during pre-treatment between straw and NaOH. More NaOH dose means more chemical reactions happen to cause the change of straw composition. This leads to the removal of more lignin, hemicellulose, and cellulose and the production of sugars and other less complex chemical components which are easier to be digested by microorganisms.

The untreated rice straw filtrate sample had around 3000 mg/L sCOD value. The value continues to get higher for pre-treated samples as the NaOH dose increases. It becomes around 10,200 mg/L for rice straw pre-treated with 10 % gNaOH/gTS. It means that the sCOD value, which represents CODs released, became 3 times higher than the value of untreated one. This is giving clear evidence of the good efficiency of NaOH pre-treatment of rice straw.

In the case of wheat straw, the sCOD value starts with 3300 mg/L for untreated wheat filtrate samples. It gets higher as the NaOH dose increases. the value reaches around 11,000 mg/L for wheat with 10 % gNaOH/gTS. This shows an increase of more than 3 times compared to the original sCOD value of untreated wheat.

For rye straw, the untreated sample shows a sCOD value of around 3600 mg/L. the same rise happens as NaOH dose increases in pre-treated samples. It gets to the value of 13,000 mg/L for 10 % gNaOH/gTS pre-treated rye sample creating more than 3 times increase of sCOD value compared to untreated sample.

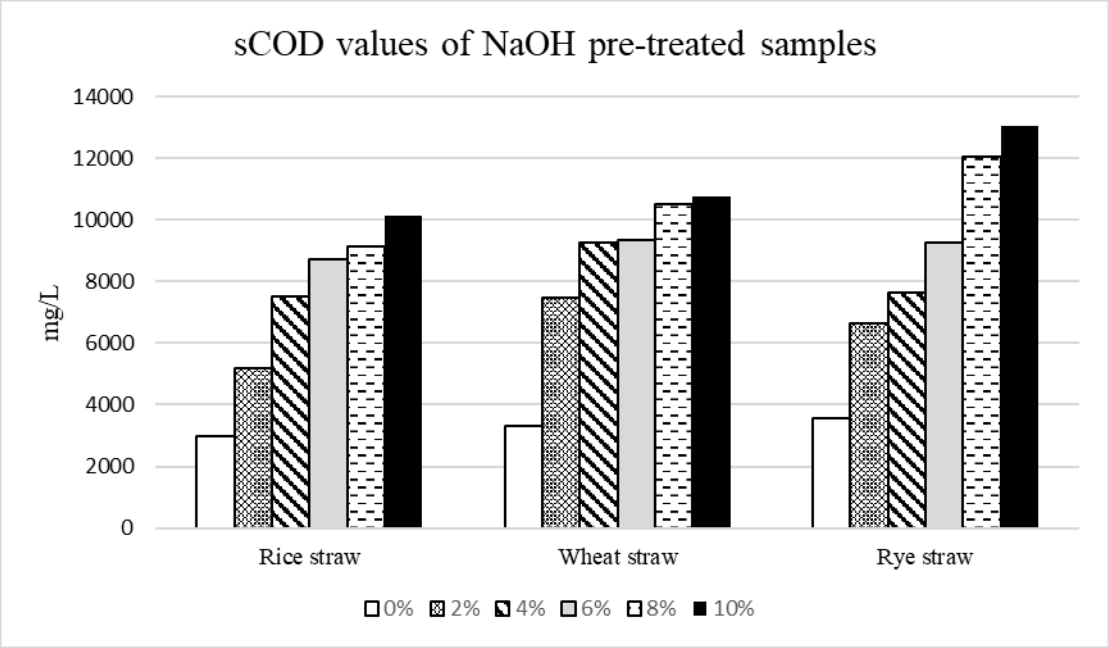

Figure 5.5. sCOD values of filtrates released after 24 h pre-treatment of rice, wheat, and rye straw.

5.3. Effect of Pre-treatment on Methane Production

After alkali pre-treatment of substrates with different NaOH dosages, they were ready to be used for BMP tests. Samples pre-treated with NaOH solution in which solid to liquid ratio was 100 gTS/L, were first filtrated. Other samples which were pre-treated with 200 gTS/L NaOH solution, were sent directly to BMP test without filtration. BMP test was conducted for 30 days with an inoculum/substrate ratio of 2 as recommended by Ozbayram et al. (2018).

BMP tests were conducted using a volumetric gas production method (AMPTS II) which calculates the cumulative methane produced from each digester in (ml methane). The methane yield in terms of (mL CH4 /g VS) for each substrate was calculated by subtracting an amount of 76 ml CH4 (obtained from anaerobic digestion of seed sludge) from the total cumulative methane produced then dividing the amount to 1.65g volatile solids which is the added amount of substrates in the set - up of digesters (Bozan, 2018).

The methane production for all digesters was nearly stabilized on the 20th day. At the end of the BMP test, we can clearly notice that the methane yield gets higher as the NaOH pre-treatment dosage increases until it reaches the highest values with 8 % and 10 % NaOH pre-treated samples for almost all substrates. The reason behind this is that alkali pre-treatment of lignocellulosic biomass causes swelling and damaging of the linkages in the structure of straw besides increasing the internal surface

area. By increasing NaOH dose which means an increase of the chemical reactions occurring between NaOH and straw during pre-treatment, it leads to more degradation of straw structure which increases the digestibility of straw. This was explained by Chandra et al. (2012) and Sambusiti et al. (2012).

By investigating the charts of methane yield for both samples pre-treated with 100 and 200 gTS/L total solids concentrations, we will notice that there are slightly higher values of methane yield produced from 200 gTS/L pre-treated samples than 100 gTS/L pre-treated ones. The increase ranges between 10 % for the lowest and 16 % for the highest. This can be explained by the filtration process conducted to samples pre-treated with 100 gTS/L. after 24 hours of pre-treatment, these samples were filtered, and the filtrate was sent for compositional analysis. The filtrate (the liquid left after pre-treatment) contained a high portion of hydrolyzed sugars (CODs released from the pre-treated straw), which was proven by the soluble COD test conducted on those filtrates. This means that samples pre-treated with 100 gTS/L NaOH solutions lost some of their CODs in the filtrates, which negatively affected their production of methane, unlike 200 gTS/L pre-treated samples which had no filtrates and lose in their CODs contents.

5.3.1. Rice Straw

By checking Figures 5.6 and 5.7, we can see the positive effect of the alkali pre-treatment of rice straw. In both cases of pre-treatment, the methane yield increases by increasing the NaOH dosage during the pre-treatment process. The yield starts at its lowest value for untreated straw then it gets higher till it reaches the highest value for straw pre-treated with 8 % gNaOH/gTS. In the first 10 days, a methane production of approximately 90 % of the total yield has been reached by almost all samples.

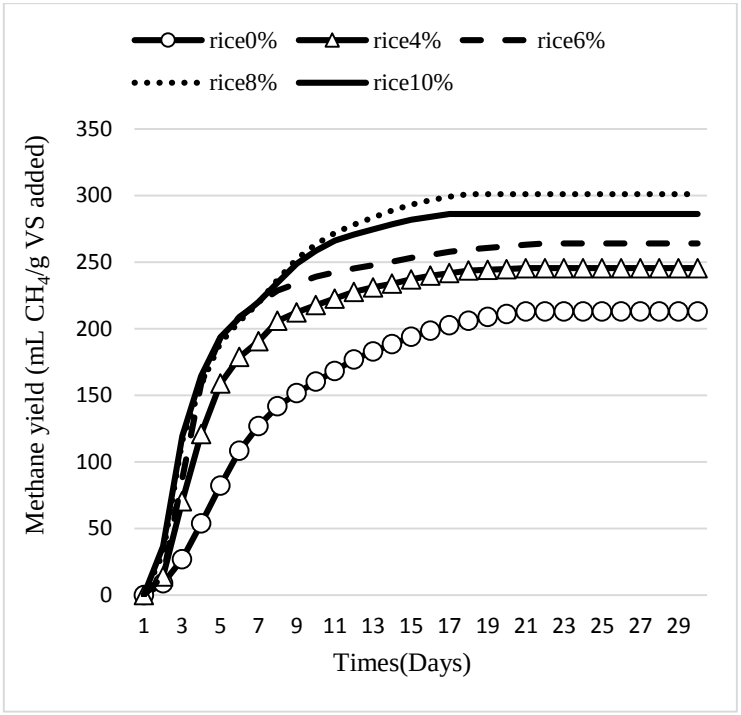

Figure 5.6. Cumulative methane yield of rice straw with different NaOH dosages and total solid concentrations of 100 gTS/L.

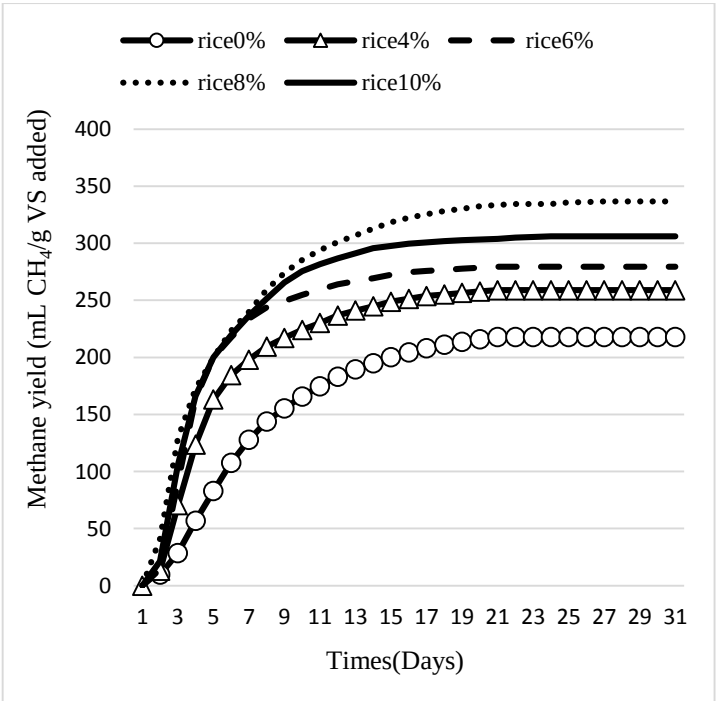

Figure 5.7. Cumulative methane yield of rice straw with different NaOH dosages and total solid concentrations of 200 gTS/L.

Rice straw pre-treated with 100 gTS/L total solid concentrations

The methane yield of untreated rice straw was around 213 mL CH4/g VS added which is almost in agreement with the data shown in past studies (He, et al., 2009) (Mussoline, et al., 2013). Then the yield gets higher with 4 %, and 6 % NaOH pre-treated straw and shows a value of 245 and 264 mL CH4/g VS added, respectively. The increase in methane yield was the highest in the case of 8 % and 10 % NaOH pre-treated rice straw with values of 301 and 286 mL CH4/g VS added, respectively. Pre-treatment with 8 % gNaOH/gTS has shown the highest performance of methane production with an increase of up to 41 % compared to untreated rice straw (Figure 5.6).

Rice straw pre-treated with 200 gTS/L total solid concentrations

The methane yield for these samples was slightly higher than those pre-treated with 100 gTS/L. The untreated rice straw shows a methane yield value of 217 mL CH_4/g VS added. The yield curve gets higher with 4 % and 6 % pre-treated straw and shows values of 258 and 279 mL CH4/g VS added, respectively. The highest values of methane yield were recorded by straw pre-treated with 8 % and 10 % by values of 336 and 306 mL CH4/g VS added, respectively. The highest increase of methane yield was recorded by 8 % gNaOH/gTS pre-treated rice straw which shows an increase of 54 % compared to untreated rice straw (Figure 5.6). It is clear that the second curve (Figure 5.7) shows higher methane yield values with an increase of around 11 % compared to the first one.

5.3.2. Wheat Straw

The anaerobic biodegradability of wheat straw has increased gradually by increasing the NaOH dose during pre-treatment. This can be seen by checking Figures 5.8 and 5.9 of total methane yields. We can also notice that the methane production has reached 90 % of the total yield in approximately 10 days. Then it starts to slightly increase till it is stabilized by day 20. Wheat straw pre-treated with 8 % and 10 % gNaOH/gTS has shown a relatively higher methane yield than other pre-treated and untreated samples with an increase of around 10-15 %.

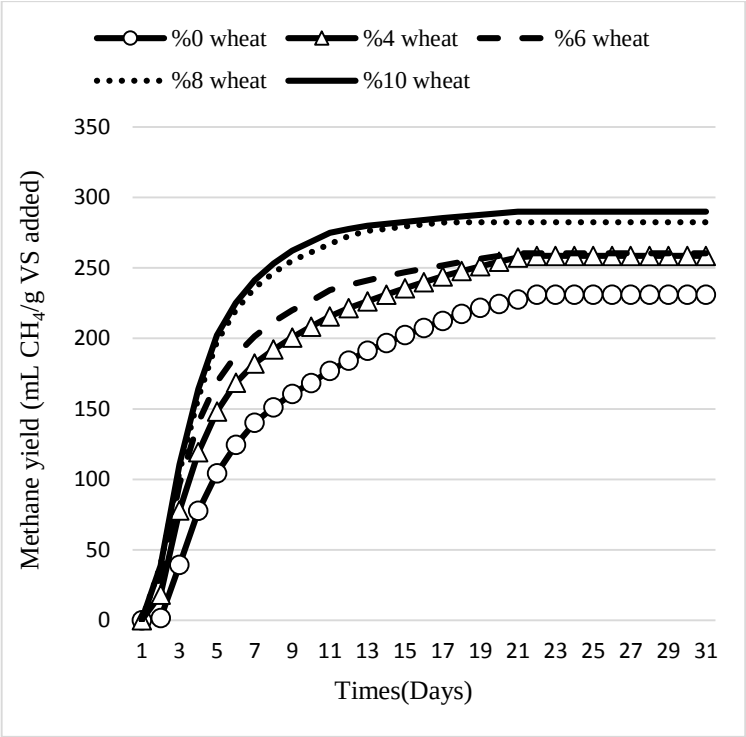

Figure 5.8. Cumulative methane yield of wheat straw with different NaOH dosages and total solid concentrations of 100 gTS/L.

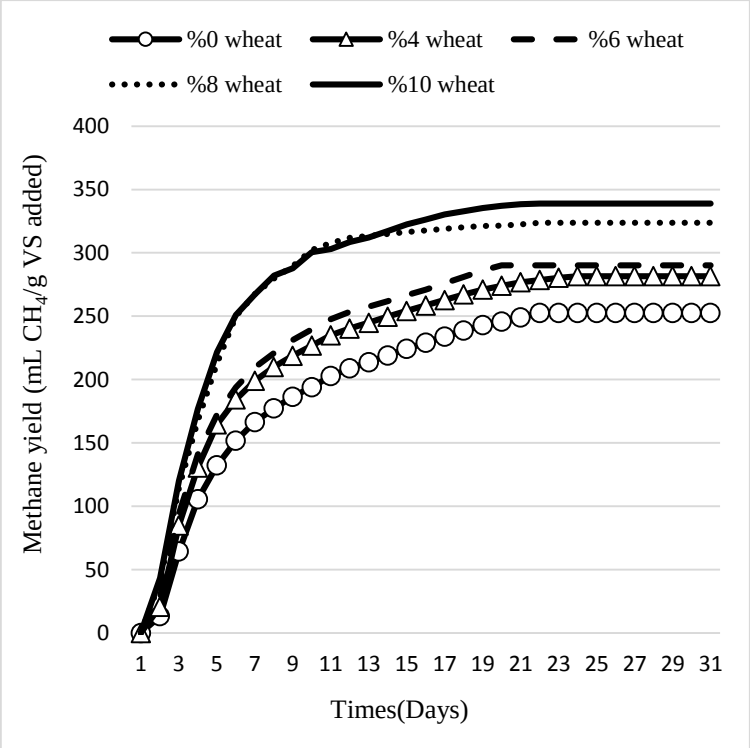

Figure 5.9. Cumulative methane yield of wheat straw with different NaOH dosages and total solid concentrations of 200 gTS/L.

Wheat straw pre-treated with 100 gTS/L total solid concentrations

The curve shows the lowest value of 231 mL CH_4/g VS methane yield in the case of untreated wheat straw which is close to the values regarded by Bolado-Rodríguez et al. (2016). The methane yield gets a slight increase of around 11 % in the case of wheat straw pre-treated with 4 % and 6 % by showing values of 258 and 260 mL CH_4/g VS, respectively. The highest methane yield is recorded by wheat straw pre-treated with 8 % and 10 % NaOH by values of 282 and 290, respectively. the total increase of the methane yield was approximately 50 mL CH_4/g VS added, which means 21.5 % compared to the untreated straw.

Wheat straw pre-treated with 200 gTS/L total solid concentrations

The curve shows relatively higher results for all samples than the previous one. The untreated rice straw for example shows a value of 252 mL CH_4/g VS added which is around 8 % higher than the untreated rice straw of the previous curve. Wheat straw pre-treated with 4 % and 6 % NaOH shows a methane yield of 281 and 290 mL CH_4/g VS added, respectively. Pre-treatment with 8 % and 10 % NaOH shows the highest yield of methane production with values of 323 and 338 mL CH_4/g VS added, respectively. The total methane yield increase was around 34 % compared to the untreated straw. Comparing both curves, we will notice that pre-treatment with 200 gTS/L total solid concentrations results in higher methane yield by an increase of 16 %.

5.3.3. Rye Straw

The methane yield of NaOH pre-treated rye straw is relatively higher than the untreated straw which proves the positive effect of alkali pre-treatment. More than 90 % of the total methane yield was achieved in the first 10 days. The production was stabilized by day 20. Rye straw pre-treated with 8 % and 10 % gNaOH/gTS shows the highest methane yield. The lowest value of methane yield is recorded by untreated rye straw.

Rye straw pre-treated with 100 gTS/L total solid concentrations

Mussoline et al. (2013) showed a value of 290 mL CH_4/g VS added methane yield caused by untreated squashed rye straw. The untreated rye straw in this study recorded a value of 226 mL CH_4/g VS methane yield as the lowest value among all tested samples. The yield gets a slight increase with 4 % and 6 % gNaOH/gTS samples and shows values of 274 and 275 mL CH_4/g VS added,

respectively. The highest methane yield was recorded by 8 % and 10 % gNaOH/gTS rye samples by showing values of 290 and 299 mL CH_4/g VS added, respectively. The total increase of methane yield caused by 10 % NaOH pre-treatment is approximately 33 % compared to untreated rye straw (Figure 5.10).

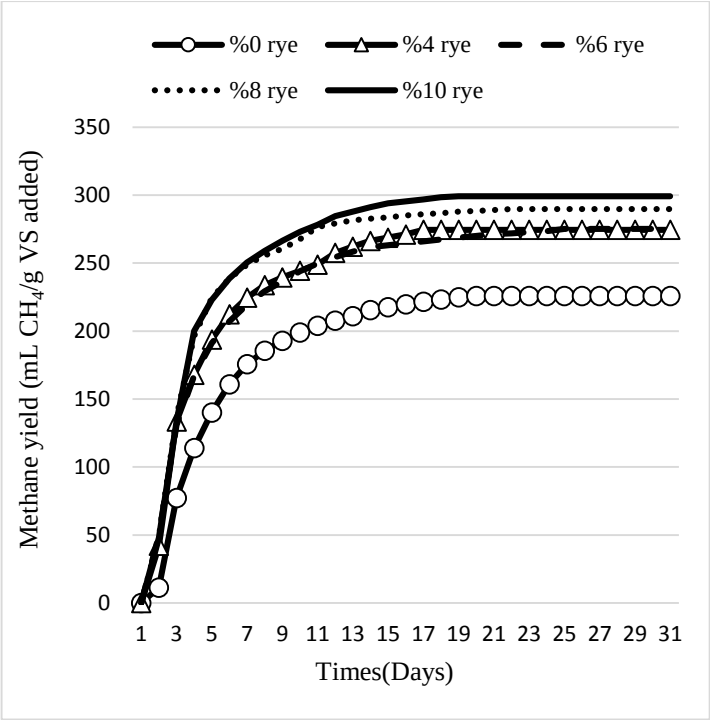

Figure 5.10. Cumulative methane yield of rye straw pre-treated with different NaOH dosages and total solid concentrations of 100 gTS/L.

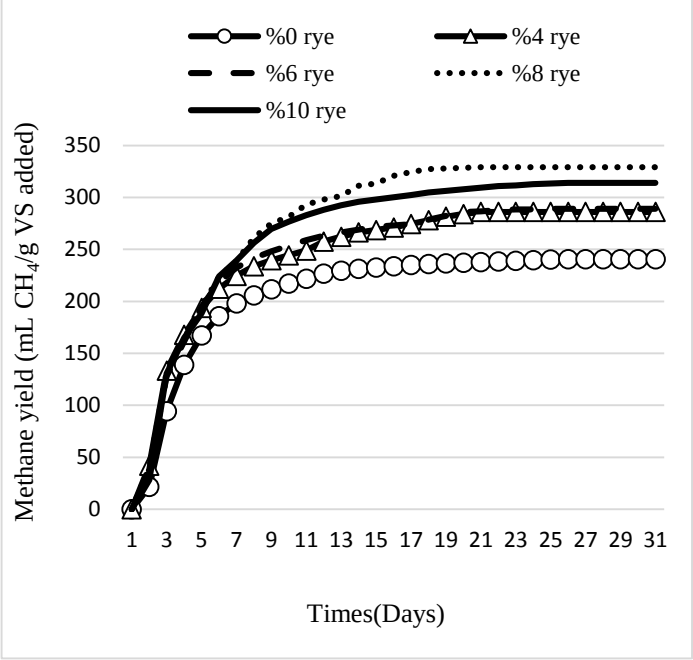

Figure 5.11. Cumulative methane yield of rye straw pre-treated with different NaOH dosages and total solid concentrations of 200 gTS/L.

Rye straw pre-treated with 200 gTS/L total solid concentrations

Pre-treatment with 200 gTS/L solid to liquid ratio has always shown better results than 100 gTS/L. In the case of rye straw and by checking Figure 5.11, we will notice relatively higher methane yield results compared to Figure 18. Untreated rye straw has shown a value of 240 mL CH_4/g VS added of methane production, while 4 % and 6 % gNaOH/gTS straw has recorded values of 286 and 289 mL CH_4/g VS added, respectively with an increase of nearly 20 %. 8 % and 10 % gNaOH/gTS rye straw samples have shown the best results of methane yield with values of 329 and 313 mL CH_4/g VS, respectively. The total increase of methane yield compared to untreated straw is around 37 %. The increase in methane yield achieved by pre-treatment with 200 gTS/L total solid concentrations is 10 %.

5.3.4. Co-digestion Test

The co-digestion BMP test was established to decide on the best set combination among all used straws (rice/wheat/rye) with the highest performance of biodegradability and methane production. Samples were prepared with equal VS amounts and with 8 % gNaOH/gTS for 24 hours. 8 % gNaOH/gTS amount of NaOH was chosen for this test as a result of its high biodegradability performance shown by single BMP tests of each substrate. In the first 10 days, more than 90 % of methane production was achieved.

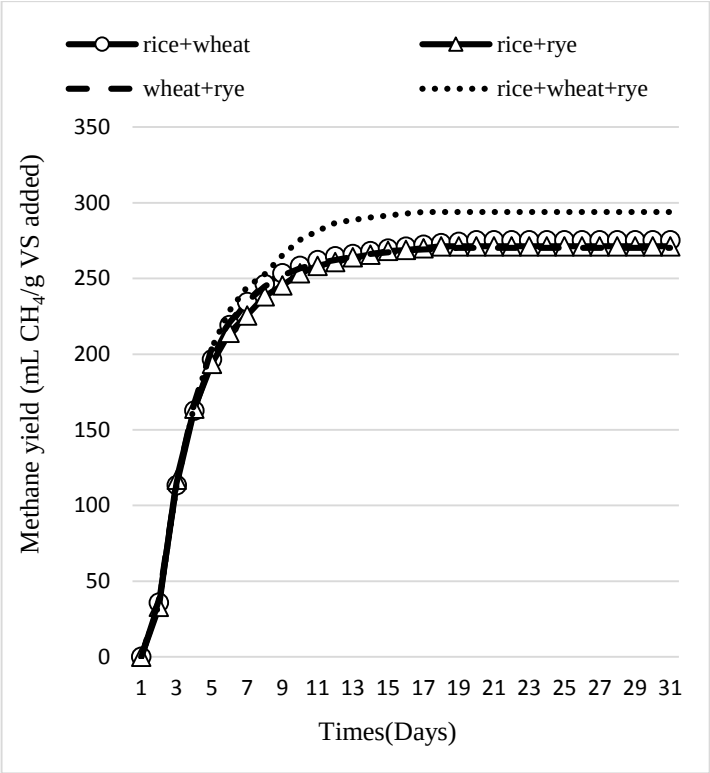

Figure 5.12. Cumulative methane yield of co-digestion test pre-treated with 8 % NaOH dosage and total solid concentrations of 100 gTS/L.

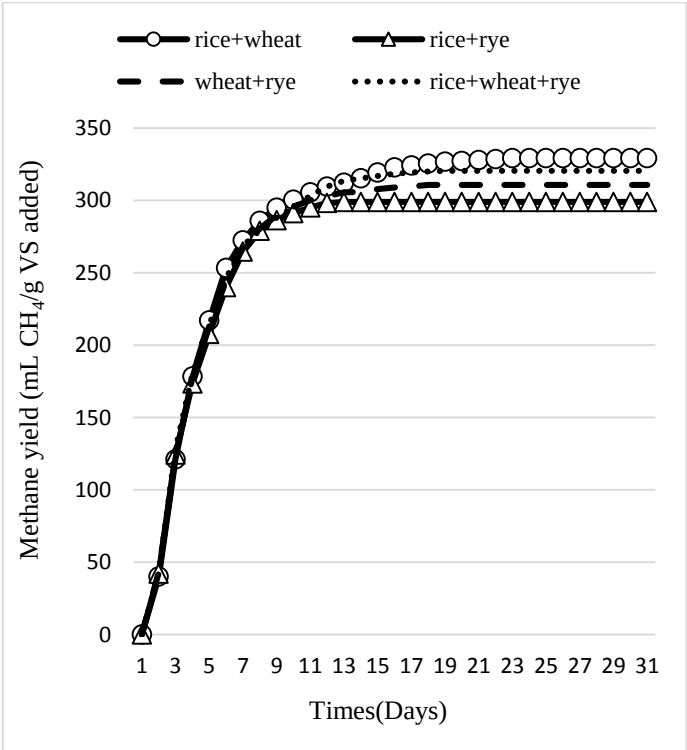

Figure 5.13. Cumulative methane yield of co-digestion test pre-treated with 8 % NaOH dosage and total solid concentrations of 200 gTS/L.

Pre-treatment with 100 gTS/L total solid concentrations

There are relatively similar results of methane yield for all sets created. The highest methane yield was recorded by the set of (rice/wheat/rye) straws with a value of 293 mL CH_4/g VS. Other combinations of substrates have resulted in almost the same values of methane yield with slight differences. (Wheat/rye) set for example is showing a value of 270 mL CH_4/g VS while a value of 275 mL CH_4/g VS is shown by the set of (wheat/rice). See Figure 5.12.

Pre-treatment with 200 gTS/L total solid concentrations

The results of methane yield are showing an increase while pre-treatment with 200 gTS/L solid to liquid ratio is applied. A value of 320 mL CH_4/g VS methane yield is recorded by the set (rice/wheat/rye) showing an increase of 9 % compared to 100 gTS/L pre-treated samples. (Rice/wheat) set has recorded the highest methane yield with a value of 329 mL CH_4/g VS, while a value of 310 mL CH_4/g VS methane yield is shown by (wheat/rye) set.

From the previous results, we can conclude that pre-treatment with 8 % and 10 % gNaOH/gTS can increase the methane yield in a range of (30-50) % compared to untreated straws. 4 % and 6 % gNaOH/gTS straws show nearly the same results of methane yield in most cases. The NaOH pre-treatment of straws with 200 gTS/L total solid concentrations shows relatively higher methane yield than pre-treatment with 100 gTS/L total solid concentrations by an increase up to 10 %. By using NaOH pre-treatment, the pH of all digesters had a slight change in a range of (7.1-7.8) which had no negative effect on the biodegradability process. The co-digestion test shows that the combination of wheat and rice straws results in the highest methane yield by producing 330 mL CH_4/g VS added after being with 8 % gNaOH/gTS.

5.4. Effect of Pre-treatment on TS/TVS Reduction Efficiencies on the 30th Day

The efficiency changes in straw biodegradability caused by NaOH pre-treatment can be observed by measuring the total solids and volatile solids variation before and after the BMP test. The increase in the removal of TS/TVS by means of pre-treatment can be a good sign of better efficiency of straw biodegradability which in turn will show a higher methane production (Chandra, et al., 2012). In this study, samples with both 100 and 200 gTS/L total solid concentrations show nearly the same results of TS/TVS removal efficiencies.

5.4.1. Rice Straw

Figure 5.14 shows the differences in TS/TVS removal efficiencies of untreated and rice straw samples on the 30th day of the BMP test. The TS and TVS removal efficiency of untreated rice straw was observed as 13.4 % and 20 %, respectively. 10 % NaOH rice straw samples showed the highest TS and TVS removal efficiencies by values of 24.1 % and 28.5 %, respectively. That means an increase of 42.5 % of the total TV removal efficiency has been reached by pre-treatment with 10 % NaOH.

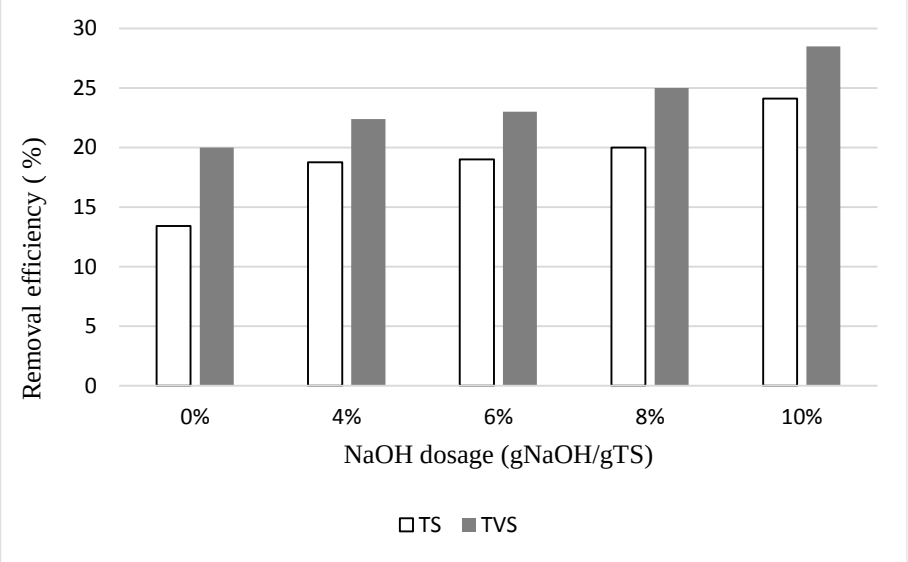

Figure 5.14. Variation of TS and TVS mass removal efficiencies of untreated and different dosages rice straw.

5.4.2. Wheat Straw

By checking Figure 5.15, we can observe that TS and TVS removal efficiencies showed their lowest values in the case of untreated wheat straw by 14.3 % and 20 %, respectively. On the other hand, wheat straw with 10 % NaOH dosage showed the highest values of TS and TVS removal efficiencies by 16.8 % and 26 %, respectively. In other words, an increase of 30 % of wheat straw TVS removal efficiency has been reached by pre-treatment with 10 % NaOH.

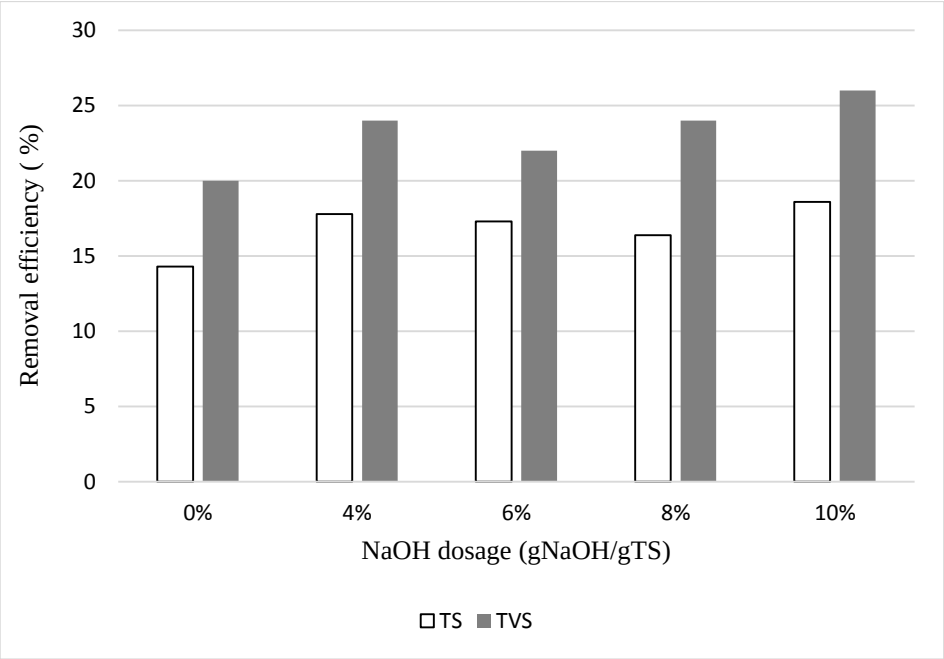

Figure 5.15. Variation of TS and TVS mass removal efficiencies of untreated and different dosages wheat straw.

5.4.3. Rye Straw

Figure 5.16 presents the values of TS and TVS mass removal efficiencies of untreated and rye straw samples on the 30th day of the BMP test. There is a slight change in the removal efficiencies for both TS and TVS caused by NaOH pre-treatment. The untreated rye straw showed the lowest values of TS and TVS removal by 19 % and 22 %, respectively. The values started to get higher but with a slight change by means of pre-treatment. The highest TS and TVS removal efficiencies were observed in case of 8 % NaOH rye straw by 23.2 % and 27 %, respectively. A total increase of 22.7 % in TVS removal of rye straw was reached by pre-treatment with 8 % NaOH.

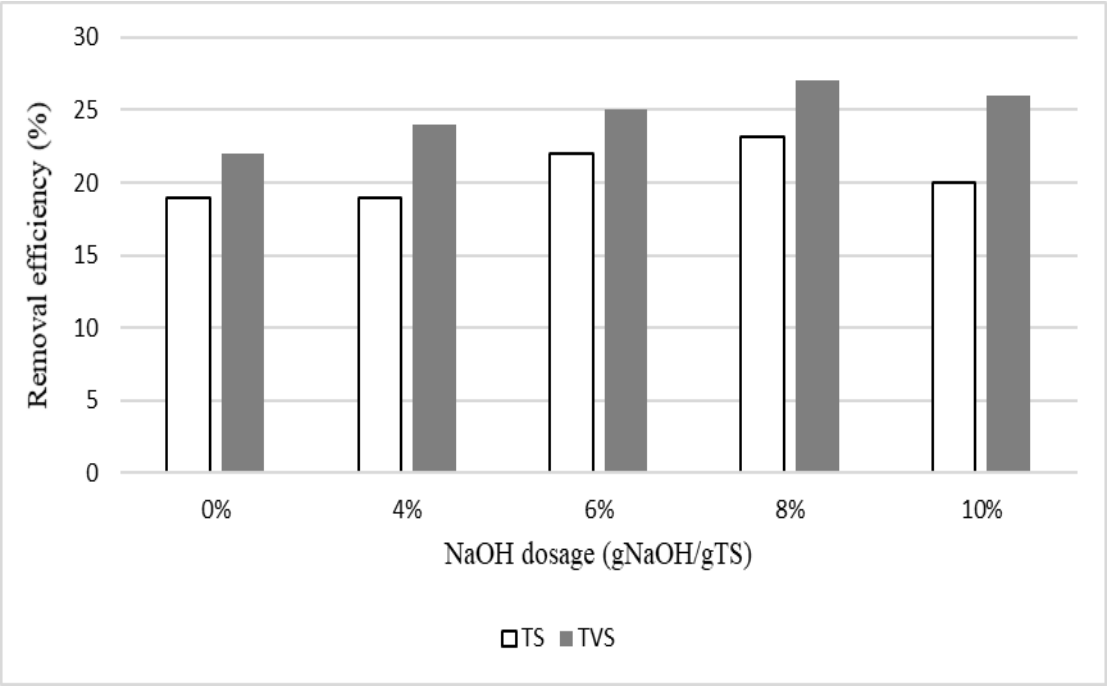

Figure 5.16. Variation of TS and TVS mass removal efficiencies of untreated and different dosages rye straw.

5.5. Effect of Pre-treatment on sCOD Removal in Anaerobic Digesters

Another way of observing the changes in the efficiency of straw biodegradability after NaOH pre-treatment is measuring the soluble chemical oxygen demand (sCOD) for all digesters before and after BMP tests. The high removal of CODs in the digester at the end of the BMP test would be a sign of a better biodegradability performance of substrates, which means a higher methane yield is expected by the digester. Therefore, sCOD test was conducted to all digesters on the 0^{th} and 30^{th} days and the CODs removed were calculated.

5.5.1. Rice Straw

Digesters' sCOD values are presented in Figures 5.17 and 5.18 for rice straw samples with 100 gTS/L and 200 gTS/L total solid concentrations, respectively. In both Figures, results show that the highest CODs values on the 0th day are obtained by 8 % and 10 % NaOH digesters. However, digesters with 200 gTS/L total solid concentrations samples show relatively higher sCOD values on the 0th day than others which are with 100 gTS/L. On the 30th day, all digesters show approximately the same range of sCOD values (250:500) mg/L with slight changes. In Figure 5.17, the highest sCOD removal is obtained by 8 % digesters by a value of 82 %, while the highest sCOD removal in Figure

5.18 is obtained from 8 % digesters by a value of 74 % with a total sCOD removal of 1086 mg/L. The lowest values of sCOD removal are recorded by untreated digesters with total sCOD removal mass values of 543 and 568 mg/L, respectively.

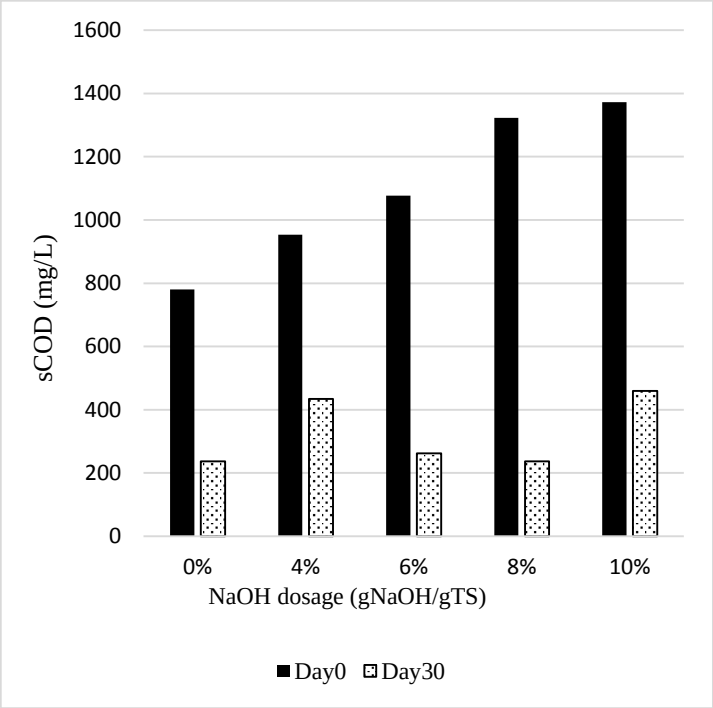

Figure 5.17. sCOD values of digesters containing rice straw with different NaOH dosages and total solid concentrations of 100 gTS/L.

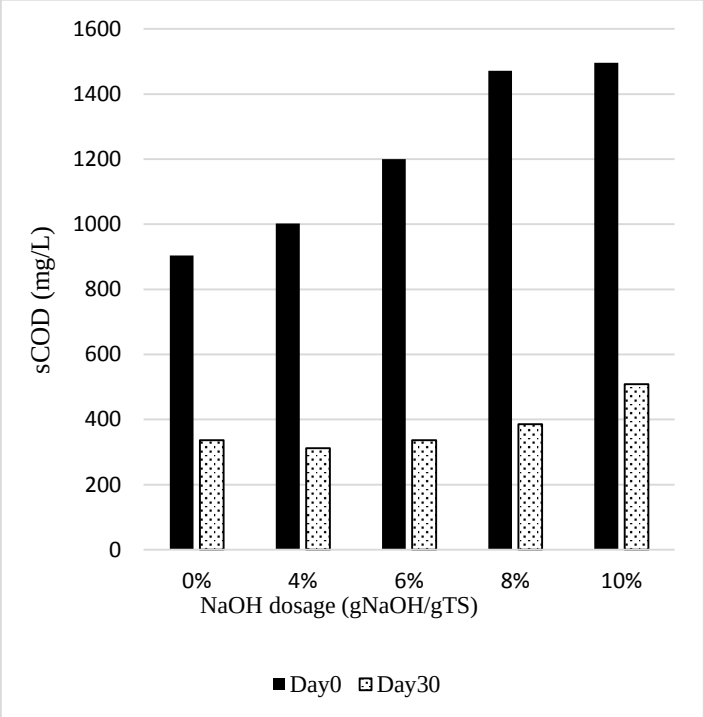

Figure 5.18. sCOD values of digesters containing rice straw pre-treated with different NaOH dosages and total solid concentrations of 200 gTS/L.

5.5.2. Wheat Straw

Figures 5.19 and 5.20 present the sCOD values of wheat straw digesters with 100 gTS/L and 200 gTS/L total solid concentrations, respectively. Similar to rice straw, the highest sCOD values on the 0th day are obtained by 8 % and 10 % digesters. A slight increase in sCOD values is observed by digesters with 200 gTS/L (Figure 5.19). In Figure 5.20, the highest sCOD removal is obtained from 10 % digesters by a value of 76 %. However, a value of 78 % sCOD removal was observed by 10 % NaOH digesters in Figure 5.19 with a total sCOD removal of 976 mg/L. The lowest sCOD removal values are obtained by untreated digesters in Figures 5.19 and 5.20 with a total sCOD removal mass of 271 and 269 mg/L, respectively.

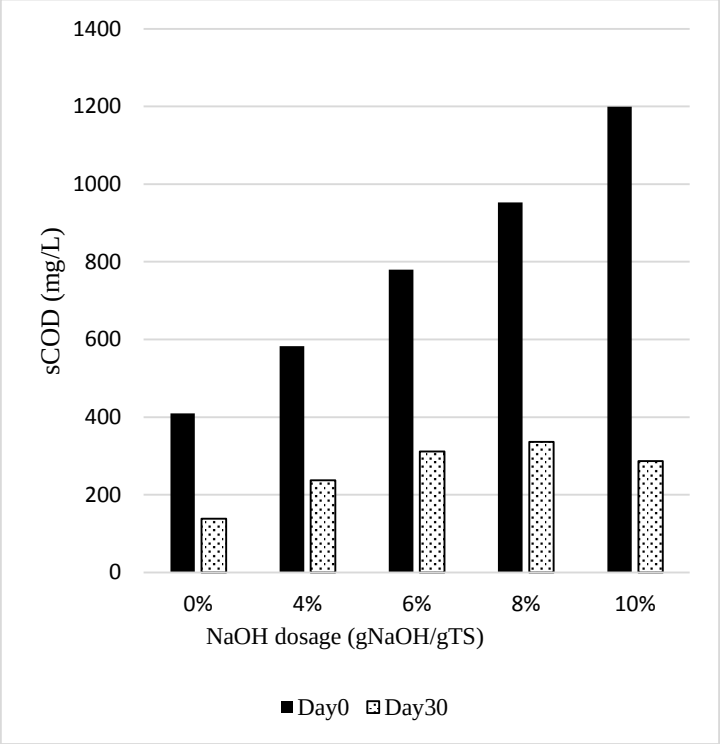

Figure 5.19. sCOD values of digesters containing wheat straw with different NaOH dosages and total solid concentrations of 100 gTS/L.

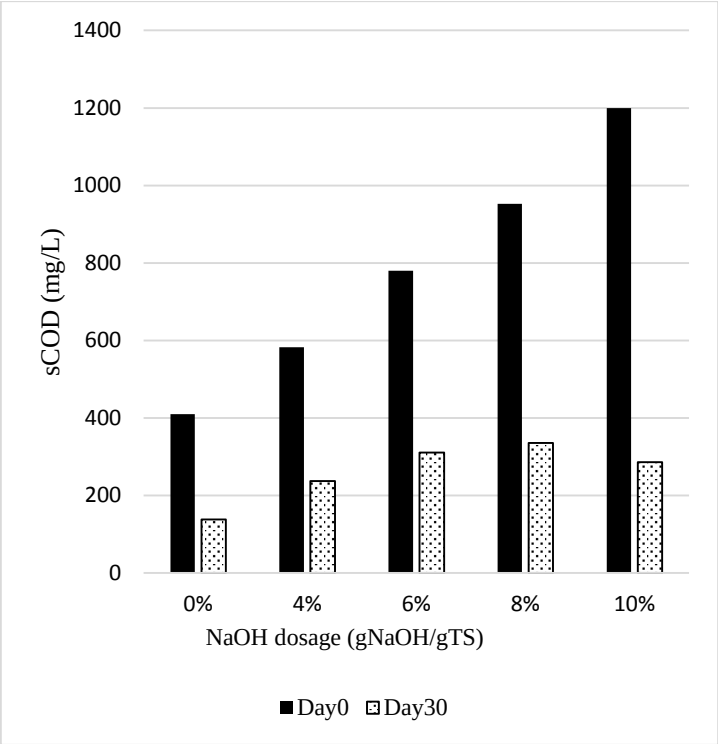

Figure 5.20. sCOD values of digesters containing wheat straw with different NaOH dosages and total solid concentrations of 200 gTS/L.

5.5.3. Rye Straw

The values of sCOD related to digesters of rye straw with 100 gTS/L and 200 gTS/L total solid concentrations are presented in Figures 5.21 and 5.22, respectively. In both Figures, the highest sCOD values on the 0th day are observed by digesters with 8 % and 10 % NaOH dosages. On the 30th day, nearly all the digesters' sCOD values are located in the same range (250:550) with slight changes. Coming to sCOD removal, the highest sCOD removal in Figure 5.21 is observed by 10 % NaOH digesters with a value of 48 %. However, a removal value of 52 % is obtained by 10 % NaOH digesters in Figure 5.22. The lowest sCOD removal values are obtained by untreated digesters in both Figures with total sCOD removal mass of 198 and 222 mg/L, respectively.

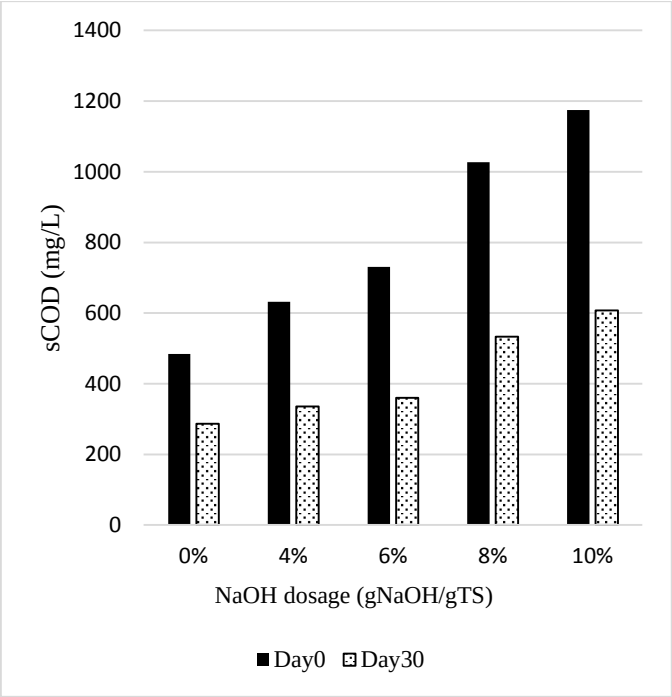

Figure 5.21. sCOD values of digesters containing rye straw pre-treated with different NaOH dosages and total solid concentrations of 100 gTS/L.

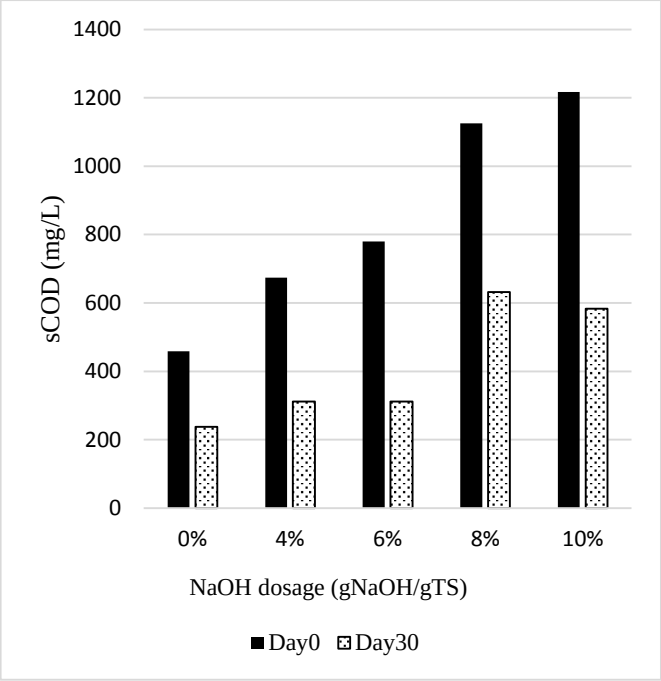

Figure 5.22. sCOD values of digesters containing rye straw pre-treated with different NaOH dosages and total solid concentrations of 200 gTS/L.

5.5.4. Co-digestion Test

Figures 5.23 and 5.24 present the sCOD values of digesters in the co-digestion test with 8 % NaOH dosage beside 100 gTS/L and 200 gTS/L total solid concentrations, respectively. The combinations of (rice/wheat) and (rice/wheat/rye) show the highest values of sCOD on day 0th in both Figures, however, in Figure 5.23 (200 gTS/L) the values are slightly higher. On the 30th day, all sCOD values are similarly close in a range of (300:550) mg/L. In Figure 5.23, the highest sCOD removal value is obtained by digester (rice/wheat/rye) by a value of 67 % with a total removal mass of 1011 mg/L. However, in Figure 5.24, The highest value is observed by (rice/wheat) digesters by a value of 70 % with a total sCOD removed mass of 1061 mg/L.

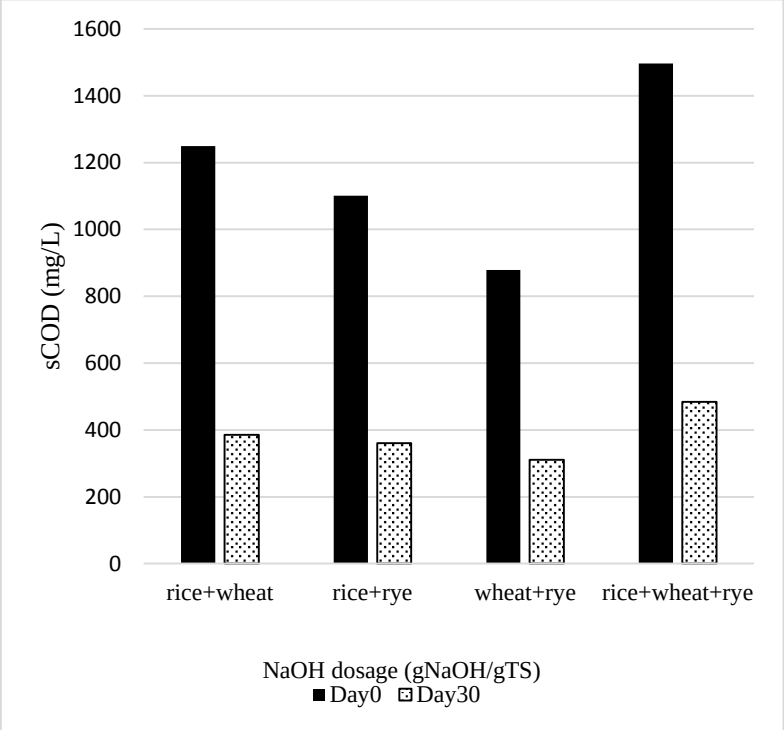

Figure 5.23. sCOD values of digesters of co-digestion test pre-treated with 8 % NaOH dosage and total solid concentrations of 100 gTS/L.

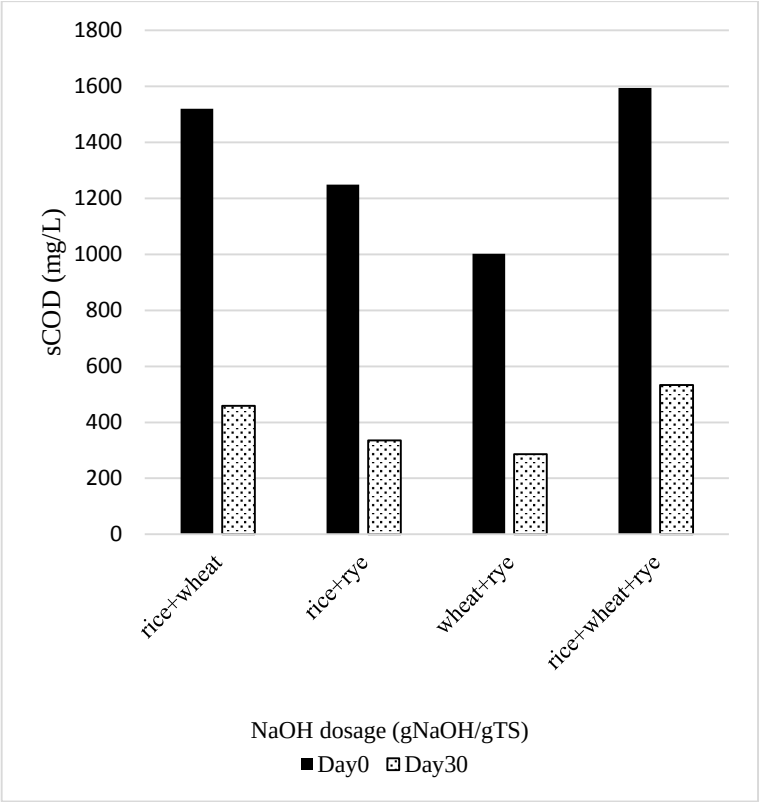

Figure 5.24. sCOD values of digesters of co-digestion test pre-treated with 8 % NaOH dosages and total solid concentrations of 200 gTS/L.

5.6. VFA Production/Removal During the Co-digestion Test

VFA concentration is one of the most important parameters that can affect methane production during anaerobic digestion. Accumulation of VFAs can lead to inhibition which causes a drop in the biological activity within the system. Wang et al. (2009) observed that acetic acid, propionic acid, and butyric acid at concentrations of 1600, 300, and 1800 mg/L, respectively, led to the maximum accumulative methane yield. However, when the propionic acid concentration was increased to 900 mg/L, significant inhibition appeared and the bacteria concentration decreased. That's why VFA concentration analysis is considered in this study.

During the co-digestion test, samples were taken from each digester on the 0^{th}, 3^{rd}, 5^{th}, and 30^{th} days and total VFA concentration was measured and calculated as total acetic acid (mg/L). Acetic, propionic, and iso-butyric acids were the common volatile fatty acids produced in the digesters along with small amounts of butyric, isovaleric, and valeric acids. Figures 5.25 and 5.26 present the total acetic acid produced from each set of substrates which were with 8 % NaOH dosage and total solid concentrations of 100 and 200 gTS/L, respectively.

We can observe that the acetic acid concentrations of digesters with 200 gTS/L (Figure 5.26) are relatively higher than those with 100 gTS/L (Figure 5.25). The highest acetic acid concentration was observed on the 0^{th} day for (wheat/rye) and (rice/rye) in both Figures. However, the highest acetic production for (rice/wheat) and (rice/wheat/rye) is observed on the 5th day. On the 0th day, all sets recorded high acetic acid production, but there are significantly higher concentrations recorded by sets with 200 gTS/L (Figure 5.26).

During the co-digestion test, an increase in VFA production was regarded by (rice+wheat) and (rice+wheat+rye) sets by values of 1930 and 1710 mg/L total acetic acid on the 5th day. The 0th day as well has witnessed an increase in VFA production for almost all sets with 200 gTS/L (solid to liquid) by more than 200 %. On the 30th day, the lowest acetic acid concentrations are observed for all sets. The highest acetic acid removal is observed by (rice/wheat) and (rice/wheat/rye) sets with a value of 93 % and 92 %, respectively (Figure 5.26).

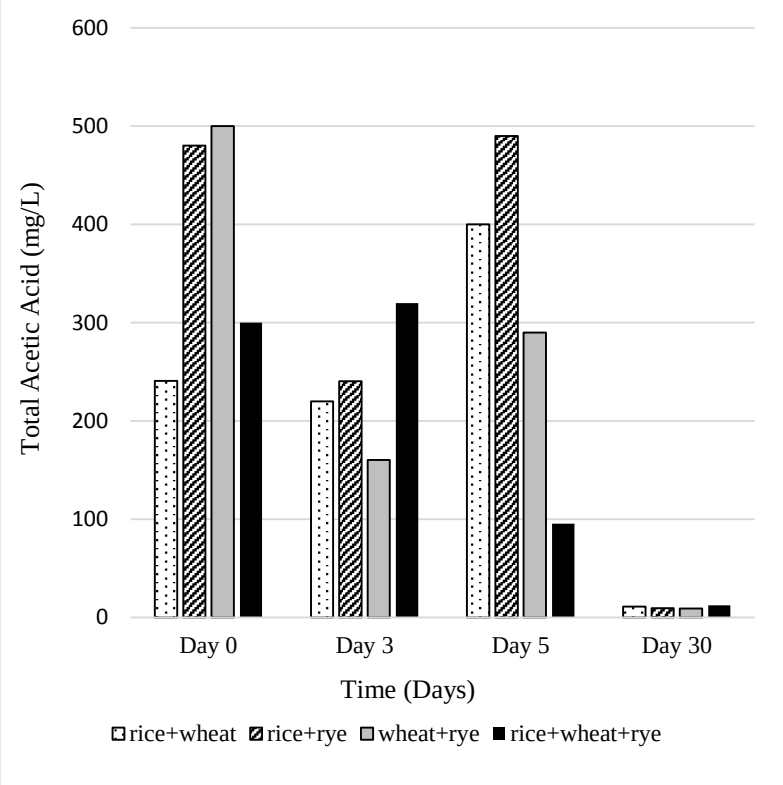

Figure 5.25. VFA concentrations of digesters in the co-digestion test with 8 % NaOH dosage and total solid concentrations of 100 gTS/L.

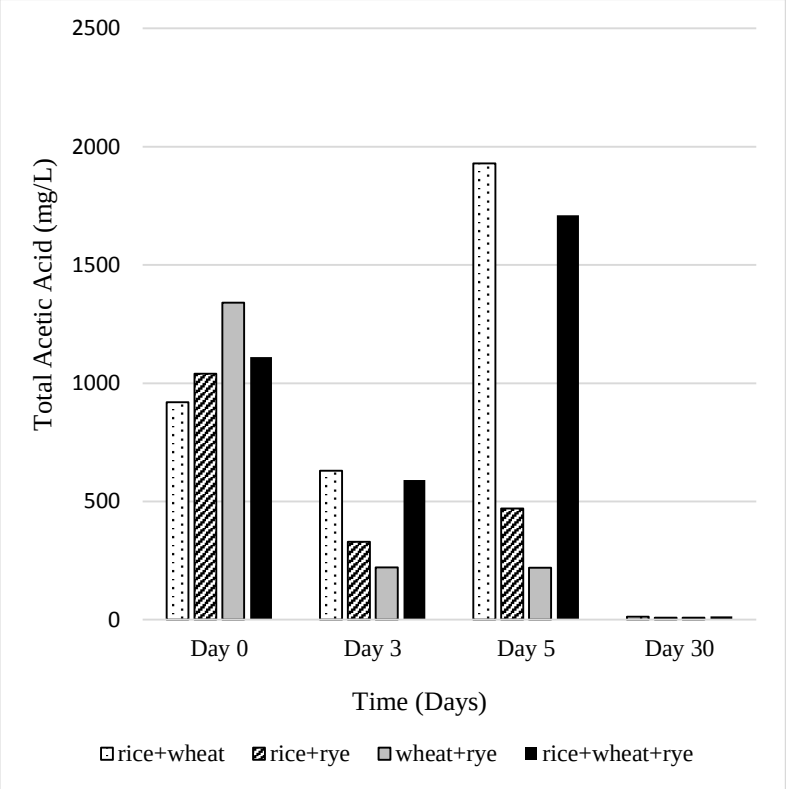

Figure 5.26. VFA concentrations of digesters in the co-digestion test with 8 % NaOH dosage and total solid concentrations of 200 gTS/L.

5.7. Microbial Community Composition and Dynamics

5.7.1. Microbial Community Pattern of Seed Sludge

The bacterial community profile of the seed sludge is shown in Figure 5.27 at the phylum, class, order, and family levels. By checking the korona chart, we can see that the seed sludge is composed mainly of 10 bacterial phyla in which the most abundant phyla are *Proteobacteria* and *Bacteroidetes* accounting for 65 % and 22 %, respectively. Furthermore, 8 % of the bacteria in the seed sludge was dominated by *Firmicutes*. At the class level, *Betaproteobacteria* and *Deltaproteobacteria* are the most dominated classes by percentages of 31 % and 20 % of the bacteria, respectively. Moreover, *Bacteroidia*, *Clostridia*, *Campylobacterales*, and *Saprospirales* account for 11 %, 7 %, 7 % and 6 % of all bacterial classes exist in the seed sludge, respectively.

At the family level, *Syntrophaceae (*18 %) and *Comamonadaceae* (12 %) were the most abundant families in the seed sludge. Besides, many other minor portions of bacterial families existed with small percentages.

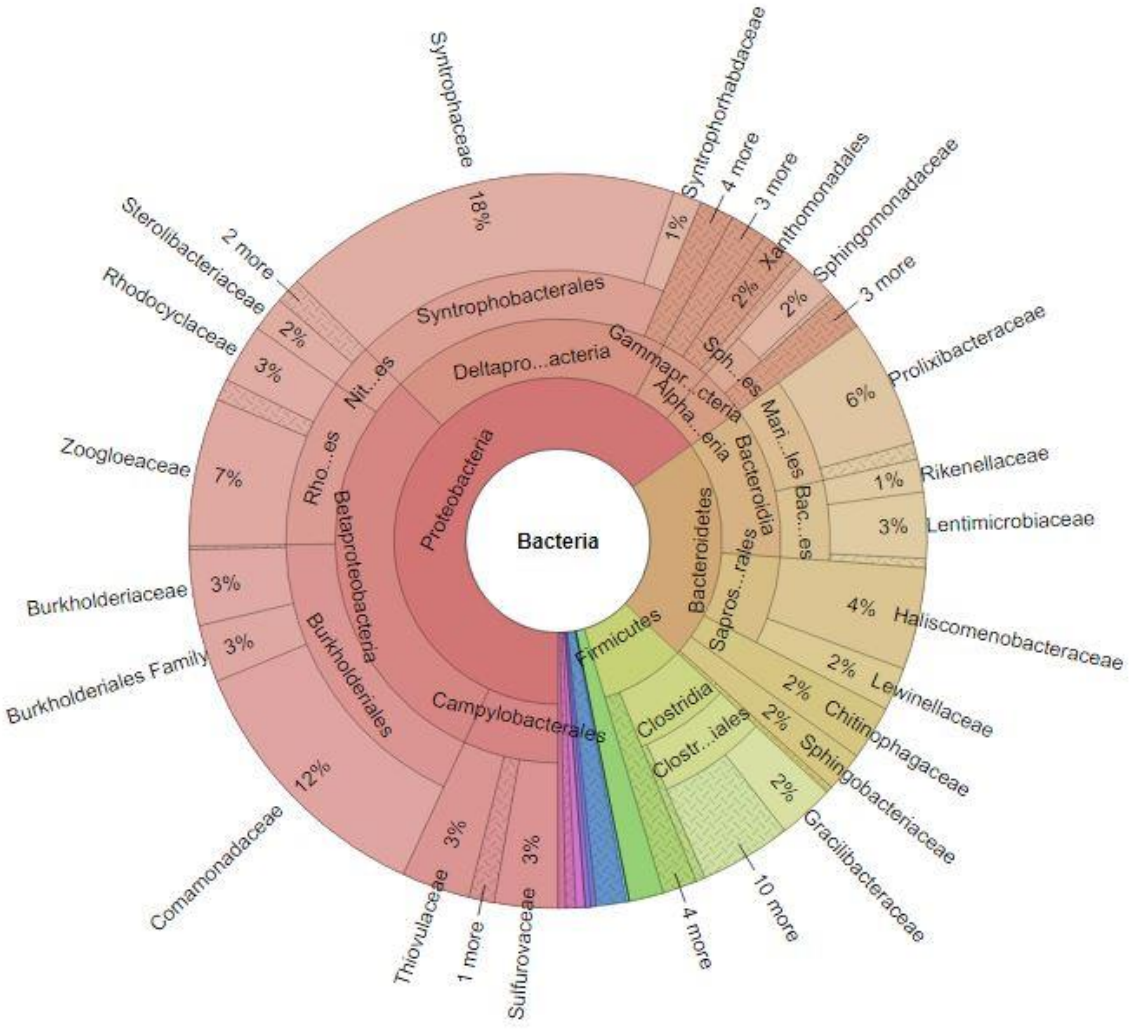

Figure 5.27. The bacterial communities in phylum, class, order, and family levels of the seed sludge.

5.7.2. Microbial Community Pattern of (8 % Rice Day 0) and (8 % Rice Day 30) Samples.

The bacterial community compositions of 8 % rice digester sample on the 0th and 30th days are shown in Figure 5.28. As in the sample taken on the 0th day, the most predominant phylum was *Proteobacteria* accounting for 93 % of all bacteria that existed, along with minor portions of *Bacteroidetes* and *Firmicutes*. While in the sample taken on the 30th day, *Proteobacteria* portion decreased to 73 % and *Bacteroidetes* increased to 17 %.

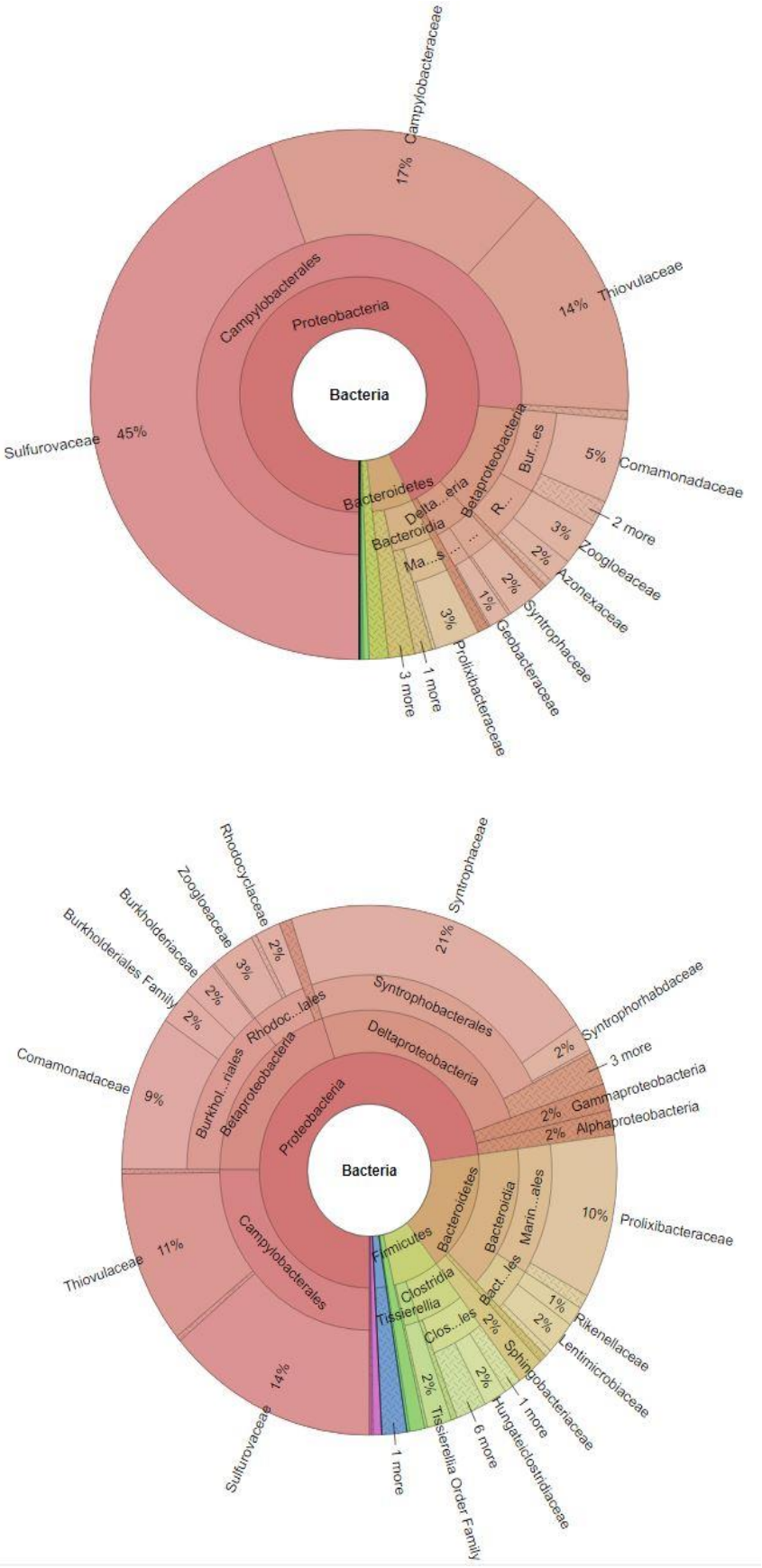

Figure 5.28. The bacterial communities in phylum, class, order, and family levels of 8 % rice digester samples on the 0th and 30th days.

At the class level, *Campylobacterales* was the most abundant bacterial class on the 0th day accounting for 77 %. Besides, some minor portions of *Betaproteobacteria, Deltaproteobacteria*, and *Bacteroidia.* On the 30th day, *Campylobacterales* class decreased to 25 % while *Betaproteobacteria* and *Deltaproteobacteria* increased to 20 % and 25 %, respectively. At the family level, *Sulfurovaceae* was the most dominant bacterial family on the 0th day accounting for 45 %. Besides *Campylobacteraceae* and *Thiovulaceae* existed with percentages of 17 % and 14 %, respectively. while on the 30th day, *Syntrophaceae, Sulfurovaceae,* and *Thiovulaceae* were the major bacterial families accounting for 21 %,14 %, and 11 %. respectively.

5.7.3. Microbial Community Pattern of (0 % Rice Day 30) and (8 % Rice Day 30) Samples.

Figure 5.29. shows the bacterial community composition of 2 different digester samples at the phylum, class, order, and family levels. The first one is taken from 0 % rice (without pre-treatment) digester on the 30th day, while the second one is taken from 8 % rice digester on the 30th day. By checking both korona charts, we can see that the major bacterial phylum in both untreated and samples is *Proteobacteria* accounting for 61 % and 73 %, respectively. *Bacteroidetes* phylum is assigned for 26 % and 17 % in both samples, respectively, along with minor portions of *Firmicutes* and *Chloroflexi.* At the class level, the most abundant bacterial classes in the untreated sample are *Deltaproteobacteria, Bacteroidia, Betaproteobacteria*, and *Campylobacterales* accounting for 29 %, 25 %, 15 %, and 13 %, respectively. While, the major bacterial classes in the sample are *Deltaproteobacteria, Campylobacterales, Betaproteobacteria*, and *Bacteroidia* accounting for 25 %, 25 %, 20 %, and 15 %, respectively. At the family level, the most dominated bacterial families in the untreated rice sample are *Syntrophaceae, Prolixibacteraceae* and accounting for 25 %, 24 %, and 12 %, respectively. On the other hand, *Syntrophaceae, Sulfurovaceae,* and *Thiovulaceae* are the major bacterial families in the sample with percentages of 21 %,14 %, and 11 %. respectively.

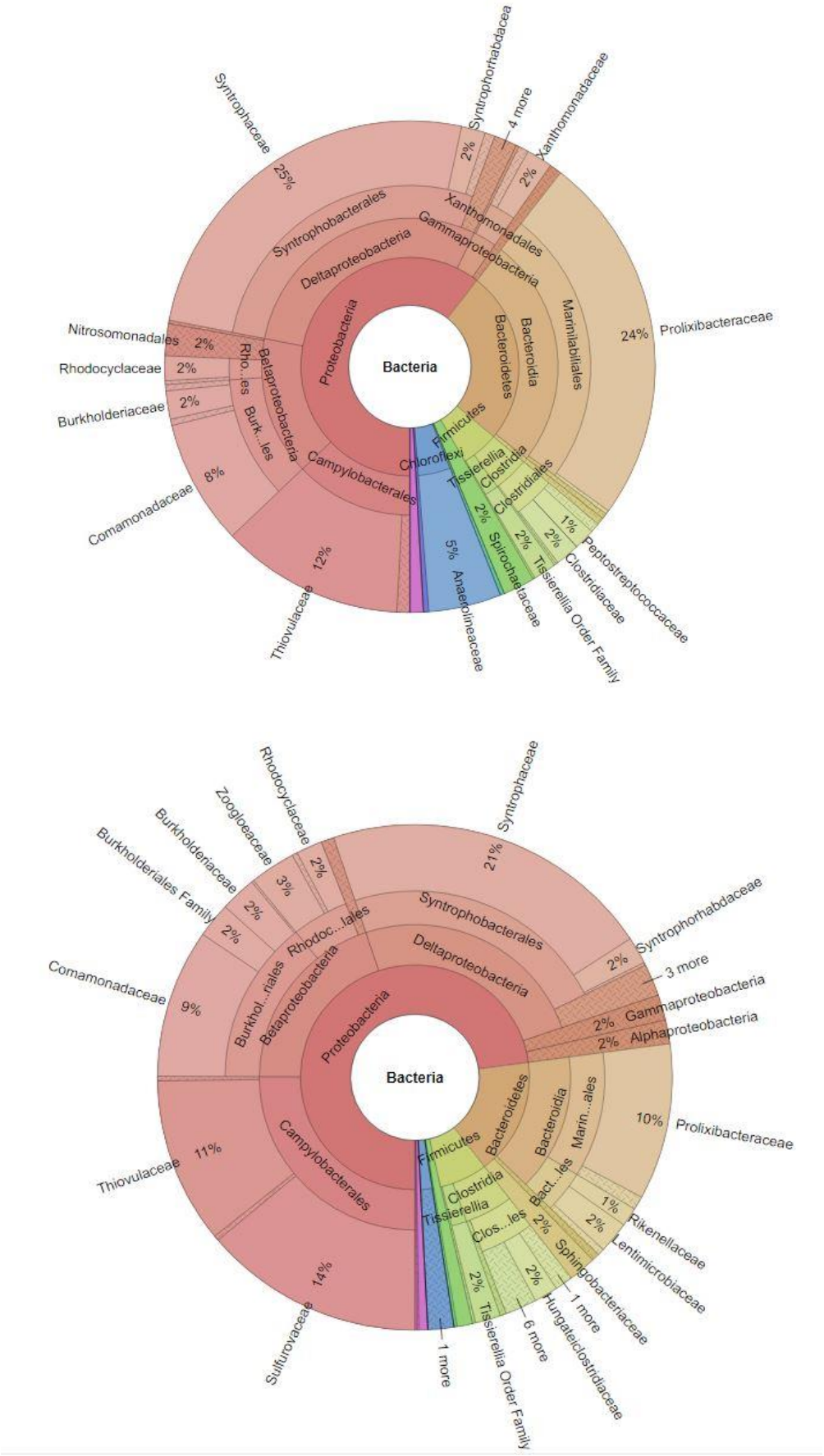

Figure 5.29. The bacterial communities in phylum, class, order, and family levels of 0 % Rice D30 and 8 % Rice D30 digester samples.

5.7.4. Microbial community pattern of 8 % (Rice+Wheat) digester samples on Days 5 and 30.

The bacterial profiles of 8 % (rice+wheat) digester samples on the 5th and 30th days are presented in Figure .30. On the 5th day, we can see that the most dominated bacterial phylum is *Proteobacteria* accounting for 82 %, together with minor portions of *Bacteroidetes* and *Firmicute.* While on the 30th day, *Proteobacteria* portion decreased to 72 % and *Bacteroidetes* increased to 17 %. At the class level, *Campylobacterales* was the most dominated on the 5th day accounting for 42 %. Then *Betaproteobacteria* came with a percentage of 22 %. On the 30th day, *Campylobacterales* decreased to 23 %, however, *Betaproteobacteria* and *Deltaproteobacteria* increased to 27 % and 19 %, respectively. At the family level, *Campylobacterales* (29 %), *Comamonadaceae* (13 %), and *Thiovulaceae* (10 %) were the most abundant families on the 5th day. While on the 30th day, the most dominated bacterial families were *Syntrophaceae* (16 %), *Comamonadaceae* (14 %), *Thiovulaceae* (11 %), and *Campylobacterales* (8 %).

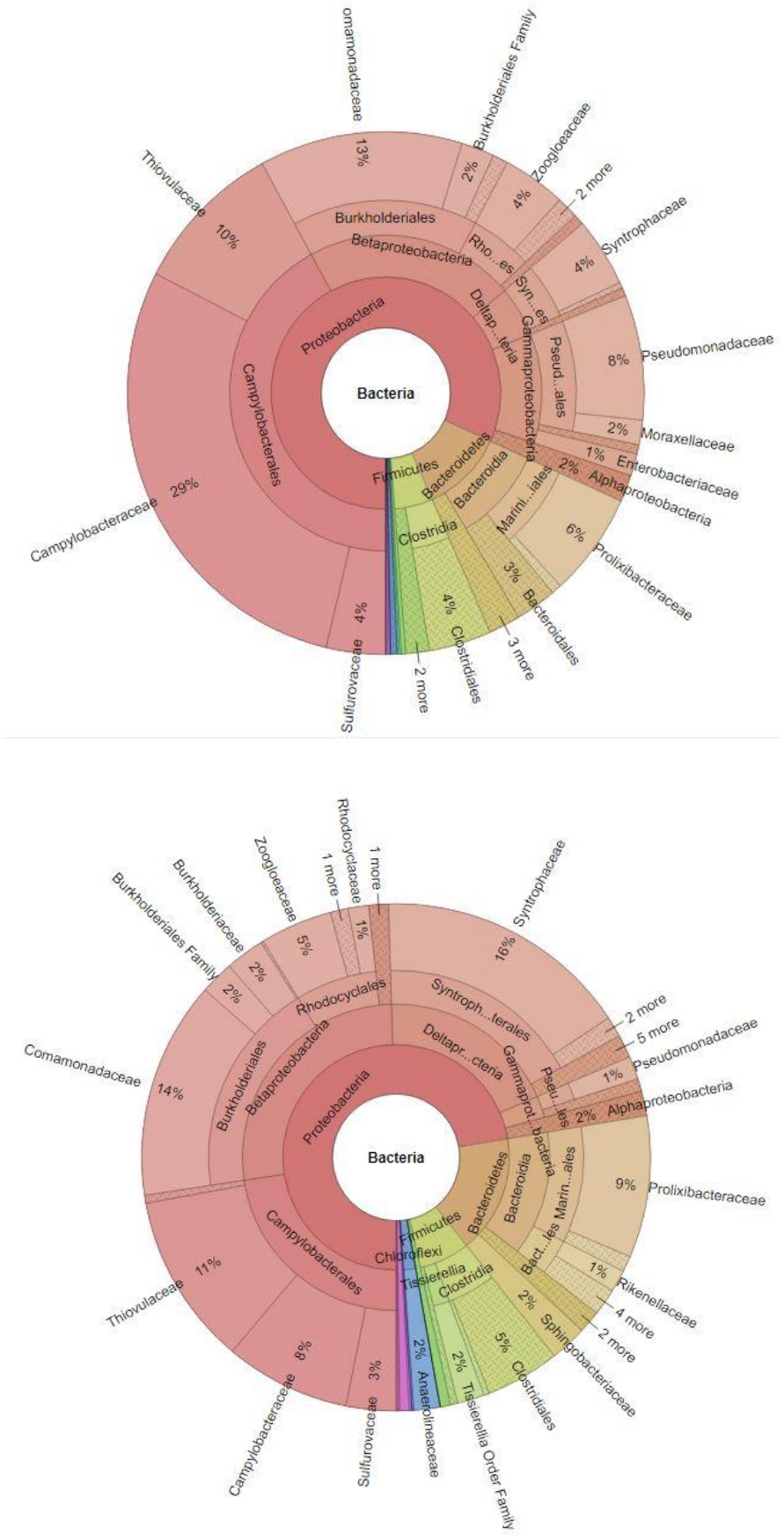

Figure 5.30. The bacterial communities in phylum, class, order, and family levels of 8 % (Rice+Wheat) digester samples on the 5th and 30th days.

5.7.5. Microbial community pattern of 8 % (Rice+Wheat+Rye) samples on Days 5 and 30.

The korona charts in Figure 5.31 present the bacterial profiles of 8 % (rice+wheat+rye) digester samples on the 5th and 30th days. *Proteobacteria* was the most abundant phylum on the 5th day accounting for 78 %. While *Bacteroidetes* assigned for 13 %. On the other hand, *Proteobacteria* decreased to a percentage of 71 % on the 30th day, and *Bacteroidetes* increased to 16 %. At the class level, *Betaproteobacteria* (43 %) was the most dominant bacterial class. Minor portions of *Gammaproteobacteria* (19 %) and *Bacteroidia* (10 %) existed as well. While on the 30th day, the major bacterial classes in the samples were *Deltaproteobacteria* (29 %), *Campylobacterales* (20 %), *Betaproteobacteria* (19 %), and *Bacteroidia* (14 %). At the family level, *Comamonadaceae* was the most abundant bacterial family on the 5th day accounting for 22 %. The second most abundant was *Pseudomonadaceae* and *Prolixibacteraceae* assigned for 11 % and 9 %, respectively. On the 30th day, the most predominant bacterial family was *Syntrophaceae* with a percentage of 24 %. *Thiovulaceae* came in second place with 16 % of all bacterial families existing.

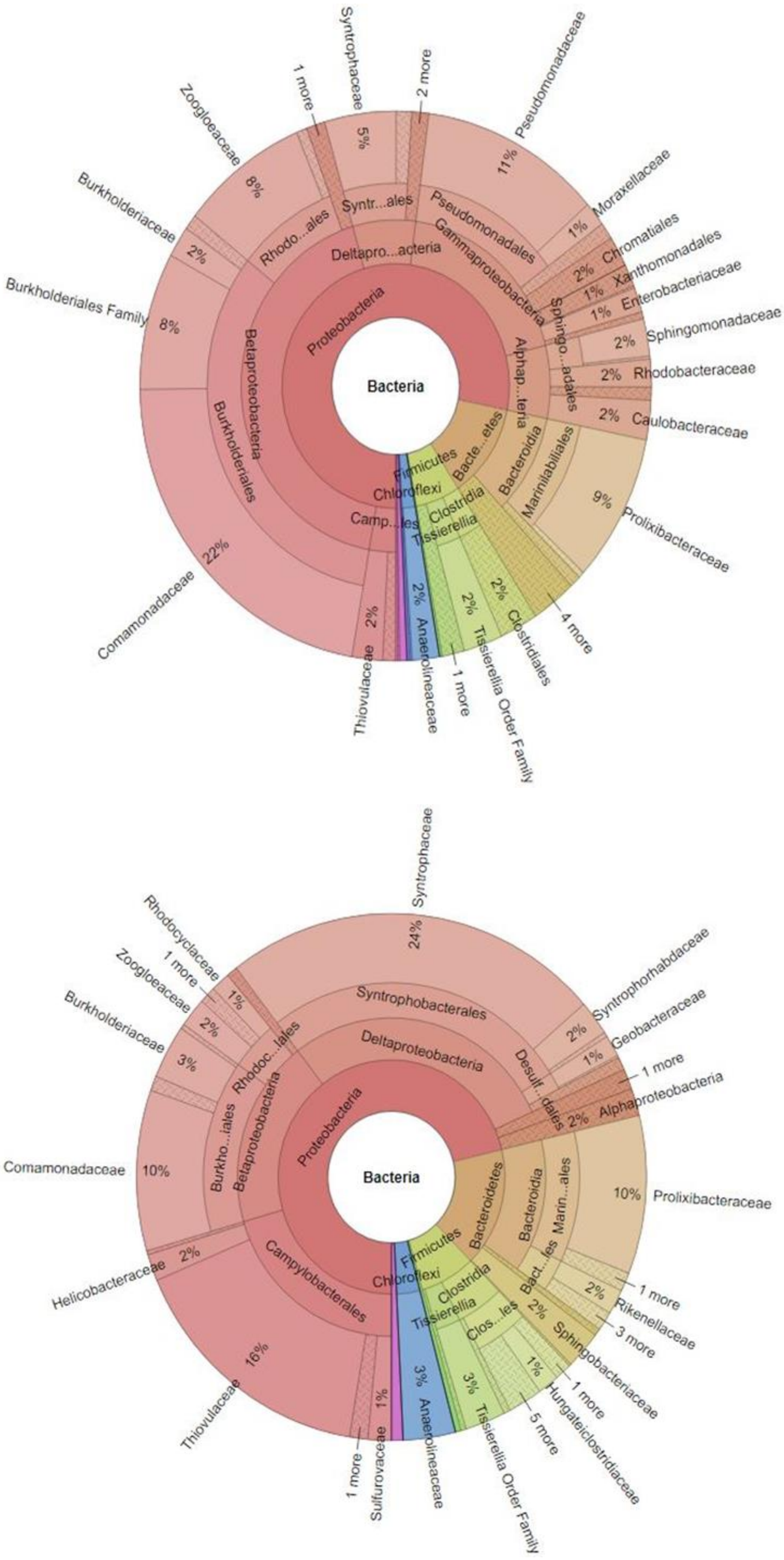

Figure 5.31. The bacterial communities in phylum, class, order, and family levels of 8 % (rice+wheat+rye) digester samples on the 5th and 30th days.

5.7.6. Methanogenic archaeal communities at the genus level

The methanogenic archaeal community of the seed sludge was dominated mainly by the genus *Methanothermobacter* and *Methanoculleus* accounting for approximately 70 % of all the archaeal communities that exist in the inoculum. Unlike the digester samples, the diversity in the methanogenic community was less as expected in the seed sludge.

the similarity in the methanogenic communities of the AMPTS digesters was noticed with more diversity than the seed sludge. Combinations of *Methanothermobacter*, *Methanosaeta*, *Methanoculleus*, and *Methanosarcina* were the most dominant methanogens in the AMPTS digesters by the 5th and 30th days. We could also notice that the digester samples had a more diverse methanogenic community than the untreated ones, indicating that the NaOH pre-treatment process has supported the enrichment of the methanogenic archaeal communities in the digesters during the anaerobic degradation process

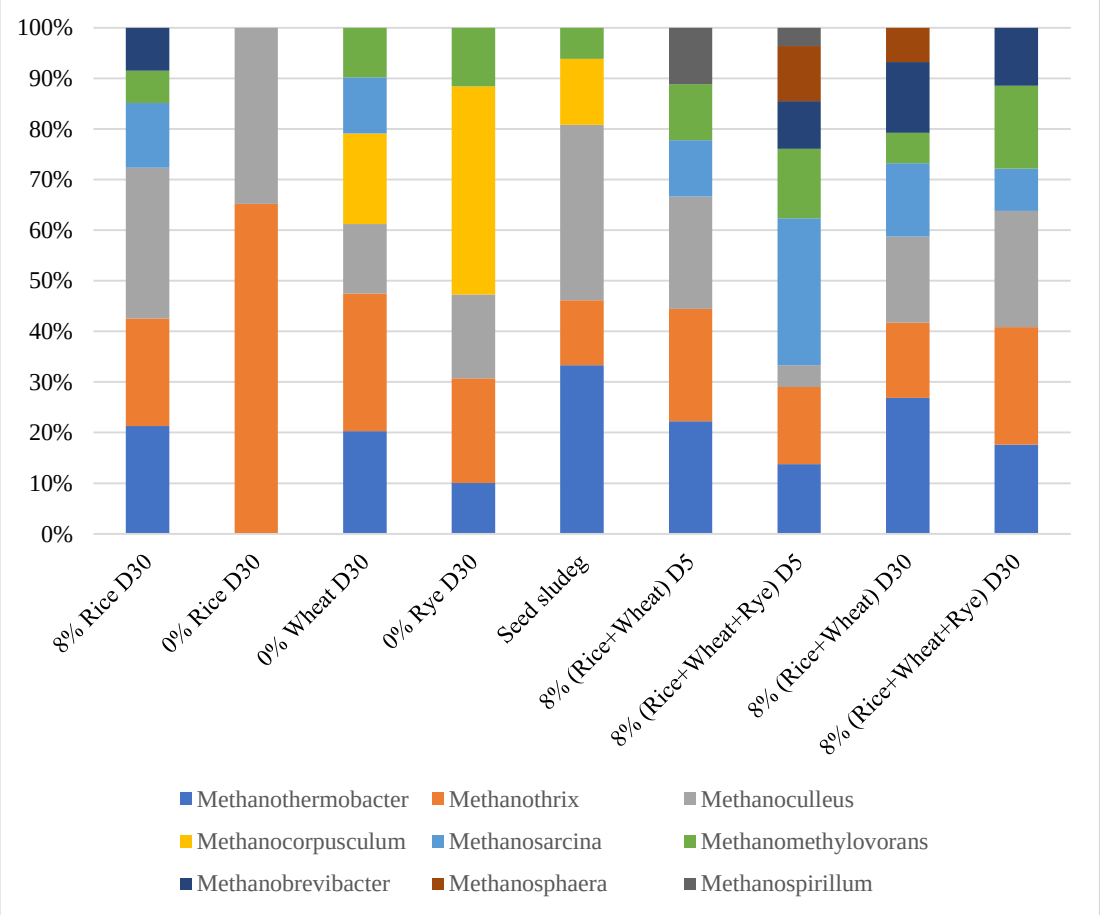

Figure 5.32. Methanogenic archaeal community composition of the seed sludge and collected digester samples.

6. CONCLUSIONS AND RECOMMENDATIONS

The main focus of this study was to find the best conditions of alkali pre-treatment of cereal straws using sodium hydroxide to ensure the highest degradation performance and methane production, along with feasibility factors through the anaerobic digestion process. Several pre-treatment conditions have been investigated and analyzed using three of the most common agricultural residues worldwide; wheat, rice, and rye straws.

The major contributions of this study are summarized below;

- Pre-treatment with 8 % (w/w) gNaOH/gTS dose has shown the best results in terms of lignin and hemicellulose decomposition along with methane production.
- Total solids concentration of 200 g TS/L, used in pre-treatment, has shown higher performance during the anaerobic digestion process.
- Approximately 90 % of the total methane production was obtained in the first 10 days of the BMP tests of the pre-treated samples.

The following outcomes were withdrawn from the mono and co-digestion studies;
During NaOH alkali pre-treatment of rice straw;

- The decomposition of hemicellulose and lignin by 81 % and 62 %, respectively.
- COD solubilization increased 3 times higher than the value of untreated straw.
- Pre-treated rice straw increased the methane yield by 55 % compared to untreated rice straw.
- Volatile solids' conversion into biogas by rice straw regarded an increase of 42.5 % more than untreated straw.
- sCOD removed by straw during BMP test was found 2 times higher than untreated straw.

During NaOH alkali pre-treatment of wheat straw;

- Hemicellulose and lignin decomposed by 86 % and 69 %, respectively.
- COD solubilization was found 3 times higher than the value of untreated wheat straw.
- Pre-treatment of wheat straw resulted in an increase in methane yield by 40 % compared to untreated straw.
- 30 % increase in volatile solids' removal during the BMP test was observed by wheat straw substrates.

- sCOD removed during anaerobic digestion has increased 2 times higher than untreated straw.

During NaOH alkali pre-treatment of rye straw;

- The decomposition of hemicellulose and lignin contents by 80 % and 74 %, respectively.
- Besides, a more than 3 times increase in COD solubilization was regarded by rye straw.
- Compared to the untreated rye straw, 46 % more methane yield was obtained with 8 % NaOH-treated rye straw.
- A total increase of 22.7 % in volatile solids' conversion of rye straw was reached by pre-treatment with 8 % NaOH.
- During anaerobic digestion, 2 times more sCOD was removed in case of 8 % NaOH-treated rye straw more than the untreated one

During Co-digestion test of NaOH alkali straws;

- Pre-treatment with 8 % gNaOH/gTS could increase the methane yield in a range of 30-50 % compared to untreated straws.
- Rice/wheat and rice+wheat+rye sets recorded the highest methane yield with values of 329 mL CH_4/g VS and 320 mL CH_4/g VS, respectively.
- A total increase of 35 % in volatile solids' conversion of rye straw was reached by pre-treatment with 8 % NaOH.

Semi-continuous lab-scale and pilot-scale digestion studies are highly recommended to be performed to confirm the efficacy and there for the potential application of BMP tests with NaOH alkali pre-treatment.

More detailed microbial analyses for the comparison of untreated and pre-treated digesters are highly recommended as well for future studies so that we can get a clearer idea of the effect of pre-treatment on the microbial communities and their activities during the anaerobic digestion process.

Other lignocellulosic substrates like barley straw, corn stover, and triticale crop residues would make a worthwhile contribution for future studies to Figure out the effect of NaOH alkali pre-treatment on their methane production.

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