ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

ANAEROBIC DIGESTION OF LIGNOCELLULOSIC WASTE USING ALKALI PRETREATMENT METHOD INTERMS OF PERFORMANCE, MICROBIAL COMMUNITY, AND COST ANALYSIS

M.Sc. THESIS

Canberk KAZANCI

Department of Environmental Engineering

Environmental Biotechnology Programme

SEPTEMBER 2024

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

ANAEROBIC DIGESTION OF LIGNOCELLULOSIC WASTE USING ALKALI PRETREATMENT METHOD INTERMS OF PERFORMANCE, MICROBIAL COMMUNITY, AND COST ANALYSIS

M.Sc. THESIS

Canberk KAZANCI
(501211803)

Department of Environmental Engineering

Environmental Biotechnology Programme

Thesis Advisor: Prof. Dr. Orhan İNCE

SEPTEMBER 2024

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

LİGNOSELÜLOZİK ATIKLARIN ALKALİ ÖN ARITIM YÖNTEMİ İLE ANAEROBİK ARITIMI, PERFORMANS, MİKROBİYAL TOPLULUK VE MALİYET ANALİZİ

YÜKSEK LİSANS TEZİ

Canberk KAZANCI
(501211803)

Çevre Mühendisliği Anabilim Dalı

Çevre Biyoteknolojisi Programı

Tez Danışmanı: Prof. Dr. Orhan İNCE

EYLÜL 2024

Canberk KAZANCI, a M.Sc. student of İTU Graduate School student ID 501211803, successfully defended the thesis/dissertation entitled "ANAEROBIC DIGESTION OF LIGNOCELLULOSIC WASTE USING ALKALI PRETREATMENT METHOD INTERMS OF PERFORMANCE, MICROBIAL COMMUNITY, AND COST ANALYSIS", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Orhan İNCE** Istanbul Technical University	.....
Jury Members :	**Prof. Dr. Süleyman ÖVEZ** Istanbul Technical University	.....
	Prof. Dr. Bülent MERTOĞLU Marmara University	.....

Date of Submission : 24 May 2024
Date of Defense : 26 September 2024

FOREWORD

I would like to express my gratitude and huge respect to my esteemed thesis advisor, Prof. Dr. Orhan İNCE, who gave me both academic and life discipline with her mentorship, supported me in every times and kept my motivation and interest alive.

I would for everything to Dear Prof. Dr. Bahar İNCE, who shed light on my study with her valuable knowledge and experience.

I would like to thank and appreciate Ömer UZUN, who helped me in every way possible with the problems I encountered, both laboratory experiments and methods of the thesis and during my studies.

I want to express my deepest gratitude to my girlfriend, Xinyue Liao. Throughout this journey, she has been by my side, offering constant encouragement, understanding, and love. Her belief in me, especially during the most challenging moments, has been a source of strength that I will forever cherish. This work would not have been possible without her, and I am endlessly grateful for her presence in my life. This thesis is as much hers as it is mine.

I am deeply thankful to my dear friend İrem GÜVEN for his kind support.

Finally, I would like to thank my dear mother, father and grandmother, who encouraged me to continue my graduate education and supported me throughout my graduate studies. This study was financially supported by the Istanbul Technical University Research Projects Coordination Department.

May 2024

Canberk KAZANCI
(Environmental Engineer)

TABLE OF CONTENTS

Page

ABBREVIATIONS

AD	**:** Anaerobic Digestion
BMP	**:** Biological Methane Potential
COD	**:** Chemical Oxygen Demand
FISH	**:** Fluorescence in situ Hybridization
LHW	**:** LIQUID HOT WATER
Q-PCR	**:** Realtime Polymerase Chain Recation
TKN	**:** Total Kjeldahl Nitrogen
TS	**:** Total Solids
VFA	**:** Volatile Fatty Acid
VS	**:** Volatile Solids

LIST OF TABLES

Page

LIST OF FIGURES

Page

ANAEROBIC DIGESTION OF LIGNOCELLULOSIC WASTE USING ALKALI PRETREATMENT METHOD INTERMS OF PERFORMANCE, MICROBIAL COMMUNITY AND COST ANALYSIS

SUMMARY

Agricultural residues with lignocellulosic structures play a crucial role among renewable bioenergy sources and are abundantly available in nature. Anaerobic digestion (AD) is considered an ideal technology for agricultural waste stabilization/treatment and the production of renewable energy carriers. However, the overall economic value of the produced methane remains low, especially when compared to the potential value of waste streams. In this project, the aim is to facilitate the transition to anaerobic fermentation using agricultural waste, specifically sunflower stalks and heads with lignocellulosic structures, to increase the production of volatile fatty acids (VFA). An alkali pretreatment method will be employed as a chemical pretreatment process to investigate the yield from the selected agricultural waste and assess its impact on acidification efficiency. The purpose of using a pretreatment process is to make lignocellulosic material which is difficult to biologically degrade, more easily digestible thereby enhancing process efficiency. Wastes subjected to pretreatment with Potassium Hydroxide (KOH) at various dosages will be thoroughly examined for VFA yield. During the research process, the taxonomic classification of the microbial community active throughout the process will be conducted using the third-generation sequencing technology provided by Oxford Nanopore MinION, targeting the 16S and 18S rRNA gene regions. Finally, a cost analysis will be developed based on the obtained data. This analysis aims to contribute significantly to research in this field by providing practical guidelines to enhance the effectiveness of anaerobic treatment systems and transform organic waste into a sustainable energy source.

LİGNOSELÜLOZİK ATIKLARIN ALKALİ ÖN ARITIM YÖNTEMİ İLE ANAEROBİK ARITIMI, PERFORMANS, MİKROBİYAL TOPLULUK VE MALİYET ANALİZİ

ÖZET

Lignoselülozik yapıya sahip tarımsal atıklar, dünya genelinde sürdürülebilir enerji üretimi ve çevresel yönetim açısından oldukça önemli bir kaynak olarak kabul edilmektedir. Bu atıklar, özellikle tarımsal üretim süreçlerinden geriye kalan organik materyalleri temsil eder ve içerdiği lignin, selüloz ve hemiselüloz gibi polimerler nedeniyle biyolojik olarak zor bozunurlar. Bu yapısal zorluk, lignoselülozik atıkların geleneksel biyokimyasal süreçlerle enerjiye veya değerli yan ürünlere dönüştürülmesini güçleştirir. Ancak, modern biyoteknolojik yöntemler, bu atıkların daha verimli bir şekilde işlenebilmesini mümkün kılmaktadır. Lignoselülozik atıkların uygun ön arıtım süreçlerinden geçirilmesiyle, biyokimyasal olarak daha kolay parçalanabilmesi sağlanarak, biyolojik dönüşüm süreçlerinde daha yüksek verimlilikler elde edilebilmektedir. Bu çerçevede, lignoselülozik biyokütlenin ön arıtım süreçleriyle işlenmesi, biyokimyasal ürünlerin elde edilmesi için kritik bir adımdır.

Bu tez çalışmasında, lignoselülozik tarımsal atıkların alkali ön arıtım yöntemi kullanılarak anaerobik sindirimi detaylı bir şekilde ele alınmıştır. Çalışmanın temel hedefi, bu atıkların biyolojik olarak daha kolay sindirilebilir hale getirilmesi ve anaerobik sindirim sürecinde uçucu yağ asitleri (VFA) üretiminin artırılmasıdır. VFA'lar, biyokimya ve biyoyakıt endüstrilerinde kullanılan değerli yan ürünlerdir ve bu nedenle, biyogaz üretimi yerine VFA üretimi çalışmanın ana odağını oluşturmuştur. VFA üretiminin optimize edilmesi, biyoyakıt üretiminde önemli bir hammadde kaynağı sağlarken, aynı zamanda çeşitli biyo-kimyasalların üretimi için de önemli fırsatlar sunmaktadır. Lignoselülozik biyokütlenin bu amaçla işlenmesi, çevresel sürdürülebilirlik ve ekonomik fayda sağlama açısından önemli bir potansiyel taşımaktadır.

Ayçiçeği sapları ve başları gibi lignoselülozik atıklar, dünya genelinde geniş ekim alanlarına sahip olan ayçiçeği bitkisinin tarımsal üretim süreçlerinden geriye kalan önemli bir biyokütle kaynağıdır. Bu atıklar, tarımsal faaliyetlerin bir yan ürünü olarak ortaya çıkmakta ve çoğunlukla tarlalarda yakılarak veya çürümeye bırakılarak bertaraf edilmektedir. Ancak, ayçiçeği sapları ve başları yüksek miktarda organik madde içermekte olup, uygun işlemlerle değerlendirildiğinde biyokimyasal süreçlerde değerli ürünlere dönüştürülebilecek potansiyele sahiptir. Bu çalışmada, ayçiçeği sapları ve başları kullanılarak lignoselülozik biyokütlenin alkali ön arıtım yöntemi ile biyolojik olarak daha kolay parçalanabilmesi sağlanmış ve anaerobik sindirim sürecinde VFA üretimi üzerine odaklanılmıştır. Alkali ön arıtım sürecinde potasyum hidroksit (KOH) kullanılmış ve çeşitli dozajlarda (%0, %6, %8, %10) uygulanan bu ön arıtım sonucunda elde edilen VFA verimi kapsamlı bir şekilde analiz edilmiştir.

Deneysel süreçte kullanılan alkali ön arıtım yöntemi, biyokütlenin yapısındaki dirençli bağları kırarak mikroorganizmaların bu biyokütleyi daha kolay parçalamasını

sağlamıştır. Özellikle lignin gibi biyolojik olarak zor bozunabilen bileşenlerin yapısının bozulmasıyla, biyokütlenin biyokimyasal süreçlerde daha hızlı ve verimli bir şekilde işlenmesi mümkün hale gelmiştir. Bu durum, VFA üretimi açısından son derece kritik bir rol oynamaktadır. Elde edilen sonuçlar, %8 KOH dozajının VFA üretiminde en yüksek verimi sağladığını ve lignoselülozik atıkların biyolojik olarak daha kolay parçalanmasını sağladığını göstermiştir. Bu bulgular, alkali ön arıtımın lignoselülozik biyokütlenin işlenmesinde ne kadar etkili bir yöntem olduğunu ortaya koymaktadır.

Anaerobik sindirim sürecinde, mikroorganizmalar biyokütleyi oksijensiz ortamda parçalayarak enerji ve çeşitli yan ürünler elde eder. Bu süreçte aktif olan mikrobiyal topluluklar, biyokimyasal süreçlerin gerçekleşmesinde ve son ürünlerin oluşmasında kritik bir rol oynar. Çalışmada, mikroorganizmaların süreç üzerindeki etkilerini anlamak amacıyla, alkali ön arıtım sonrası aktif olan mikrobiyal toplulukların taksonomik yapısı incelenmiştir. Oxford Nanopore MinION dizileme teknolojisi kullanılarak yapılan analizler, mikrobiyal toplulukların süreç boyunca nasıl değiştiğini ve farklı KOH dozlarının mikrobiyal çeşitliliğe etkisini ortaya koymayı amaçlamıştır. 16S ve 18S rRNA gen bölgeleri hedef alınarak yapılan dizileme çalışmaları sonucunda, metanojenik arkeaların baskılandığı ve VFA üreten bakterilerin baskın hale geldiği gözlemlenmiştir. Bu durum, metan üretimi yerine VFA üretimini ön plana çıkaran asit üretici bakterilerin süreç sonunda baskın hale gelmesiyle elde edilmiştir. Alkali ön arıtımın mikrobiyal topluluklar üzerindeki bu etkisi, lignoselülozik biyokütlelerin anaerobik sindirimi sürecinde önemli bir avantaj sağlamaktadır.

Geleneksel biyogaz üretim süreçlerinde, metanojenik arkealar baskın türler arasında yer alır ve bu mikroorganizmalar metan gazının üretiminden sorumludur. Ancak, bu çalışmada metan üretimi yerine VFA üretimi hedeflenmiştir. Bu nedenle metanojenik arkeaların baskılanması ve VFA üreten bakterilerin baskın hale gelmesi sağlanmıştır. Metanojenik arkeaların baskılanmasıyla birlikte, VFA üretimi sürecin ana odağı haline gelmiş ve bu mikroorganizmaların faaliyetleri süreç sonunda asidik ortamın oluşmasına ve VFA üretiminin artmasına olanak tanımıştır. Özellikle %8 KOH dozajında, mikrobiyal toplulukların VFA üretimini destekleyecek şekilde evrildiği ve metanojenik türlerin süreç boyunca baskılandığı gözlemlenmiştir.

Mikrobiyal toplulukların dinamiklerinin yanı sıra, süreç boyunca çeşitli performans parametreleri de değerlendirilmiştir. Çözünebilir kimyasal oksijen ihtiyacı (sCOD), toplam katı madde (TS) ve uçucu katı madde (VS) giderimi gibi parametreler, alkali ön arıtımın lignoselülozik atıkların biyolojik olarak daha kolay parçalanmasına nasıl katkı sağladığını göstermektedir. Deneysel veriler, alkali ön arıtımın lignoselülozik biyokütlelerin biyokimyasal süreçlerde daha verimli kullanılmasını sağladığını ve bu süreçlerde elde edilen VFA miktarını artırdığını açıkça ortaya koymaktadır. Bu bulgular, lignoselülozik atıkların biyokütle bazlı enerji üretimi ve biyo-kimyasal ürünlerin üretimi için nasıl optimize edilebileceğine dair önemli ipuçları sunmaktadır.

Bu çalışmada elde edilen bulgulara dayanarak, alkali ön arıtım yönteminin ekonomik olarak uygulanabilirliği de değerlendirilmiştir. Yapılan maliyet analizleri, %8 KOH dozajının hem VFA üretimi açısından en verimli sonuçları verdiğini hem de maliyet etkinliği açısından en uygun seçenek olduğunu göstermiştir. Lignoselülozik atıkların bu şekilde işlenmesi, geleneksel atık bertaraf yöntemlerine kıyasla çok daha sürdürülebilir ve ekonomik bir çözüm sunmaktadır. Maliyet etkinliği, özellikle endüstriyel ölçekli uygulamaların yaygınlaştırılması için kritik bir faktör olarak öne çıkmaktadır. Bu çalışmada elde edilen sonuçlar, lignoselülozik tarımsal atıkların biyokimyasal proseslerle nasıl daha verimli bir şekilde değerlendirilebileceğine dair

önemli ipuçları sunmakta ve bu süreçlerin büyük ölçekli uygulamalara entegrasyonu için önemli bir potansiyel barındırdığını ortaya koymaktadır.

Sonuç olarak, bu çalışma lignoselülozik yapıya sahip tarımsal atıkların alkali ön arıtım yöntemi kullanılarak anaerobik sindirimi üzerine kapsamlı bir inceleme sunmaktadır. Elde edilen bulgular, alkali ön arıtım yönteminin lignoselülozik atıkların biyolojik olarak daha kolay bozunabilir hale getirilmesi ve VFA üretimi açısından ne kadar etkili olduğunu göstermektedir. Aynı zamanda, mikrobiyal toplulukların süreç boyunca nasıl değiş tiği, alkali ön arıtımın bu topluluklar üzerindeki etkileri ve süreç verimliliğini artıran mekanizmalar da detaylı bir şekilde incelenmiştir. Bu bulgular, atık yönetimi, biyokimyasal üretim süreçleri ve çevresel sürdürülebilirlik açısından önemli katkılar sunmakta olup, lignoselülozik atıkların daha etkin bir şekilde değerlendirilmesi için gelecekte yapılacak araştırmalara önemli bir temel sağlamaktadır.

Gelecekteki araştırmalar, farklı tarımsal atık türlerinin benzer ön arıtım yöntemleriyle değerlendirilmesi, bu süreçlerin endüstriyel ölçekte uygulanabilirliğinin incelenmesi ve lignoselülozik biyokütlenin enerji üretimi dışındaki biyokimyasal ürünler için nasıl optimize edilebileceğine dair daha geniş kapsamlı çalışmaların yapılması gerekmektedir. Aynı zamanda, mikroorganizmaların bu süreçlerdeki rolü ve etkilerinin daha derinlemesine araştırılması, anaerobik sindirim süreçlerinin verimliliğini artırmak açısından büyük önem taşımaktadır. Bu çalışma, lignoselülozik tarımsal atıkların enerji üretimi ve biyo-kimyasallar elde etmek için optimize edilmesine yönelik önemli bir temel oluşturarak, sürdürülebilir biyoekonomi ve çevresel yönetim stratejileri açısından değerli bir katkı sunmaktadır.

1. INTRODUCTION

Annually, over 15 billion tonnes of carbon dioxide are emitted into the Earth's atmosphere, much of which is attributed to the combustion of fossil fuels. The rise in CO_2 levels has been directly connected to global warming. The harmful impact of greenhouse gas emissions on the environment and widely acknowledged, in conjunction with the diminishing reserves of petroleum and the potential energy security implications for the future [1].

The current state of fossil fuels, coupled with their rapid depletion, has underscored the need for alternative, sustainable, and cost-effective sources of energy. Biofuels, including bio-hydrogen, bio-methane, bioethanol, biomethanol, biobutanol, among others, have emerged as viable options to address this challenge. These renewable energy sources have the potential to substantially decrease reliance on fossil fuels and mitigate their adverse effects. As such, exploring and investing in the development of biofuels is critical to achieving a sustainable and secure energy future [2].

The utilization of sustainable raw materials presents a viable option in mitigating the effects of climate change. This is due to comparatively lower greenhouse gas emissions than fossil fuels, and their ability to bind carbon dioxide over the long run when utilized for materials. Energy crops are widely utilized as feedstocks for biogas amount, connected to their high energy potential. However, they account for a significant percentage of the total biogas production cost, typically ranging from 30-35%. As a result, they may not be economically feasible without substantial benefits. To mitigate such costs, agricultural waste materials are increasingly viewed as a promising solution to maintain biogas production potential while reducing overall production costs. Unlike energy crops, agricultural residues do not compete with food production and are readily available in substantial quantities at relatively low costs. Lignocellulosic waste materials, in particular, are attractive for biogas production because of their plentiful supply and cost-effectiveness.The most prevalent renewable raw material is lignocellulosic biomass, with a global production volume of approximately 182 billion tons annually. However, only around 8 billion tons of this

biomass are currently utilized. Lignocellulosic biomass contains three of the most abundant natural polymers on earth, namely cellulose, lignin, and hemicellulose [3]. Biomass comprises a composite material wherein polysaccharides and lignin are interconnected through covalent cross-linkages. Anaerobic digestion (AD) has emerged as a promising approach for the treatment of organic solid waste and wastewater, as it enables the combination of waste treatment with energy recovery. Lately, there has been a notable uptick in utilizing anaerobic digestion (AD) for processing highly biodegradable wastes like lignocellulosic materials, animal manure, kitchen waste, and municipal sewage sludge. A reliable pretreatment is vital in cellulose conversion processes, as it's instrumental in modifying the structure of cellulosic biomass to enhance the accessibility of cellulose to the enzymes responsible for converting carbohydrate polymers into fermentable sugars [4].

Alkalines are efficacious in eliminating cellulose accessibility inhibitors, such as acetyl groups, lignin, and uronic acid substitutions, which impede enzymatic saccharification. Additionally, they catalyze the solubilization of polysaccharides like cellulose, hemicellulose, lignin, and silica in the cell walls of lignocellulosic biomass through enzymatic hydrolysis. This process leads to the disruption of crosslinks between various components, thereby increasing the porosity of the biomass. Sodium hydroxide, calcium hydroxide, potassium hydroxide, and ammonia are among the most commonly employed alkalis [2].

The residues of sunflower, specifically the sunflower stalks, sunflower heads constitute a type of lignocellulosic biomass that is produced on a global scale, ranging from 78 to 182 million tons per annum, as reported by the Food and Agriculture Organization (FAO) in 2013. It is common practice to dispose of these residues as waste or to incinerate them in fields, resulting in environmental pollution. However, the high cellulose and hemicellulose content of sunflower stalks renders them a viable feedstock for the production of renewable energy [5].

The objective is to process lignocellulosic wastes, which are organic wastes with significant importance, using suitable pretreatment methods. The aim is to establish a proper balance between degradation efficiency and energy efficiency. When the highest degradation efficiency is achieved, the efficiency of volatile fatty acid formation will also increase. In this context, the fundamental objectives include blocking methane formation and increasing the efficiency of volatile fatty acid

formation. Sunflower stalks were chosen due to their lack of usability in agriculture and livestock. A chemical pretreatment will be carried out using the alkaline pretreatment method, with potassium hydroxide (KOH) as the preferred chemical. The goal is to conduct a study that is low-cost and high-yielding based on known information. The impact of the pretreatment method used in the study on acidification efficiency will be examined in detail. Additionally, at the end of the study, the dominant microbial community will be addressed through DNA sequencing, thereby contributing to biological treatment studies.

2. LITERATURE REVIEW

2.1 Anaerobic Digestion

The current state of sustainable development is beleaguered by two predominant global concerns, namely the energy crisis and environmental degradation. Fossil fuels, which comprise over 80% of current energy consumption, are widely acknowledged as major contributors to both climate change and global warming, as well as the rapid depletion of natural energy supplies. It is imperative that we address these concerns in order to ensure a sustainable future [6]. Bioenergy has the potential to play a pivotal role in promoting renewable energy alternatives. Biogas, which is produced through anaerobic digestion, represents a promising means of addressing global energy needs while also delivering numerous environmental benefits. Moreover, from a socio-economic perspective, biogas significantly reduces the costs associated with waste treatment and boasts a relatively low feedstock cost. Additionally, biogas is competitively priced relative to diesel and petrol. These examples serve to illustrate that biogas is widely utilized as a renewable source of energy [7]. Biogas is primarily composed of methane (CH_4) and carbon dioxide (CO_2) in concentrations ranging from 50% - 70% and 30% - 50%, respectively. This renewable energy source is derived from organic matter, such as agricultural waste, landfills, and sewage sludge, and can be used as a fuel for heating, electricity generation, and transportation. The production of biogas offers numerous benefits, including reducing greenhouse gas emissions and providing a sustainable alternative to fossil fuels [8].

Anaerobic digestion technology is gaining global recognition for its economic and environmental advantages. The use of biogas as an alternative to natural gas presents several crucial benefits. Biogas is produced from renewable resources, offering a sustainable energy source. It does not contribute to greenhouse gas emissions, making it an eco-friendly option. The generation of biogas locally reduces reliance on foreign oil or gas supplies. This technology contributes to reducing the pollution produced by organic waste, which accounts for most freshwater pollution. Biogas production can help mitigate waste management issues, presenting a sustainable solution for energy

production. The adoption of anaerobic digestion technology can significantly enhance the economic and environmental sustainability of various industries [1]. Lignocellulosic biomass, an abundant organic material, is a potential source for sustainable bioenergy and biofuels, such as biogas. Biogas, consisting primarily of CH_4 (50-75%) and CO_2 (25-50%), is a promising renewable energy source. Anaerobic digestion (AD) is a well-recognized and economical approach for generating bioenergy and biofuel from organic substances. It's employed in the commercial production of electricity, heat, and compressed natural gas (CNG). Nevertheless, the utilization of lignocellulosic biomass for biogas production via anaerobic digestion hasn't been extensively embraced due to the intricate composition of the plant cell wall, making it resilient to microbial degradation. Consequently, pretreating lignocellulosic biomass is imperative to attain optimal biogas production in the AD process [9].

2.2 Anaerobic Digestion Process

Anaerobic digestion (AD) is an extensively utilized process for stabilizing waste and wastewater, and more recently for producing biofuels. This process occurs naturally in various environments, including cow's stomachs, marshes, and swamps. Within landfills, organic waste undergoes decomposition via the AD process. Biogas, a methane-rich gas, is produced as a result of anaerobic digestion of organic materials in the digester, a biological engineering structure. The presence of air-tight digesters is critical to the biogas production process, as they are essential components that facilitate the conversion of organics to simple organics and gaseous biogas products. The AD process is therefore incomplete without these key components [10].

Organic material decomposition via anaerobic digestion (AD) is a complex process that comprises four distinct stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. This process demands anaerobic conditions and relies on the coordinated activity of a diverse microbial community to convert organic matter into primarily carbon dioxide (CO_2) and methane (CH_4). Hydrolysis, the first stage in this process, is often the rate-determining step. Despite the process's multi-step nature, maintaining strict anaerobic conditions is essential for its success. The process's intricate nature also necessitates a complex microbial association for optimal results. The microbial association's coordinated activity is responsible for the efficient conversion of organic material into primarily CO_2 and CH_4. Overall, hydrolysis is

typically the slowest step and the most critical to the success of the AD process, despite the presence of four stages [11]. Figure 2.1. shows the steps involved in anaerobic digestion.

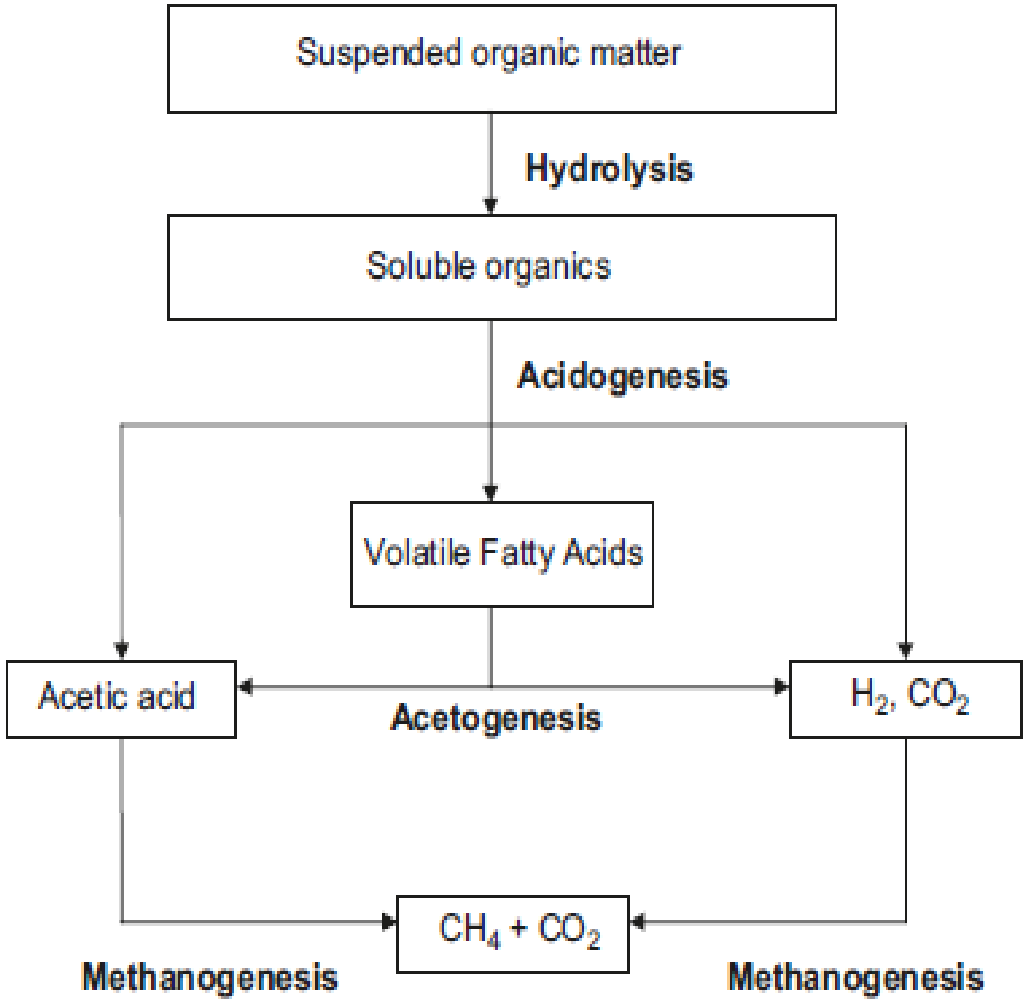

Figure 2.1 : Subsequent steps in the anaerobic digestion process [11].

2.2.1 Hydrolysis stage

The decomposition process involves the degradation of particular substances such as cellulose, proteins, and fats, into smaller units known as monomers. Exoenzymes produced by facultative and obligately anaerobic bacteria play a crucial role in the breakdown process. These enzymes catalyze the cleavage of covalent bonds through hydrolysis, a chemical reaction involving water. Carbohydrates can be broken down within a few hours, whereas proteins and lipids may take several days. However, the degradation of lignocellulose and lignin is slow and incomplete. Facultative anaerobic microorganisms consume dissolved oxygen, reducing the redox potential, which is

essential for obligately anaerobic microorganisms. During hydrolysis, carbohydrates are (converted into) sugars, lipids into fatty acids, as well as amino acids [1].

2.2.2 Acitogenesis stage

In the second stage of anaerobic digestion, anaerobic digestion, the breakdown products like amino acids, long-chain fatty acids, and simple sugars undergo conversion into diverse small organic compounds, predominantly volatile fatty acids (VFAs) such as acetate (CH_3COOH), propionate (CH_3CH_2COOH), butyrate ($CH_3CH_2CH_2COOH$), valeric ($CH_3CH_2CH_2CH_2COOH$), formic ($HCOOH$), lactic ($C_3H_6O_3$), along with hydrogen (H_2), carbon dioxide (CO_2), and ammonia. This process is predominantly facilitated by fermentative microorganisms. The end-products of the process are subject to the prevailing conditions in the reactor medium. For instance, the presence of H_2-scavenging organisms such as methanogens effectively eliminates H2, leading to acetate as the primary end-product. Conversely, if methanogenesis is suppressed and H_2 accumulates, more reduced products, including propionate and butyrate, are likely to appear. Consequently, the effluents of overloaded or disturbed anaerobic reactors often contain these more reduced intermediate products, leading to an acidic environment. Hydrogen, carbon dioxide, and acetic acid produced during the acidogenesis process bypass the acetogenesis process and are directly utilized by the methanogenic microorganisms in the final stage [12].

2.2.3 Acetogenesis stage

The generation of acetate extends beyond the fermentation of soluble organic compounds to include acetogenesis, which occurs during the acidogenic phase. In this stage, acids and alcohols such as butyrate, propionate, and ethanol, which are generated during the acid-forming phase, undergo degradation, eventually leading to the formation of acetate. The aforementioned acetate serves as an essential substrate for methane-forming bacteria. The intricate process of acetate production is directed by the activity of acetogenic or acetate-forming bacteria [13].

2.2.4 Methanogenesis stage

Methanogenesis stage is an ultimate phase for the anaerobic digestion, where methanogenic bacteria generate methane and carbon dioxide. Methanogenic microbes

are strictly anaerobic and can only survive in an oxygen-free environment. Unlike fermenting type of microorganisms, which have lower sensitivity and can exist within a pH range of 4.0 up to 8.5, methane forming bacterias are highly sensitive to pH, requiring an optimal pH around 6.5. Due to their vulnerability to environmental changes, this stage is regarded as the limiting factor of the process [14].

2.3 Critic Parameters of Anaerobic Digestion

Process of anaerobic digestion inside anaerobic environments is influenced by several parameters that impact the rates of individual steps. These parameters include pH and alkalinity, temperature, and retention times. It is crucial to note that any variations in these parameters can affect the overall efficiency of the process. Thus, it is necessary to maintain optimal conditions to ensure the desired outcomes. In summary, the interplay of pH and alkalinity, temperature, and retention times plays a critical role in determining the success of anaerobic digestion [15].

2.3.1 Temprature regime

Thermophilic anaerobic digestion (AD) operates at elevated temperatures, typically ranging between 55-70 degrees Celsius. It offers several benefits compared to mesophilic digestion, including quicker reaction rates and higher capacity for handling loads, resulting in increased efficiency. However, it can also lead to challenges such as acidification, reduced stability, lower quality effluent, increased toxicity, susceptibility to environmental factors, greater initial investments, suboptimal methanogenesis, and higher energy input. Moreover, it is more responsive to environmental variations compared to the mesophilic process. The ideal AD conditions would involve thermophilic hydrolysis/acidogenesis combined with mesophilic methanogenesis, suggesting a two-phase AD process. Nevertheless, AD microorganisms are highly sensitive to temperature fluctuations, impacting hydrogen and methane production, as well as organic material decomposition, and consequently, the overall efficiency and yield of the process. Hence, careful consideration of these factors is crucial when determining the suitable conditions for AD [16].

2.3.2 pH and volatile fatty acids

Volatile fatty acids (VFAs), primarily composed of acetic acid, propionic acid, butyric acid, and valeric acid, serve as key intermediate products in the anaerobic digestion

(AD) of organic waste. Typically, these VFAs are converted into methane (CH_4) and carbon dioxide (CO_2) by syntrophic acetogens and methanogenic bacteria. However, under high organic loading conditions, VFAs may accumulate, leading to a decrease in pH and potentially causing AD failure. Acetic and propionic acids, among the VFAs, are particularly influential in biogas production, and their concentrations can serve as indicators of AD performance [17].

The population of methanogens is significantly influenced by the pH levels of the system. A pH value that falls outside the range of 6.0-8.5 may trigger toxic effects on the methanogens population. The pH level of the system is primarily determined by the rate at which intermediates are produced during fermentation. When the pH level drops below 6.6, the methanogens' activities are adversely affected, and a pH level of 6.2 is considered toxic. Despite the pH level reaching this level, acid production continues as the acidogenic bacteria continues to produce acid until the pH level drops to 4.5-5.0 [1].

2.3.3 Nutrients

The process of producing biogas through anaerobic digestion (AD) is reliant upon the stimulatory effect of both macronutrients and trace elements. These elements act as cost-effective and eco-friendly accelerants in comparison to chemical reagents, which require significant energy inputs. The significance of macronutrients and trace elements is particularly relevant in the anaerobic digestion of energy crops, animal excreta, crop residues, and the organic fraction of municipal solid waste, which are deficient in these elements. Consequently, their presence is crucial to the efficient and profitable production of biogas [18].

Nitrogen and phosphorus are the two macronutrients of paramount importance in any biological treatment process. Methane-forming bacteria absorb these nutrients in the form of ammonia nitrog (NH_4+–N) and orthophosphate-phosphorus (HPO_4—P). However, this nutritions are only biologically available to bacteria in a soluble form. In addition to these macronutrients, micronutrients, particularly cobalt, iron, nickel, and sulfide, play a vital role in the process. The enzymes of methane-forming bacteria rely on these trace elements, along with other elements such as selenium and tungsten. Micronutrients are indispensable to ensure making degrade of substrate and efficient operation of the digester. Cobalt, iron, nickel, and sulfide are mandatory food as

mathane producing bacteria require them to convert acetate to methane. Consequently, it is imperative to consider the needs of both macronutrients and micronutrients to guarantee the optimal functioning of methane-forming bacteria [19].

Table 2.1 : Nutrients in the AD process.

	Element	Feedstock	Function
Micronutrients	Iron	Municipalsolid waste	CODH, Recips sulfides Constituent of enzymes
	Nickel	Energy Crops	CODH, other hydrogenases
	Selenium	Animal excrete	F430, Benzoyl-COA
		Crops Residues	
		Food Waste	
	Tungsten	Stillage-fed	FDH
	Zinc	Wastewater	FDH, CODH, other Hydrogenases
	Chromium		
	Molybdenum		FDH
	Cobalt		Corrinoids CODH
Macronutrients	Carbon		Energy and Cell Material
	Nitrogen		Protein Synthesis
	Potassium		Cell Wall permeability
	Phosphorus		Nucleic acid synthesis
	Sulfur		Numerous enzymes
	Magnesium		

2.3.4 Mixing

Mixing the contents of an anaerobic digester is vital to enhance the digestion process by evenly distributing bacteria, substrate, and nutrients while maintaining a consistent temperature. Acetate-and methane producing bacteria require close spatial proximity, which can be achieved through gentle mixing. Mixing also facilitates hydrolysis of wastes and production of organic acids and alcohols by acid-forming bacteria. Mechanical mixing and gas recirculation are two mixing methods used to ensure optimal results. Mechanical mixing can be accomplished through various means, such as external pumps, gas injection or recirculation from the floor or roof of the digester, propellers or turbines, and draft tubes. However, mechanical mixers are prone to clogging or fouling with digester solids. Therefore, it is essential to approach mixing with care and precision to ensure the best possible outcome [19].

2.3.5 Toxicity

Thorough mixing of the contents within anaerobic digesters is vital for optimizing the digestion process. It ensures the even distribution of bacteria, substrate, and nutrients throughout the digester, thereby promoting uniform temperature. Close spatial contact

between acetate-forming and methane-forming bacteria is crucial for their metabolic activities, facilitated by gentle mixing. This mixing also aids in efficient waste hydrolysis and the production of organic acids and alcohols by acid-forming bacteria. Various mechanical methods or gas recirculation techniques are employed to achieve this mixing. It's crucial to closely monitor and regulate levels of free oxygen and oxygen-containing compounds, such as NO_3-, H_2O_2, and SO_4-$_2$, during the process, as they can hinder microbial activity. Elevated levels of sulfate compounds can promote the growing mechanism of sulfae chemical reducer bacteria, leading to undesirable outcomes like H_2S production. Additionally, high ammonia concentrations can be toxic to the system. Therefore, meticulous management of free ammonia nitrogen levels is essential throughout the process. Overall, careful management of toxic substances is critical for ensuring the effectiveness of the anaerobic digestion process.

2.4 Volatile Fatty Acids Production from Lignocellulosic Biomass

Lignocellulosic biomass is a significant source for biofuel production due to its abundance, high energy value, and non-competition with food production. The high carbohydrate content of this biomass makes it a valuable approach for converting less useful biomass to useful fuels and chemicals through low-cost technology. However, the hard structure of lignin, one of the primary components of lignocellulosic biomass, presents a significant obstacle to its economical use. The conversion of lignocellulosic biomass into useful fuels and chemicals is a promising approach due to its noncompetition with food production and its high energy value. This process involves breaking down the complex structure of the biomass into its constituent parts, including lignin, cellulose, and hemicellulose. While cellulose and hemicellulose can be readily converted to fuels and chemicals, the hard structure of lignin poses a significant challenge. To overcome this obstacle, researchers have focused on developing cost-effective technologies to break down lignin into useful products. These processes involve the use of chemical, physical, or biological methods to break down the lignin structure and extract valuable compounds. Despite progress in this field, further research is necessary to develop effective and sustainable methods for converting lignocellulosic biomass into useful fuels and chemicals [20].

AD is a progress that can be divided into four main parts, namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis. During this process, a range of

symbiotic microorganisms containing acidogens and methanogens work collaboratively to make v fatty VFAs and methane. Acidogenic bacteria are primarily responsible for the production of VFAs during the first three stages of AD. VFAs have significant value, as they can be utilized as precursors to produce biofuel and value-added products. The addition of hydrogen to VFAs results in the production of mixed alcohols, while biodiesel can be obtained as a carbon source for many oleaginous microorganisms. Therefore, understanding the bacterial community that plays a vital role in the AD process can provide essential insights into enhancing the acidogenic performance [21].

In the context of anaerobic digestion (AD), volatile fatty acids (VFAs) and methane are both important end products of the process. However, VFAs are often considered more valuable than methane for several reasons: While methane is recognized as a valuable renewable energy source, volatile fatty acids (VFAs) offer several advantages over methane. VFAs possess a higher energy density, meaning they contain more energy potential per volume or mass compared to methane, making them a more efficient energy source. Additionally, VFAs exhibit flexibility in end use, as they can be converted into various valuable products such as biofuels, biochemicals, and platform chemicals through fermentation or chemical conversion processes. These versatile properties make VFAs appealing for a wide range of industrial applications beyond energy production. Furthermore, VFAs serve as essential chemical building blocks for synthesizing organic compounds like acetic acid, which finds widespread use in polymer, solvent, and pharmaceutical production. The accumulation of VFAs during anaerobic digestion not only indicates process stability and efficiency but also enables adjustments to optimize digestion operations by balancing microbial communities and substrate feedstocks. Moreover, utilizing VFAs as feedstock for value-added products contributes to the circular economy by reducing reliance on fossil fuels and minimizing waste streams, thus promoting sustainable waste management practices and resource recovery.

Overall, while methane is a valuable renewable energy source, VFAs offer additional advantages in terms of energy density, versatility in end use, chemical potential, process stability, and environmental benefits. Therefore, maximizing the production and utilization of VFAs through anaerobic digestion can enhance the overall efficiency and sustainability of bioenergy production systems [22].

2.4.1 Lignocellulosic biomass

Lignocellulosic biomass, a type of organic resource derived from agricultural residues, jungle waste, municipal solid waste, and other sources, is a plentiful and renewable feedstock. This type of biomass is comprised of three types of polymers, namely cellulose, hemicellulose, and lignin. The carbohydrate-based components of lignocellulosic biomass, cellulose and hemicellulose, can be fermented after hydrolysis, resulting in a product that is well-suited for bioenergy production. However, the inherent characteristics of native lignocellulosic biomass, such as its structural and chemical properties, render it resistant to biodegradation by enzymes and microbes [9]. Table 2.2. provides a comprehensive summary of the typical composition of lignocellulosic feedstocks that are commonly utilized.

Table 2.2 : Percent composition of cellulose, hemicelluloses and lignin in agricultural residues and wastes.

S. No.	Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
1	Hardwood stems	40-55	24-40	18-25
2	Softwood stems	45-50	25-35	25-35
3	Nut Shells	25-30	25-30	30-40
4	Corn Cobs	45	35	15
5	Grasses	25-45	35-50	10-30
6	Paper	85-99	0	0-15
7	Wheat straw	30	50	15
8	Sorted refuse	60	20	20
9	Leaves	15-20	85	0
10	Cottonseed hairs	80-95	5-20	0
11	Newspaper	40-55	25-40	18-30
12	Wastepaper	60-70	10-20	5-10
13	Swine waste	60	28	N/A
14	Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
15	Coastal Bermuda Grass	25	35.7	6.4
16	Switchgrass	45	31.4	12
17	Sunflower stalk	34-42	19-21	12-30
18	Corn stover	37-39	23-31	14-18
19	Eucalyptus	34-44	18-19	19-30
20	Maize stems	36-38	10-30	3.5-10.5
21	Poplar	40-43	12-26	18-25
22	Rice straw	27-44	14-34	13-26

2.4.2 Properties of lignocellulosic biomass

Lignocellulosic biomass is the dry matter of plants that primarily consists of cellulose, hemicellulose, and lignin. This biomass is predominantly secured from the agriculture, forestry, and industry sectors. The various types of lignocellulosic biomass, along with their examples, are presented in Table 2.3. Agricultural waste and forest residues are the most abundant and cost-effective feedstocks for producing energy [25].

Table 2.3 : Lignocellulosic biomass types for energy generation.

Supply Sector	Type	Examples
Agriculture	Lignocellulosic energy crops	Herbacecous (e.g. switchgrass, miscanthus, reed)
	Crop residues	Crop straw (e.g. rice straw wheat straw corn stalk cotton stalk)
	Oil, sugar and starch energy crops	Rape seed, sugarcane, corn
Forest	Dedicated forestry	Shor rotation plantations (e.g. willow, poplari eucalyptus)
	Foresty by products	Barks; Wood blocks, Wood chips from tops and branches, Wood chips from thinning, Logs from thinning
Industry	Lignocellulosic agro-industrial residues	Rice husk, Sugarcane bagasse, Corn cob
	Wood industry Residues	Industrial waste wood, Sanddust from sawmills
Other	Lignocellulosic waste	Lignocellulosic residues from parks and gardens (e.g. prunings, grass)

Previously, lignocellulosic biomass was mainly utilized for cooking and heating, causing environmental issues like land degradation and desertification. Nowadays, it can be converted into energy or energy carriers through thermochemical or biochemical processes. Thermochemical conversion employs heat and chemical reactions to generate energy products, including combustion, pyrolysis, gasification, and liquefaction. In contrast, biochemical conversion involves using bacteria, microorganisms, or enzymes to decompose biomass into gaseous or liquid fuels such as biogas or bioethanol. Figure 2.2. showcases different biomass conversion technologies, along with their primary products and end-uses [23].

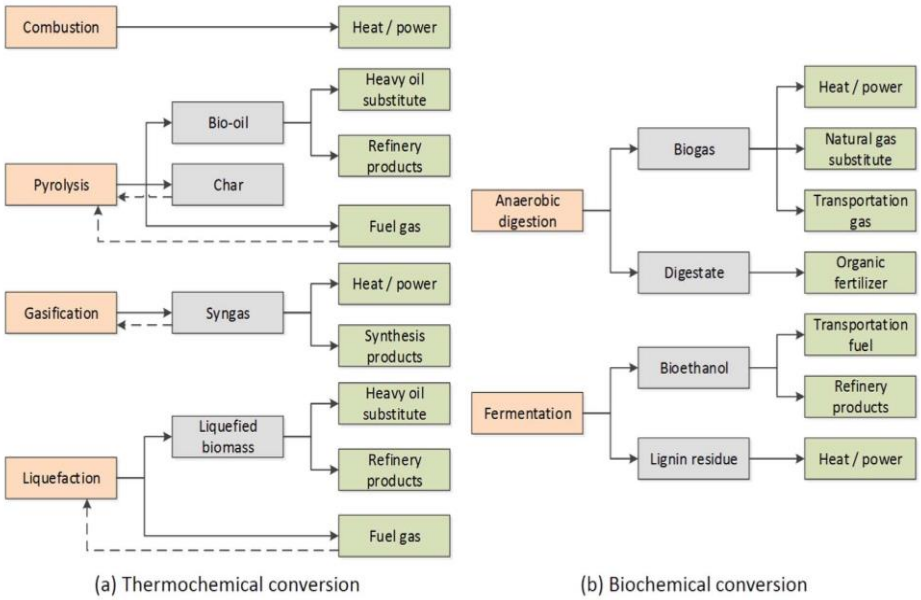

Figure 2.2 : Thermochemical and biochemical methods for converting lignocellulosic biomass.

2.4.3 Structure of lignocellulosic biomass

Lignocellulosic biomass, produced through photosynthesis, consists of polymeric sugar blocks and is also known as lignocellulose. This carbon-neutral organic energy source is considered a promising alternative to fossil fuels. Depending on the processing method, lignocellulose can be classified as either energy crops or virgin biomass and waste biomass. Through thermochemical, chemical, and biological methods, lignocellulose can be transformed into value-added bioproducts or into liquid, gaseous, and solid fuels. Its composition includes cellulose (35–60%), hemicellulose (25–41%), lignin (15–20%), acetyl and phenolic groups, minerals, and trace amounts of other compounds. These polymers vary in complexity, non-uniformity, three-dimensional structure, and composition. Cellulose, the primary (structural homopolymer), significantly contributes to lignocellulose's strength, with each unit consisting of a chain of repeating β-D-glucopyranose units linked by glycosidic bonds, providing rigidity and stability to the cell wall. The complex, non-uniform, and three-dimensional arrangement of these polymers is also influenced by lignin's hydrophobicity and the encapsulation of lignocellulose within the lignin–hemicellulose matrix by covalent and hydrogen bonds [23].

2.4.3.1 Cellulose, hemicellulose and lignin

Cellulose is composed of D-glucose subunits that are linked by b-1,4 glycosidic bonds. In plants, cellulose exists in two different forms: a crystalline structure, and an amorphous structure which is not well-organized. The cellulose strands are bundled together to form cellulose fibrils or cellulose bundles. These fibrils are mostly independent and weakly bound through hydrogen bonding [14].

Hemicellulose and cellulose are two distinct substances, with the former characterized by the presence of branches composed of various types of sugars. These branches include pentoses like rhamnose, arabinose, and xylose, as well as hexoses such as galactose, glucose, and mannose, along with uronic acids. The backbone of hemicellulose can be either a homopolymer or a heteropolymer, with short branches connected through B-(1,4)-glycosidic bonds, and occasionally B-(1,3)-glycosidic bonds. Some hemicelluloses can also be partially acetylated, such as heteroxylan. In contrast to cellulose, the polymers of hemicellulose are easily hydrolyzable. Moreover, these polymers do not aggregate, even when co-crystallized with cellulose chains.

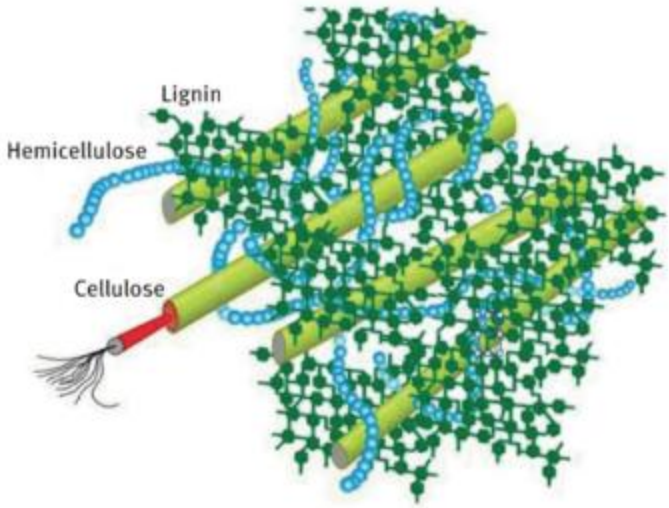

Figure 2.3 : Cellulose, hemicellulose and lignin in plant cells.

Lignin is a significant polymer found in nature, ranking third in abundance after cellulose and chitin. It is a structural component of plant cell walls that provides rigidity and impermeability, making plants resistant to microbial attack and oxidative stress. Lignin is a complex network of phenyl propane units held together by various linkages, making it an amorphous heteropolymer. It is widely accepted as the "glue" that binds the different components of biomass together, thus making it insoluble in water. Despite its critical role in plant structure and function, lignin is a major deterrent to the efficient use of lignocellulosic biomass for bioenergy production. Its close association with cellulose microfibrils makes it a significant barrier to enzymatic and microbial hydrolysis of biomass. This is due to its nonspecific adsorption of hydrolytic enzymes, interference with cellulolytic enzymes, and toxicity to microorganisms. Different feedstocks contain varying amounts of lignin. Therefore, it is crucial to remove this polymer through pretreatment to enhance biomass digestibility. Pretreatment causes the lignin to melt and coalesce upon cooling, altering its properties. Delignification, which is the extraction of lignin using chemicals, causes biomass swelling, disruption of the lignin structure, increases in internal surface area, and increased accessibility of cellulolytic enzymes to cellulose fibers [24].

2.4.4 Lignocellulosic biomass as anaerobic digestion feedstocks

Agricultural products, predominantly lignocellulosic materials, have garnered significant interest as feedstock for anaerobic digestion due to their plentiful availability and renewability. Lignocellulosic materials possess a complex structure comprising a substantial amount of cross-linked polysaccharide networks,

glycosylated proteins, and lignin. Cellulose microfibrils are long and interconnected by hemicellulose polysaccharides, with pectins and lignins filling the spaces in between. During anaerobic digestion, microorganisms can break down the lignocellulosic carbohydrates such as cellulose and hemicellulose. Cellulose, the primary component of lignocellulosic material, is a linear homopolysaccharide of glucose featuring strong β-1,4-glycosidic linkages. These cellulose molecules form microfibrils with hydroxyl groups that create hydrogen bonds both within and between them. Cellulose can be divided into amorphous and crystalline regions based on the different orientations of its molecules. Besides hydrogen bonds, cellulose microfibrils are connected by hemicellulose and pectin and are encased in lignin.Consequently, this intricate structure renders cellulose impervious to both chemical and biological degradation [2].

In principle, anaerobic microorganisms can digest cellulose and hemicellulose, which are integral components of lignocellulosic materials. Notwithstanding, untreated lignocellulosic feedstocks are unwieldy and cumbersome to introduce into conventional biogas digesters. When introduced, these substrates tend to float and can only be partially broken down during the process. Additionally, the hydrolysis phase remains the rate-limiting step throughout the lignocellulosic digestion process, as hydrolytic microorganisms, which initiate the anaerobic digestion process, are incapable of efficiently breaking down these compounds. As a result, enhancing the biodegradability of lignocellulosic materials via pretreatment is an indispensable element for biogas plants operating on agro-wastes to become economically viable. From a technical perspective, the pretreatment phase is a crucial pre-requisite stage incorporated in biogas production processes to circumvent this issue [26].

2.5 Pre-Treatment Methods for Improving the Digestibility of Lignocellulosic Biomass

The primary objective of the pre-treatment process is to dismantle the naturally resistant carbohydrate-lignin barriers that prevent enzymes and microbes from accessing cellulose and hemicellulose. Additionally, pre-treatment seeks to reduce the structural crystallinity and enhance the porosity of the lignocellulosic biomass, thereby improving its biodegradability and increasing biogas production. Successful pre-treatment methods can facilitate the conversion of lignocellulosic biomass into

biofuels, chemicals, and other value-added products. The effectiveness of pre-treatment processes can be measured by their ability to remove or modify the lignocellulosic barriers, which ultimately improves the efficiency of downstream processes [27].

The development of an optimal pretreatment method for Lignocellulosic Biomass (LCB) must take into account the inherent complexity and variability of the LCB structure to ensure that its structural and compositional properties are not compromised. An efficient LCB deconstruction process should achieve several objectives, including delignification of LCB without significant alteration of the lignosellulosic structure, low energy consumption, cost-effective operation, reduction of the cellulose crystallinity index, reduction of particle size to improve enzymatic hydrolysis, ability to pretreat different types of LCB feedstocks, avoidance of enzyme inhibitor production, and use of eco-friendly chemicals. The pretreatment approaches can generally be categorized into physical, chemical, and physicochemical methods, and each has its advantages and disadvantages. In the subsequent sections, we provide an account of recent advances in technologies for the pretreatment of LCB along with their respective merits and limitations [27].

2.5.1 Physical pre-treatment

Physical pretreatment technologies are routinely employed to modify the physical and partial chemical properties of lignocellulosic biomass. Such modifications may include altering the crystallinity of cellulose, the degree of polymerization, and the specific surface area. A range of physical methods are typically utilized for lignocellulosic pretreatment, including mechanical size reduction, irradiation, ultrasound, and electron beams. Mechanical size reduction is a key method that involves the mechanical reduction of lignocellulosic biomass particle size through techniques such as chipping, milling, or grinding, and which serves to deconstruct the microstructural void in the material [28].

Physical pretreatment is a commonly used method to prepare plant materials for further processing. This method involves grinding, shearing, and stirring and is typically applied to herbaceous plants with low lignin content such as wheat straw and meadow. By utilizing extrusion grinding, cutting, and ball milling, the size and degree of polymerization of these substrates can be significantly reduced. This pretreatment

method has become increasingly popular in recent years due to its ability to effectively process low lignin content plant materials. Moreover, mechanical pretreatment can be used alone or in combination with other pretreatment methods, such as chemical or biological pretreatment, to improve the efficiency of the overall process. In summary, mechanical pretreatment is a valuable method for the preparation of plant materials for further processing. It has proven to be effective in reducing the size and degree of polymerization of herbaceous plants with low lignin content and can be used in conjunction with other pretreatment methods for increased efficiency [9].

2.5.2 Chemical pre-treatment

Chemical pretreatment pertains to the utilization of chemical agents, such as acids, bases, and ionic liquids, to modify the physical and chemical properties of lignocellulosic biomass. Within the realm of pretreatment, chemical procedures have attracted considerable research interest. Table 2.4. encapsulates various chemical treatments that have been explored for bioenergy generation and are elaborated upon below [9].

Table 2.4 : Common chemical pre-treatment techniques.

Chemical Pretreatment	Feedstock	Pretreatment Conditions	Results
Alkaline	• Agricultural residuals; Wheat straw, rice straw, corn stover, sugar beet, leaves, maize, ensiled hay, sugarcane, bagasse, rapeseed, sunflower stalks • Forest residuals ; Fallen leaves • Hardwood ; birch • Softwood ; spurce and pine • Grass ; switch grass, smooth cordgrass • MSW; paper pulp sludge	• Chemicals; NaOH , $Ca(OH)_2$, CaO, KOH, $NH_3.H_2O$ • Chemical loading = 1-10% (g/g dry matter) • Temprature = -15 – 170 C • Time = 1h to 10 days	Positive effect in most cases with 3.2% to 2.3 folds increase of methane yield. Negative effects also occurred in very few cases. In general, it is more effective on biomass containing lignin
Acid	• Agricultural residuals; sunflower oil cakes, greenhouse residues, sugar cane bagasse, herbal extraction residue, sunflower stalks, coconut fiber, rapeseed, sunflower meals, straws, and bracken • Grass ; hay	• Chemicals: H_2SO_4 , HCL, HNO_3, H_3PO_4 ,acetic acid, maleic acid • Chemical loading = 1-4% (g/g organic matter) (organic acid could be much higher, e.g. acecit acid = 35-80%) • Temprature = ambient temprature – 170 C • Time = a few minutes to hours even 30 days	Positive effect in most cases with 20-200% of methane yield. Negative effects also accured in very few cases (e.g. '% H_2SO_4 on rape seed).

Table 2.4 (continued) : Common chemical pre-treatment techniques.

Chemical Pretreatment	Feedstock	Pretreatment Conditions	Results
Catalyzed steam explosion	• Agricultural residuals ; digested biofiber, hemp, wheat straw • MSW : paper tube residuals	• Chemicals: H_2SO_4 , SO_2 , H_3PO_4 , NaOH with and without H_2O_2 • Chemical loading = 0.5-4% (g/g dry matter • Temprature = 155-220 C • Time = 5 min to 1 h	18-107% enhancement of methane yields
Wet Oxidation	• Agricultural residuals : wheat straw , digested biowaste, cornstalks, winter rye straw, oilseed rape straw , faba bean straw • Hardwood : willow • Grass : Miscanthus • MSW : waste newspaper and yard waste	• Temptrature = 180-220 C • Oxygen pressure = 0-1.2 Mpa (H_2O_2 could be added) • Time = a few minutes	34- 136 % enhancement of methane yield
Oxidative pretreatment with peroxides	• Agricultural residuals : rice straw and sunflower stalks, sorghum, sida hermaphrodita • MSW : OFMSW and paper tube residuals • Grass : Mischantus	• Chemicals primarily include H_2O_2 with dose 1-4% (g/g dry matter) NaOH (0-2%) was also used for methane yield improvement • Time = a few minutes to 7 days • Temprature = 25-220 C	33-120% of methane yield. Negative effects were also observed
Ionic liquids (Ils)	• Agricultural residuals : defatted and bleached cotton linter, straw residuals of manure, wheat straw, rice straw, and triticale straw (a hybrit of rye and wheat) • Grass : water hyacinth • Softwood : spruce	• Ils = N-methylmorpholine- N-oxide monohydrate (NMMO) and 1-n-butyl-3 methylimidazolium chloride (BMIMCl) • Chamical loading = 70-85% • Temprature = 90-130 C • Time 1-15h	16-1200% improvement of methane yield (e.g. methane yield of softwood spruce increased 1200% with untreated material)

2.5.3 Acid pre-treatment

Lignocellulosic biomass undergoes acid pre-treatment to enhance its biodegradability. This involves exposing the biomass to either concentrated acid, typically at concentrations of 30-70% and low temperatures (40°C), or dilute acid, usually at concentrations of 0.1% and high temperatures (230°C). Dilute acid pre-treatment is preferred over concentrated acid pre-treatment due to the latter's high toxicity, corrosiveness, and requirement for specialized handling materials. Various organic and inorganic acids, such as sulfuric acid (H_2SO_4), hydrochloric acid (HCl), nitric acid (HNO_3), phosphoric acid (H_3PO_4), and acetic acid, can be used for dilute acid pre-treatment. Dilute acids can hydrolyze up to 100% of hemicellulose into its constituent

sugars, like xylose, arabinose, and galactose, and can significantly disrupt lignin. This results in increased biodegradability of cellulose and hemicellulose by enzymes and microbes.

2.5.4 Alkali pre-treatment

Alkaline pretreatment is generally carried out at low pressure and temperature, sometimes even under ambient conditions. However, this process is time-consuming, often requiring several hours or even days to complete. The effectiveness of alkaline pretreatment is significantly influenced by temperature, and increasing it can accelerate the reaction. In lime pretreatment, some calcium can be lost to non-recoverable salts or become incorporated into the biomass. Other alkaline pretreatment methods use calcium, potassium, sodium, barium, lithium, and ammonium hydroxides, depending on the biomass type. Among these, sodium hydroxide is the most commonly used, followed by lime, due to its cost-effectiveness, safety, and the ease of recovery from water as insoluble calcium carbonate by reacting with carbon dioxide. Adding extra air or oxygen can further improve the delignification of feedstocks. Alkaline pretreatment of feedstocks is similar to Kraft pulping, effectively removing lignin and enhancing the reactivity of polysaccharides. Alkaline hydrolysis efficiently removes acetyl groups and uronic substitutions from hemicelluloses, making their surfaces more accessible to hydrolytic enzymes. However, the overall results of alkaline pretreatment need to be carefully evaluated in terms of operational ease, downstream process costs, and the trade-offs between various expenses, including operational, capital, and biomass costs. Thus, cost-effective pretreatment of lignocellulosic biomass poses a significant technical challenge in cellulose conversion and multicomponent recovery. This chapter aims to evaluate the suitability of the widely used alkaline pretreatment method in enhancing biomass fractionation and the digestibility of complex biomass into fermentable sugars, based on relevant studies of this pretreatment strategy [19].

2.5.5 Physicochemical pretreatment techniques

Physicochemical pretreatments encompass a fusion of physical processes with chemical processes. The foremost techniques employed include steam explosion, microwave-chemical pretreatment, and liquid hot water pretreatment.

The pretreatment of cellulosic substrate through steam explosion involves subjecting it to high pressure, resulting in its destructuration, leading to the degradation of hemicellulose and transformation of lignin, thereby increasing the potential of cellulose hydrolysis. This process offers several advantages, including low capital investment, high sugar recovery, and low environmental impacts. However, it is susceptible to various factors, including moisture content, temperature, residence time, and substrate size. On the other hand, microwave-chemical pretreatment presents a more effective alternative to conventional heating chemical pretreatment. Microwave irradiation heats the entire sample volume, accelerating reactions during pretreatment, disrupting cellulose ultrastructure, degrading hemicelluloses, and increasing substrate accessibility. The microwave-chemical method, although effective, requires complex operational procedures, high energy consumption, and meticulous equipment monitoring. On the other hand, the liquid hot water (LHW) process stands out as a physicochemical pretreatment method praised for its environmental friendliness compared to other chemical and physicochemical treatments. This method does not use catalysts, making it non-toxic and more cost-effective, and it avoids secondary pollution associated with the high water consumption needed to clean the pretreated material. LHW involves maintaining water at high temperatures between 160 and 240°C under pressure, generating organic acids like acetic acid that catalyze the degradation of hemicelluloses into monosugars, thereby enhancing process efficiency [29].

2.6 Alkali Pre-Treatment on Sunflower Waste Using Potassium Hydroxide

The escalating demand for fuel has led to an upsurge in crude oil prices, necessitating a search for alternative sources of bioenergy. The demand for alternative bioenergy sources is expected to increase substantially in the coming years. Lignocellulosic materials have been recognized as the main source for biofuels and other value-added products. Most of the world's biomass, which includes agricultural, industrial, and forest residues, is considered lignocellulosic. Therefore, they hold immense potential as a renewable resource for bioenergy production. Sunflower residues, namely stalks, leaves, and heads, are often left in the fields following seed harvesting, leading to large quantities of organic waste. In light of the absence of suitable alternative reuse options, these residues could potentially serve as a promising renewable resource for the

purpose of bioenergy production via anaerobic digestion. Thus, it is worth exploring the feasibility of utilizing sunflower residues in this manner, given their potential to mitigate waste and provide a sustainable source of energy [30].

Various pretreatment techniques, including mechanical, thermo-chemical, chemical, and biological methods, have been employed to enhance the biodegradability of lignocellulosic residues. Among these methods, dilute-acid and alkaline chemical pretreatments have emerged as the most commonly used techniques for sunflower residues, owing to their cost-effectiveness.

2.6.1 Sunflower Heads and Stalks characteristics

In a study conducted by Zhurka et al., both the Heads and Stalks were subjected to oven drying, resulting in a low humidity level of approximately 10% for each residue. The Stalks were found to have a higher concentration of Chemical Oxygen Demand (COD) in comparison to the Heads, at a rate of 20.8%. This was in agreement with the higher Volatile Solid (VS) content of the Stalks, which accounted for 79.9% of Total Solids (TS) as opposed to 87.7% of TS for the Heads. Conversely, the Heads were found to possess more than two-fold higher levels of Total Kjeldahl Nitrogen (TKN) and lipids when compared to the Stalks, as demonstrated in Table 2.5.

Table 2.5 : Composition of the sunflower heads and stalks (raw) as well as their solid and liquid fractions (SF and LF respectively) after alkaline pretreatment.

	Raw	SF Pretreated	LF Pretreated	Raw	SF Preated	LF Preated
Humidity (%)	9.46 ± 0.4	1.3 ± 1.8	-	8.1 ± 0.4	2.5 ± 1.0	-
Total solids ; TS (%WM)	90.5 ± 0.4	98.7 ± 17.6	-	91.9 ± 0.3	97.5 ± 1.0	-
Volatile solids ; VS (%WM)	72.4 ± 0.5	87.8 ± 10.4	-	80.6 ± 0.2	83.3 ± 11.5	-
VS (%TS)	79.9 ± 0.5	88.9 ± 0.5	-	87.7 ± 0.1	85.5 ± 0.3	-
Chemical oxygen demand ; COD (Sf g kg^{-1} TS or LF g L^{-1})	890 ± 26	967 ± 28	8.3 ± 0.3	1075 ± 34	1067 ± 56	6.2 ± 0.1
TKN (g kg^{-1} TS)	13.3 ± 0.3	13.8 ± 0.3	-	6.3	5.6 ± 0.2	-
Lipids (g kg^{-1} TS)	2.5 ± 0.1	-	-	0.99 ± 0.06	-	-
Phenols (mg Gallic Acid L^{-1})	-	-	214 ± 4	-	-	112 ± 2

WM : wet matter, DM : Dry Matter.

The biomass potential of agricultural waste has been explored for a variety of applications, including the production of methane gas. In this context, a biochemical methane potential (BMP) test was conducted using an alkaline pretreatment approach. The results indicate a methane yield of 31% and 28% for the stalk and head residues, respectively. These outcomes are consistent with previous studies that have applied alkaline pretreatment under the same conditions. In particular, these studies have reported high lignin removal of 22% after pretreatment at 55°C (A). These findings highlight the potential of alkaline pretreatment as an effective method for enhancing the biodegradability of sunflower waste and increasing the methane yield as well as the VFA potential [31].

2.7 Molecular Methods Applied for Microbial Analyses in Anaerobic Digesters

Anaerobic digestion (AD) is a biological process wherein organic waste is converted into biogas by a wide range of microorganisms. The success of this process hinges on establishing a balanced microbial community. Various methods, including Realtime Polymerase Chain Reaction (Q-PCR), Fluorescence In Situ Hybridization (FISH), and Metagenomics, have been employed to analyze the microbial ecology of digesters. Properly characterizing the microbial community is crucial for optimizing the anaerobic digestion process [13].

2.7.1 Real-time Polymerase Chain Reaction (Q-PCR)

Quantitative real-time PCR (Q-PCR) is a commonly used analytical technique for identifying specific microorganisms in environmental samples. (Influence of DNA isolat) There are different types of Q-PCR methods available, with basic Q-PCR primarily used for gene detection without measuring its expression level. In the PCR reaction, data is collected to track the quantity of PCR product, and parameters like melting temperature can be determined. To study gene expression, reverse transcriptase PCR can be employed, although this necessitates RNA extraction and conversion into cDNA prior to the Q-PCR reaction [13].

2.7.2 Fluorescence in situ hybridization (FISH)

In addition to gene-based quantification methods like quantitative real-time PCR, another commonly used technique for quantifying microorganisms in environmental samples involves the hybridization of microbial cells with fluorescently labeled

oligonucleotides targeting 16S ribosomal RNA (16S rRNA), known as fluorescent in situ hybridization (FISH), followed by microscopic cell counting. This method offers the advantage of quantifying microorganisms based on individual cells, allowing for assessment at various taxonomic levels depending on the degree of conservation of the probe target sequence (Development of a flow-fluoresc). The Fluorescence in situ hybridization (FISH) method enables the identification of specific groups of microorganisms and can provide insights into both culturable and unculturable microorganisms. It offers multiple ways to categorize microorganisms in a sample, including by domain, family, genus, and species. Additionally, FISH can offer insights into the function and structure of complex microbial communities. However, observing these microorganisms under fluorescence microscopy can be challenging due to the unique characteristics of their cell walls [13].

3. MATERIAL AND METHODS

3.1 Substrate and Inoculum Characterization

In this research endeavor, sunflower residues, prevalent in specific regions of Turkey, have been designated as the substrate of interest. These residues were procured subsequent to the harvest in the Trachia District during the summer of 2023. The primary rationale behind selecting this substrate pertained to its complete disposal status, rendering it obsolete for any further industrial or agricultural applications. Consequently, the sunflower remnants, comprising stalks and heads, had reached a state of disuse subsequent to the harvesting process.

Figure 3.1 : Sunflower waste.

Following the collection of sunflower waste, the specimens were promptly transferred to the controlled environment of Boğaziçi University's esteemed Microbial Ecology Laboratory, where they were stored at a temperature of +4 degrees Celsius pending subsequent analyses. With a kitchen blender, the samples underwent pulverization into fine particulates, which were then homogenized to ensure a consistent mixture, as illustrated in Figure 3.1. Furthermore, post-blending, the samples underwent a rigorous decontamination procedure involving a 15-minute exposure to ultraviolet radiation, aimed at eradicating any viable microbial entities present within the samples.

3.1.1 Anaerobic seed sludge

The inoculum sludge employed in the anaerobic process was sourced from a fully operational wastewater treatment facility located in Antalya, Turkey. Following collection, the sludge was diligently preserved at a temperature of +4 degrees Celsius until its utilization in the experimental procedures.

3.1.2 Analytical determinations

The characterization of substrates and inoculum sludge conducted for Total solids (TS) and Volatile Solids (VS), according to Standart Methods. After that ph, alkalinity, TKN and C/N were measured. Table 3.1. Shows the measured initial conditions for the samples, and Figure 3.2. shows the process of measuring TS,VS.

Table 3.1 : Initial parameters of samples.

Samples	ph	TS (%)	VS (%)	VS/TS	Alkalinity (mg $CaCO_3$/g)	TKN (mg/g)	C:N
Sunflower	7,65	93	83	0,89	3200	336	17
Seed Sludge	7,80	1,2	0,86	0,72	5150	550	7

Figure 3.2 : The process of measuring TS,VS.

3.2 Experimental Procedures

3.2.1 Alkaline pretreatment

In this study, potassium hydroxide (KOH) was employed as a pretreatment agent aimed at disrupting lignocellulosic bonds, thereby enhancing the substrate's

accessibility to microorganisms during anaerobic digestion. Sunflower waste underwent pretreatment using four distinct dosages of KOH specifically, 6%, 8%, and 10% calculated relative to the dry matter content of the substrates. Additionally, a control sample was prepared using deionized water, denoted as 0% KOH.

The solutions were prepared by mixing the substrates with liquid in a ratio of 100 gTS per liter, maintaining a total solids concentration of 10%. Then, each sample was placed into its designated solution and left at room temperature for 24 hours, as illustrated in Figure 3.3. This timeframe was chosen to allow sufficient time for the process to take place, based on similar studies previously conducted.

Figure 3.3 : Samles kept 24 hours without stirring during pretreatment.

Following the pretreatment procedure, all samples underwent filtration using filters with a pore size of 0.2 mm, in conjunction with coffee filters, to facilitate the separation of solid and liquid fractions.

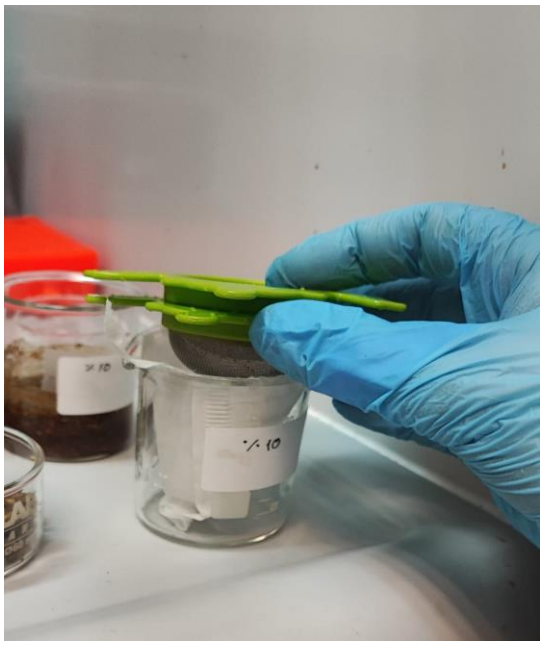

Figure 3.4 : The sieves and coffee filter used for seperation on the sample after pretreatment.

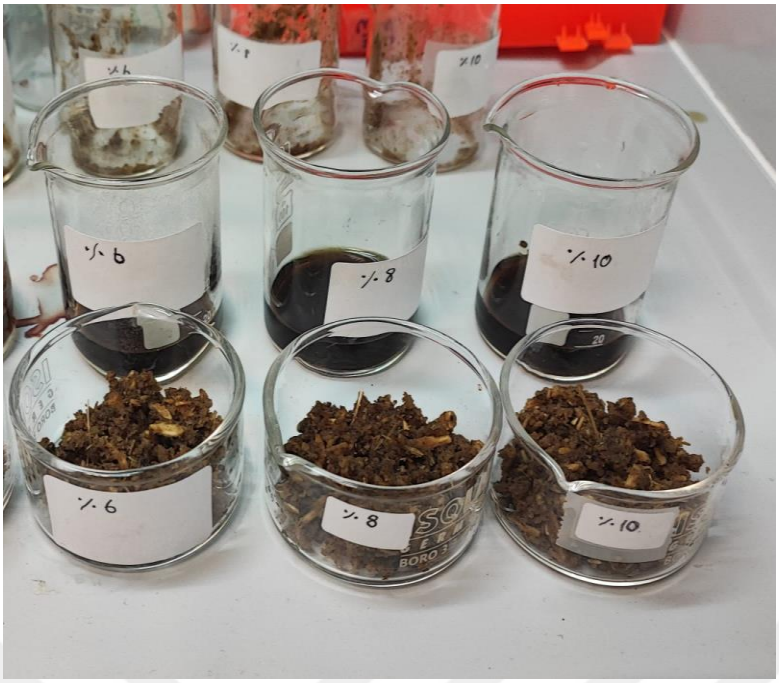

Figure 3.5 : The liquid and solid fractions after the seperations of samples.

Upon the completion of sample separation, the respective fractions were stored at a controlled temperature of +4 degrees Celsius pending subsequent processing steps.

3.2.2 Setup of the anaerobic digestion process

The solid fractions, subsequent to separation, were quantified by weight and subsequently introduced into 100 mL bottles, where they were combined with the inoculum sludge. In each digester, a specified amount of 0.86 gVS, corresponding to a volume of 100 mL of seed sludge, was mixed with 0.44 gVS of each sample, thereby achieving an inoculum-to-substrate ratio of 1:2. Detailed calculations for these proportions are presented in.

Following the calculations, the bottles were filled with the inoculum and respective samples, with the pH adjusted to 5.5 to ensure optimal acidification conditions. To maintain anaerobic conditions, all bottles were tightly capped and purged with N_2 gas for a duration of 5 minutes. To enhance reliability, all experimental procedures were conducted in triplicate. Subsequently, the bottles were incubated in a room maintained at 37 degrees Celsius for a period of 30 days, as depicted in Figure 3.6. Additionally, a 50 mL sample from the inoculum was extracted and preserved at -20 degrees Celsius for subsequent analyses.

Figure 3.6 : Anaerobic digestion setup.

3.2.3 Sampling and controlling pH

Throughout the experimental duration, systematic sampling occurred at predefined intervals: days 0, 1, 2, 3, 4, 7, 10, 15, and 30. Each day, two of 2 ml tubes were employed for sampling, utilizing 2 to 5 ml syringes, while ensuring the integrity of the bottles remained intact. Extracted samples were promptly fixed with 10N phosphoric acid and were subsequently stored at +4 degrees Celsius to maintain their compositional integrity until VFA analyses were conducted. Additionally, pH measurements were performed daily using the Hach Pocket Pro+ pH meter to sustain the acidification process at the optimal level. The representation of the anaerobic digestion sampling technique is shown in Figure 3.7.

Figure 3.7 : Sampling from anaerobic digestion setup.

3.2.4 COD analysis

To assess the impact of pretreatment on Chemical Oxygen Demand (COD) levels, analyses were conducted on the liquid fraction of the separated samples. Initial sample extraction involved the two tubes, each containing 2 milliliters of liquid, from all dosage variants, which were labeled for identification.

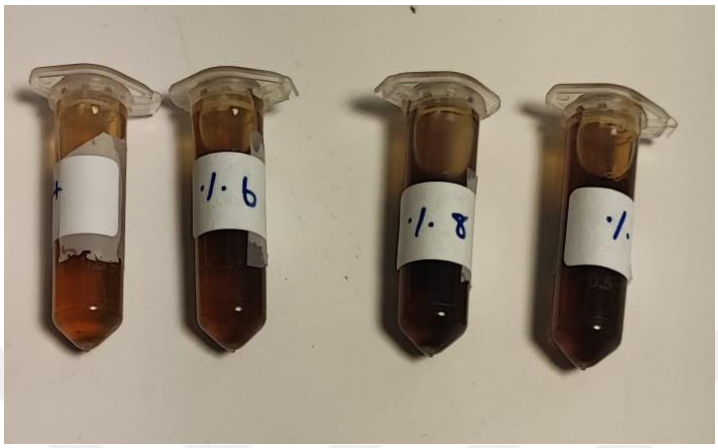

Figure 3.8 : Different dosages of pretreatment liquid samples.

To assess the variance in COD levels between treated and untreated samples, soluble COD tests were carried out on the filtrates obtained after 24 hours of pre-treatment. A control sample was prepared using a 0% KOH solution. For the testing procedure, the filtrates were initially passed through 0.22 μm-sized filters to isolate soluble COD amounts. Subsequently, the filtrates were diluted with deionized water at a ratio of 1:100 to facilitate the COD testing process.

3.2.5 VFA analysis

During the VFA analysis stage, the samples collected on specific days underwent centrifugation using the (name of the device) for 15 minutes at 12,000 rpm to separate the solid and liquid components. Following centrifugation, the liquid portion was carefully separated from the solids using a 2 mL syringe and transferred to a separate 2 mL tube for further processing. The seperated liquid from the collected samples is shown in Figure 3.9.

After the seperation process, samples were taken into a VFA measuring device Shimadzu GC-2025 Gas Chromatograph. And the formation of VFA’s noted.

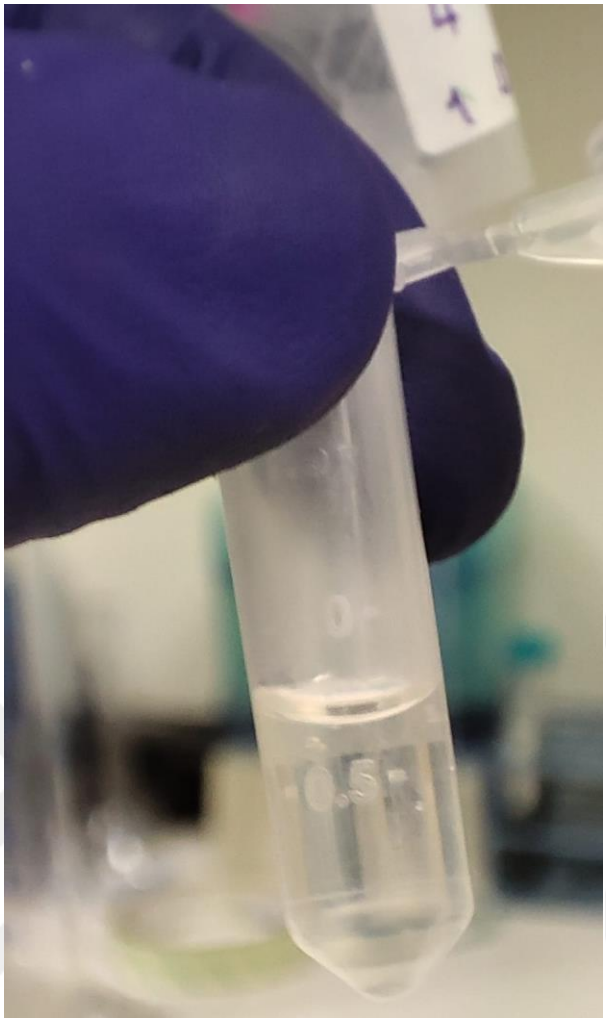

Figure 3.9 : Liquid seperated from anaerobic digestion samples.

3.2.6 Microbial community analysis

In this study, microbial diversity was analyzed by targeting 16S rRNA, 18S rRNA, and archaeal communities. The 16S rRNA gene region was used for identifying bacterial communities, the 18S rRNA gene region for eukaryotic microorganisms, and specific archaeal primers were utilized to assess archaeal diversity.

3.2.6.1 Sample collection and DNA extraction

Samples were collected from mix of subtrate and seed sludge to investigate microbial communities through 16S rRNA gene and Archaea-specific gene analysis. The DNA was extracted using a standardized protocol designed to yield high-quality genomic DNA suitable for sensitive downstream applications, including PCR. The specific method of DNA extraction is critical, although it is not explicitly detailed in the documents, ensuring the integrity and purity of the DNA was maintained for the subsequent amplification processes.

3.2.6.2 16S rRNA gene amplification

The amplification of the 16S rRNA gene was performed using the 2× HiFi Taq Master Mix, which is specifically designed for high-fidelity DNA amplification [32]. This master mix contains HiFi Taq Polymerase, a high-fidelity enzyme with an error rate that is 53 times lower than conventional Taq polymerase and 6 times lower than Pfu polymerase. The inclusion of a special extension factor and specificity-enhancing factors within the master mix ensures robust amplification even for long DNA fragments.

The PCR reaction setup was prepared in a final volume of 50 µL, carefully combining all necessary components. Specifically, 25 µL of 2× HiFi Taq Master Mix was added, along with 2 µL of forward primer and 2 µL of reverse primer, each at a concentration of 10 µM. Depending on the type of template DNA used, an appropriate amount was included: 50-400 ng for genomic DNA, 10 pg-30 ng for plasmid or viral DNA, or 1-5 µL for cDNA. The volume was adjusted to a final 50 µL using nuclease-free water. All reaction components were thoroughly mixed before being aliquoted into PCR tubes for subsequent thermal cycling.

The PCR was performed using a thermal cycler (e.g., Bio-Rad, USA) under specific cycling conditions to ensure optimal amplification. The reaction began with an initial denaturation at 95℃ for 3 minutes to ensure complete denaturation of the DNA template. This was followed by a denaturation step at 95°C for 15 seconds, facilitating the separation of DNA strands. The annealing step, set between 56-72°C for 15 seconds, was optimized based on the melting temperatures (Tm) of the primers used. Extension was carried out at 72℃ for 30-60 seconds per kilobase of the target DNA, allowing the HiFi Taq Polymerase to synthesize new DNA strands efficiently. A final extension at 72℃ for 5 minutes ensured the complete extension of all amplified products. For long-fragment PCR, where the amplification of extended sequences was necessary, an alternative cycling protocol was employed. This included a denaturation step at 95℃ for 15 seconds, followed by an extension at 68℃ for 60 seconds per kilobase, a protocol essential for amplifying DNA fragments larger than typical 16S rRNA gene targets [33].

3.2.6.3 Archaea-specific PCR amplification

The PCR amplification targeting Archaea-specific genes was also conducted using the 2× HiFi Taq Master Mix [32]. Given the lower abundance and higher specificity required for Archaea detection, the reaction conditions were meticulously optimized.

The reaction components for the Archaea-specific amplification mirrored those used for 16S rRNA, with the primers specific to Archaea being added in place of the universal 16S rRNA primers. The concentration of the Archaea-specific primers was optimized to enhance specificity and reduce non-specific amplification.

The thermal cycling conditions were specifically adjusted to suit the requirements of the Archaea primers, ensuring precise and effective amplification. The process began with an initial denaturation at 95℃ for 3 minutes, followed by a denaturation step at 95℃ for 15 seconds. The annealing temperature was carefully optimized based on the primer Tm to enhance specificity, though the exact temperature was tailored to the experiment. The extension phase was conducted at 72℃, with the duration adjusted according to the length of the target product. A final extension at 72℃ for 5 minutes was included to ensure the complete synthesis of all amplified DNA fragments. This careful optimization of conditions was crucial in maximizing the amplification of Archaea DNA while minimizing the unintended amplification of non-target DNA.

3.2.6.4 Minion sequencing device

The DNA is extracted from bacterial and archaeal samples using a suitable method that ensures high purity. The DNA concentration and purity are measured using a Qubit fluorometer, ensuring that the OD 260/280 ratio is around 1.8–2.0. The NEBNext Ultra II End Prep reagents are thawed on ice and mixed well. In a PCR tube, 11.5 µL of extracted DNA (130 ng) is added, and the volume is made up to 15 µL with nuclease-free water. Then, 1.75 µL of End-prep reaction buffer and 0.75 µL of End-prep enzyme mix are added and mixed well. The mixture is incubated in a thermal cycler at 20℃ for 5 minutes, followed by 65℃ for 5 minutes. The Native Barcodes (NB01-24) and NEB Blunt/TA Ligase Master Mix are thawed on ice. Then, 7.5 µL of end-prepped DNA is mixed with 2.5 µL of a unique barcode and 10 µL of ligase master mix. The reaction is incubated at room temperature for 20 minutes, and after incubation, 2 µL of EDTA is added to stop the reaction. To clean up the barcoded DNA, 0.4X volume of AMPure XP beads is added to the reaction mixture, and it is

incubated at room temperature on a Hula mixer for 10 minutes. The tubes are then placed on a magnetic rack to pellet the beads, and the beads are washed twice with 80% ethanol. The DNA is eluted by resuspending the beads in 10 µL of nuclease-free water, and the eluate is transferred to a new tube. For adapter ligation, the NEBNext Quick Ligation Reaction Module reagents are thawed and mixed. Then, 30 µL of pooled barcoded samples, 5 µL Native Adapter, 10 µL Ligation Buffer, and 5 µL Quick T4 DNA Ligase are added. The mixture is incubated at room temperature for 20 minutes, and the ligation reaction is cleaned up using AMPure XP beads, with the DNA eluted in 15 µL of Elution Buffer. For flow cell preparation and library loading, the flow cell is primed by loading 800 µL of priming mix (Flow Cell Flush and Tether). The DNA library is prepared by mixing 12 µL of the DNA library with 37.5 µL of Sequencing Buffer and 25.5 µL of Library Beads. Then, 75 µL of the prepared library is loaded onto the flow cell via the SpotON sample port. Sequencing is initiated using the Minion software, and the run is monitored to ensure data acquisition and basecalling are proceeding correctly. After sequencing, the data is analyzed using bioinformatics tool to identify bacterial and archaeal communities [33].

4. RESULTS AND DISCUSSION

The main objective of this study is to investigate the effect of KOH akali pre-treatment on agricultural waste in terms of VFA proction. In this study, sunflower hads and sunflowers stalks are choosen as an agricultural waste due to abundance and no effective use after crop. The waste pretreated with KOH for 24 hours and anaerobically digested at 37 degree celcius.

Alkali-based pretreatment technology is regarded as one of the most promising methods among the developed pretreatment approaches. This is primarily due to its high effectiveness in degreading lignocellulosic biomass and the resulting high total sugar yields [34]. Alkali pretreatment typically involves the use of strong alkaline solutions, such as NaOH or KOH, which break the ester bonds between hemicellulose and lignin. This process disrupts the α-O-4 and β-O-4 bonds, facilitating the removal of lignin and hemicellulose. Among these agents, NaOH is the most commonly used. Concentrated NaOH pretreatment can increase the cellulose content of lignocellulose from 30%-40% to 60%-65%, with the cellulose content generally rising as the alkali concentration and treatment time increase. However, the discharge of large amounts of sodium ions into the environment can pose a stress to other organisms and cause ecological problems. To mitigate these environmental hazards, KOH found to be used as a substitute for NaOH [34].

The pretreatment was done in sealed beherglasses at room temprature. The substrates were soaked into KOH solutions at different dosages as %0, %6, %8, %10 with a solid to liquid ratio of 100gTS/L. Pretreatment conducted for 24 hours. After the process, samples were seperated to solid and liquid parts for further analysis and anaerobic digestion process.

Determining the most efficient KOH pretreatment dosage, comparisons were made between the samples. This evaluation considered various factors, including the pretreatment's impact on the substrate's chemical composition, soluble chemical oxygen demand, volatile fatty acids production, and TS/VS removal.

Characterization studies of bacterial and archaeal communities were performed on the inoculum and on the samples with the highest VFA production. Sequencing methods were then employed to uncover the diversity of microbial communities within each digester. Subsequently, bioinformatics analyses were conducted to assess and compare bacterial diversity.

4.1 Effect of Pre-treatment on Soluble COD of Samples

During the pretreatment process, samples were saked into KOH solutions for 24 hours at root temprature. After, all different dosages of samples were seperated into solid and liquid fractions as can bee seen in Figure 4.1.

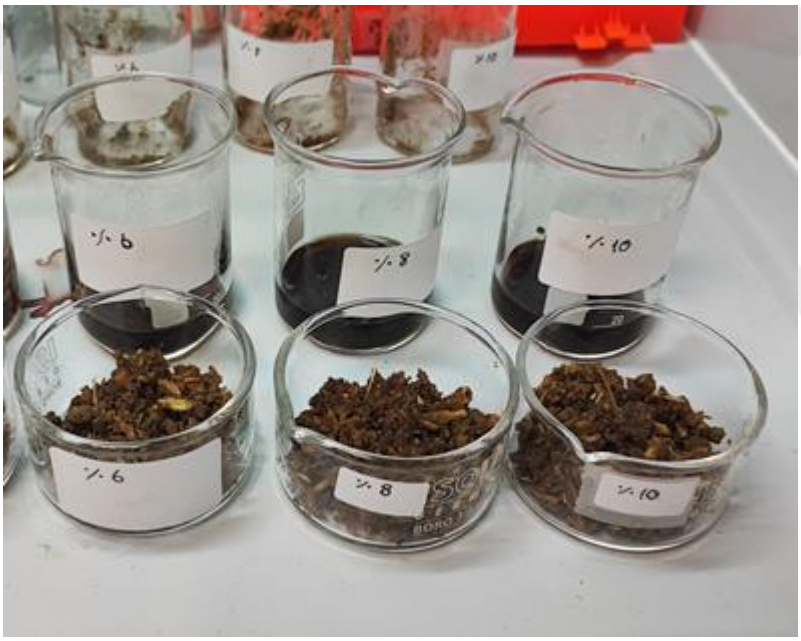

Figure 4.1 : Seperated sunflower samples after pretreatment.

During pretreatment, the swelling and hydrolysis of hemicelluloses and cellulose led to a noticeable increase in the release of chemical oxygen demand (COD) in the form of sugars and other organic compounds in the filtrates. This increase was evident in the color difference between the filtrates of treated and untreated samples, as shown in Figure 4.2. To quantify the COD released from the treated and untreated samples, soluble COD tests were conducted on the filtrates collected after 24 hours of pretreatment. A control sample with 0% KOH solution (using only deionized water) was used. For the test, the filtrates were diluted with deionized water at a 1:100 ratio.

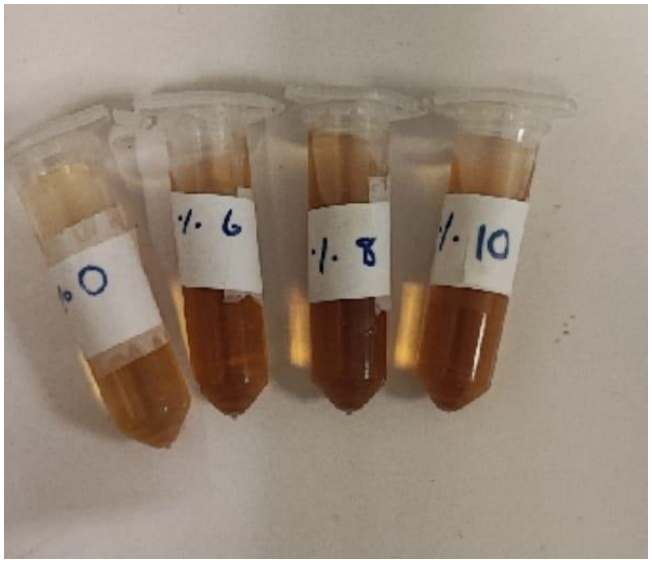

Figure 4.2 : Filtrates after seperation and dillution with 1:100 raito.

Color difference was observed through the samples are getting darker compared to %0 KOH solution pretreatment. This shows that the increase of KOH dosages also increases the released CODs to higher levels up to a certain point.

The sCOD analysis proved to be an effective method for evaluating pretreatment efficiency by measuring the COD levels released in filtrates from treated and untreated straw samples. A chart was created using these sCOD values to compare the impact of different KOH dosages on each straw sample, as shown in Figure 4.3. The chart reveals that the sCOD value for each sample increases with the KOH dosage, up to a certain point. Starting from its lowest value in untreated samples, the sCOD value rises as the KOH dosage increases, peaking at an 8% KOH concentration. This trend is attributed to the chemical reactions occurring during pretreatment between the substrate and KOH. At the 8% KOH dosage, more extensive chemical reactions take place, altering the substrate composition. This leads to greater removal of lignin, hemicellulose, and cellulose, and the production of sugars and other simpler chemical compounds, which are more readily digested by microorganisms.

The untreated sample which is %0 KOH, turns out to have 13200 mg/L sCOD. This amount increased up to 22200 mg/L with the %8 KOH pretreated sample and started to decrease to 17400 mg/L with %10 KOH sample. Highest sCOD value shows that more CODs released compared to other samples and became %60 higher than untreated sample.

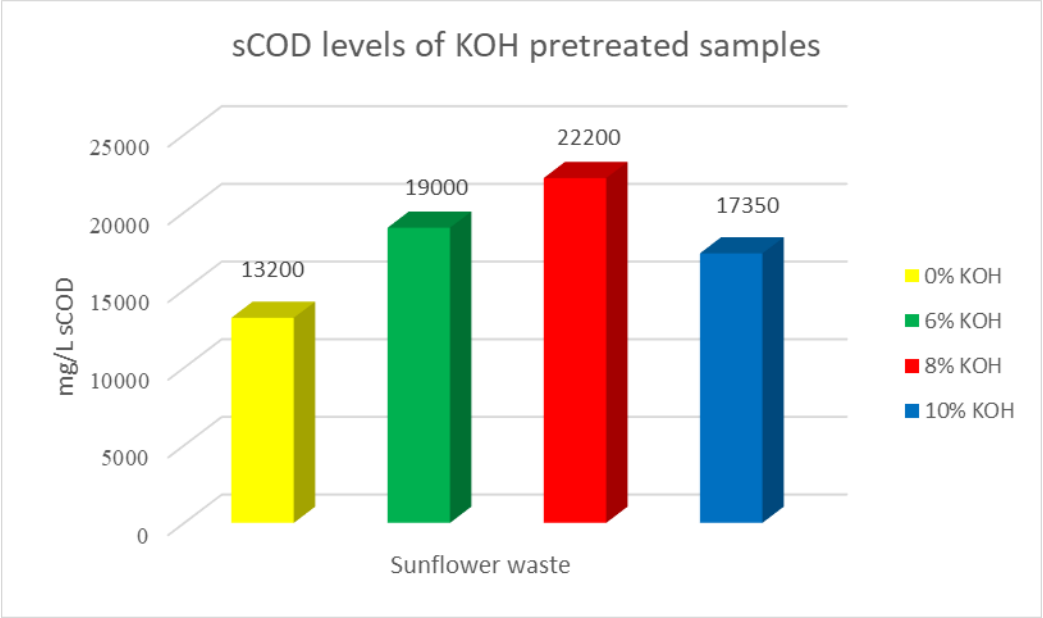

Figure 4.3 : sCOD values of pretreated samples.

The increase in sCOD with the application of KOH pretreatment can be attributed to the enhanced solubilization of organic matter in the samples. KOH is known to be effective in breaking down complex organic structures, such as lignocellulosic materials, thereby releasing soluble organic compounds that contribute to the sCOD. The observed trend is consistent with findings from previous studies, which have shown that alkaline pretreatment can significantly enhance the solubilization and subsequent degradation of organic matter [35, 36].

The peak sCOD level at 8% KOH indicates that this concentration is optimal for maximizing the release of soluble organics under the conditions tested. The 67.7% increase in sCOD compared to the control suggests that 8% KOH is highly effective in breaking down organic material, making it more accessible for further biological or chemical processing.

The sCOD level at 10% KOH was lower than at 8%, indicating that higher concentrations of KOH may lead to diminishing returns or even adverse effects. This could be due to excessive solubilization or degradation of organic matter, which might lead to the formation of inhibitory compounds or the loss of critical structural components necessary for microbial processing [37]. This finding highlights the importance of optimizing KOH concentration to balance the benefits of solubilization with the potential risks of over-treatment.

The results indicate that 8% KOH is the optimal concentration for maximizing sCOD levels in the samples, leading to improved solubilization of organic matter. This concentration not only enhances the availability of soluble organics but also potentially boosts VFA production, making it highly suitable for applications in waste treatment and bioenergy production. However, caution is needed when increasing KOH concentrations beyond this point, as the potential for negative effects becomes more pronounced.

4.2 TS/VS Removal

The examined the effect of selected KOH concentrations and control sample on the Total Solids (TS) and Volatile Solids (VS) of the samples. In Table 4.1. the results showed that the addition of 8% KOH had a noticeable impact on the removal efficiency of solids. Specifically, the Total Solids (TS) decreased slightly from 1.28 on Day 0 to 1.19 on Day 30, while the Volatile Solids (VS) also dropped from 0.86 on Day 0 to 0.77 on Day 30.

Table 4.1 : Removal percentages of samples in term of TS and VS.

Type	TS	VS
Control D0	1.28	0.86
Control D30	1.21	0.79
8% KOH D30	1.19	0.77

In Figure 4.4 results indicated that the addition of 8% KOH significantly enhanced the removal efficiency of solids. Specifically, the TS increased from 5.47 to 7, and the VS rose from 8.13 to 10.5. This represents a 28% increase in TS and a 29% increase in VS. The improvement in removal efficiency is closely linked to the enhanced production of Volatile Fatty Acids (VFAs), which are critical intermediates in the anaerobic digestion process and agricultural waste biodegradability [1].

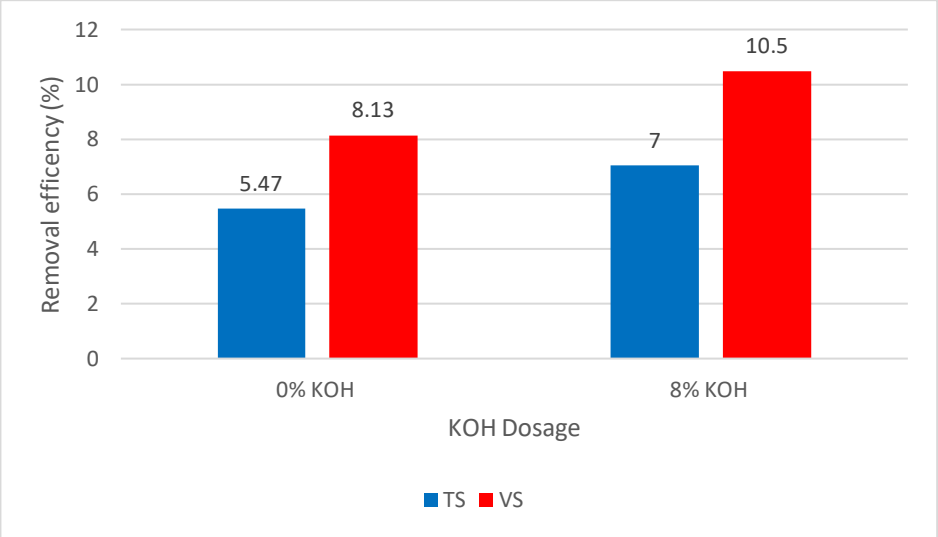

Figure 4.4 : Removal efficencies of anaerobic digestion setups.

The connection between the increased removal efficiency and VFA production is particularly noteworthy. VFAs are crucial intermediates in the anaerobic digestion process, serving as substrates for methane production in the final stages of digestion. The elevated levels of TS and VS observed in the samples treated with 8% KOH likely contribute to a higher production of VFAs, thereby improving the overall efficiency of the anaerobic digestion process. This is consistent with findings from other studies, where alkaline pretreatment has been shown to enhance the production of VFAs by breaking down complex organic materials into simpler, more readily degradable compounds [35, 37].

The role of KOH in improving VFA production is linked to its ability to disrupt the lignocellulosic structure of organic materials, thereby increasing the availability of soluble compounds that can be converted into VFAs. This mechanism is supported by the significant increase in both TS and VS, indicating that a higher concentration of degradable solids was made available for VFA production [38].

As a result, the use of 8% KOH significantly improves the removal efficiency of solids, with the observed increases in TS and VS being closely tied to enhanced VFA production.

4.3 VFA Production During Acidification

Volatile fatty acids (VFAs) can also be generated through microbial fermentation, as these acids are the final products of various fermentation pathways. Increasingly,

biological methods for VFA production are gaining attention due to their ability to utilize renewable carbon sources as raw materials, making these processes more environmentally sustainable [39].

Volatile fatty acids (VFAs), primarily consisting of acetate, propionate, isobutyrate, n-butyrate, iso-valerate, and n-valerate, can serve as a carbon source in biological nutrient removal processes [39]. During anaerobic digestion, samples were collected from digesters on days 0, 1, 2, 3, 4, 7, 10, 15, and 30, and the total VFA concentrations were measuredand calculated as the ppm and equivalent of total acetic acid.

In Figure 4.5. is visible that The 8% KOH pretreated samples exhibit a significantly higher cumulative VFA production compared to the control at every measured time point, with the final cumulative VFA reaching 15,738 mg/L sCOD equivalent, as opposed to 7,396 mg/L in the control. This clearly demonstrates that KOH pretreatment substantially enhances VFA production. The pretreated samples show a rapid increase in VFA concentration during the initial days (D0 to D4), with values rising from 2,838 mg/L to 9,808 mg/L, indicating that KOH pretreatment accelerates the degradation of organic matter in the early stages of the process. Following this initial surge, the rate of VFA production begins to stop, with a less pronounced increase by upcoming days, suggesting that most of the easily degradable material has been processed early in the treatment.

In contrast, the control samples (0% KOH) demonstrate a more moderate VFA production throughout the process, eventually reaching 7,396 mg/L sCOD equivalent by day 30. The gradual increase in VFA production suggests that, in the absence of KOH, the breakdown of organic matter is slower and less efficient. Unlike the pretreated samples, the control does not exhibit a rapid initial surge in VFA production. This indicates that organic matter is less accessible to microbial activity without KOH, which significantly affects the overall efficiency of VFA production. The comparison underscores the substantial impact of KOH pretreatment in enhancing both the rate and yield of VFA production, making the process far more efficient than in the absence of KOH.

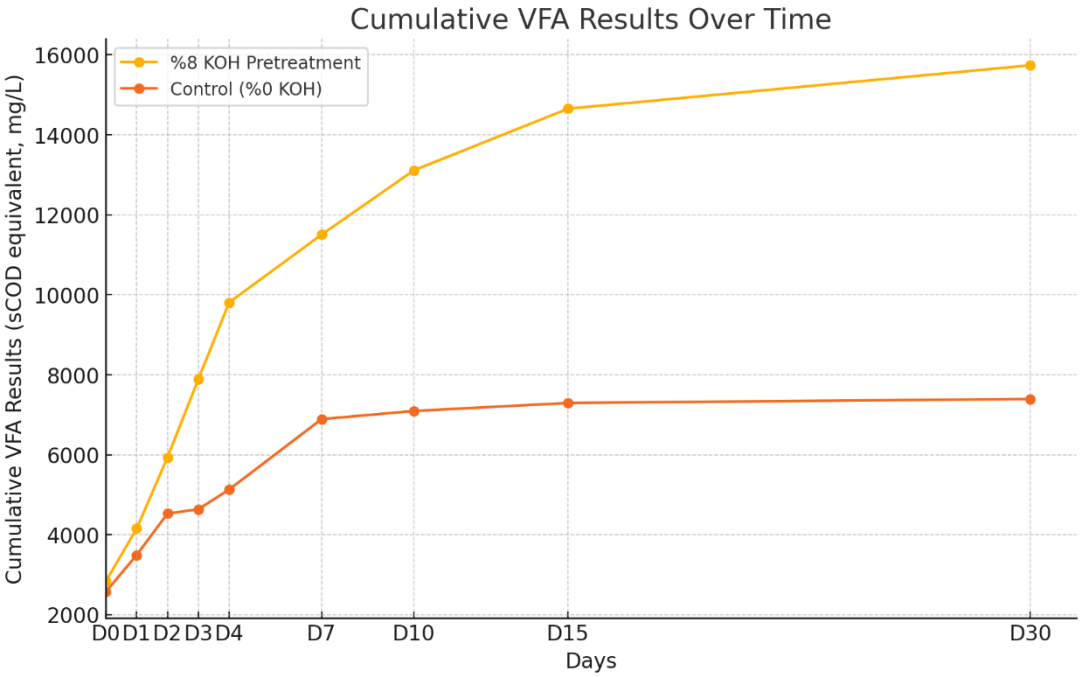

Figure 4.5 : Cumulative VFA result of pretreated and unpretreated samples.

The efficiency of 70.98% achieved for the 8% KOH pretreated sample indicates a significant conversion of soluble organic matter (sCOD) into volatile fatty acids (VFA) by Day 30. This result underscores the efficacy of KOH pretreatment in enhancing the anaerobic digestion process. KOH pretreatment effectively disrupts the lignocellulosic structure of biomass, increasing the accessibility of cellulose and hemicellulose to microbial activity, which in turn enhances VFA production.

Such findings are consistent with previous studies that have reported similar enhancements in VFA yield following alkaline pretreatment. For instance, Zhang et al. (2020) demonstrated that alkaline pretreatment significantly improves the solubilization of organic matter, thereby increasing the yield of VFAs during anaerobic digestion (46). Moreover, research by Jang et al. (2019) highlighted the role of KOH in breaking down lignocellulosic barriers, facilitating more efficient microbial degradation and leading to higher VFA production efficiencies [48].

These results suggest that 8% KOH is an optimal concentration for pretreatment, offering a balance between effective solubilization. The high conversion efficiency observed supports the continued use and exploration of KOH pretreatment in the enhancement of anaerobic digestion processes.

The data in Figure 4.6. represets the production of various Volatile Fatty Acids (VFAs) over 30 days from samples pretreated with 8% KOH. The VFAs analyzed include

acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid, caproic acid, and heptanoic acid. As a detailed analysis of each acid's production:

Acetic Acid: Acetic acid showed a substantial increase in concentration over the study period, starting with an initial concentration of 20.95 mg/L on Day 0 and reaching a final concentration of 416.27 mg/L by Day 30. The most significant growth occurred between Day 3, when the concentration was 179.05 mg/L, and Day 4, where it increased to 258.84 mg/L. By Day 30, acetic acid had increased approximately 19.9 times from its initial concentration.

Propionic Acid: Propionic acid exhibited the most significant increase among all VFAs, starting from an initial concentration of 168.69 mg/L on Day 0 and reaching a final concentration of 1601.55 mg/L by Day 30. The largest increments were observed between Day 1 (320.67 mg/L) and Day 2 (491.16 mg/L) and then again between Day 15 (1421.44 mg/L) and Day 30 (1601.55 mg/L). This nearly tenfold rise made propionic acid the most dominant VFA produced in the study.

Isobutyric Acid: Isobutyric acid also showed a significant increase in concentration, beginning at 161.93 mg/L on Day 0 and rising to 1418.2 mg/L by Day 30. A particularly sharp increase was noted between Day 3 (602.31 mg/L) and Day 4 (773.63 mg/L). By the end of the study period, isobutyric acid’s concentration was approximately 8.8 times higher than at the start, indicating robust production.

Butyric Acid: Butyric acid closely followed propionic acid in terms of its increase, with the concentration rising from 155.59 mg/L on Day 0 to 1513.90 mg/L by Day 30. Notable growth was seen between Day 3 (683.51 mg/L) and Day 4 (867.60 mg/L) and later between Day 15 (1393.52 mg/L) and Day 30 (1513.90 mg/L). The overall increase was nearly tenfold from the initial concentration.

Isovaleic Acid: Isovaleric acid production was significant, increasing from 196.97 mg/L on Day 0 to 1572.69 mg/L by Day 30, which is about an eightfold increase. The sharpest rise occurred between Day 15 (1392.50 mg/L) and Day 30 (1572.69 mg/L), suggesting that isovaleric acid may be a key component during the later stages of VFA production.

Valeic Acid: Valeric acid saw its concentration rise from 151.90 mg/L on Day 0 to 1056.92 mg/L by Day 30, reflecting an approximate sevenfold increase. Notable jumps

in concentration were observed between Day 3 (477.87 mg/L) and Day 4 (600.53 mg/L).

Isocaproic Acid: Isocaproic acid showed a more moderate increase compared to other VFAs, starting at 135.93 mg/L on Day 0 and rising to 533.67 mg/L by Day 30. This represents about a fourfold increase, with steady growth and significant changes particularly between Day 3 (324.32 mg/L) and Day 4 (370.37 mg/L).

Caproic Acid: Caproic acid followed a trend similar to isocaproic acid, with its concentration increasing from 167.36 mg/L on Day 0 to 643.62 mg/L by Day 30. This steady rise represents about a 3.8-fold increase over the study period.

Heptanoic Acid: Heptanoic acid showed the least increase among the VFAs, with its concentration rising from 149.05 mg/L on Day 0 to 434.47 mg/L by Day 30. The growth was steady but less pronounced compared to the other acids, resulting in a threefold increase from the initial concentration.

The study demonstrates that pretreatment with 8% KOH is highly effective in enhancing the production of volatile fatty acids (VFAs) over a 30-day period. The VFAs of greatest significance in this process include propionic acid, butyric acid, and isovaleric acid. These three acids reached the highest concentrations by the end of the study, indicating their prominence in the production process. Among them, propionic acid stands out as the most dominant, with a final concentration of 1601.55 mg/L by Day 30. This substantial increase suggests that propionic acid is particularly favored under the conditions provided by the KOH pretreatment.

The steady and substantial increase in propionic acid, as well as the significant production of butyric and isovaleric acids, can be explained by several factors. One key factor is the alkaline environment created by the KOH pretreatment, which likely improves the breakdown of complex organic materials into simpler substrates. These simpler substrates are more easily converted into VFAs by microbial communities. Previous studies have shown that alkaline pretreatment methods, like using KOH, enhance the accessibility of lignocellulosic biomass, thereby boosting VFA production during anaerobic digestion [41]. This increase in substrate availability could explain the high concentrations of propionic, butyric, and isovaleric acids observed in this study.

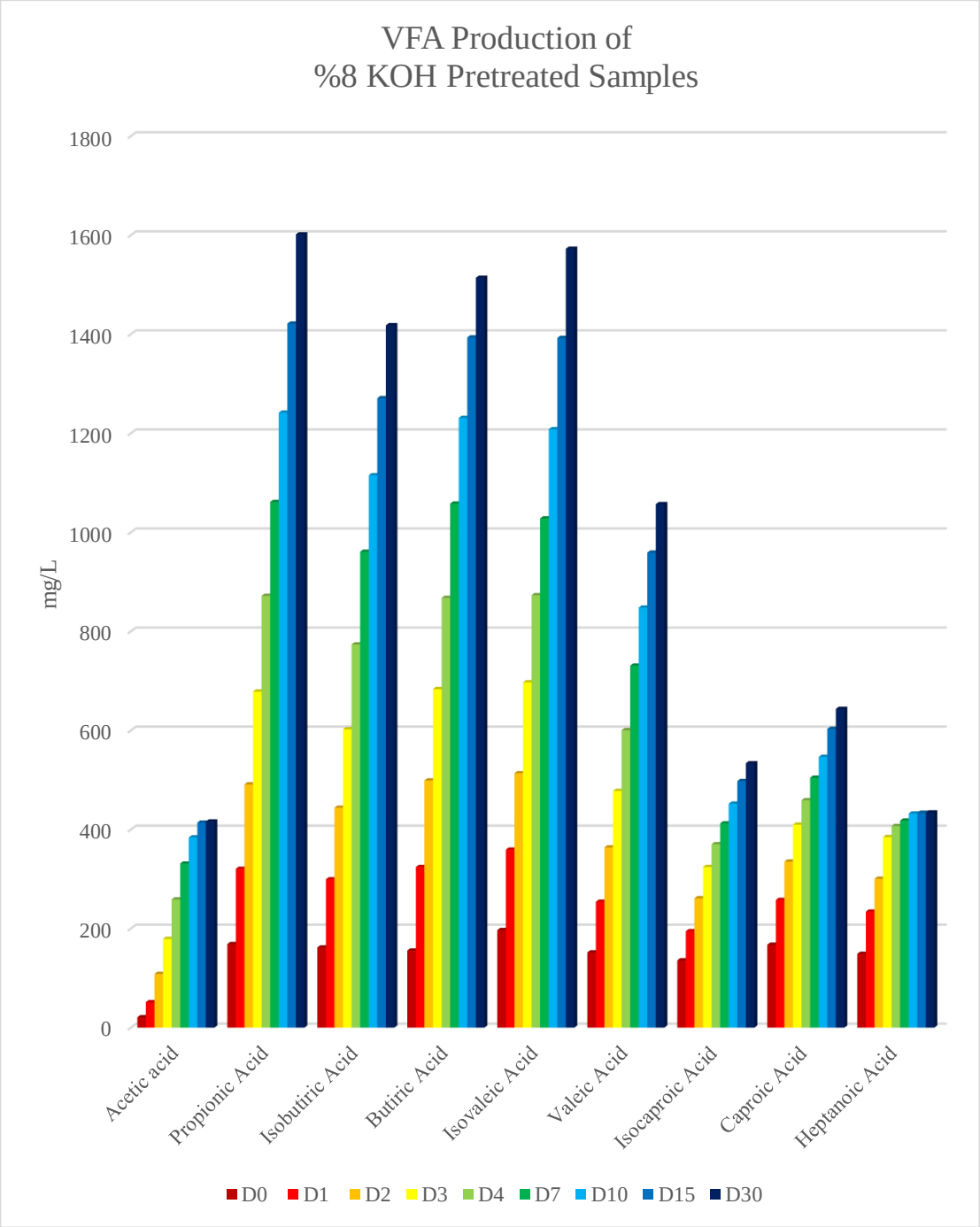

Figure 4.6 : VFA Production of %8 KOH pretreated samples.

Propionic acid, which proved to be the most dominant VFA, is well-regarded for its industrial importance, particularly as a preservative in the food industry and in the production of bioplastics [42].

Butyric acid, which closely followed propionic acid in terms of production, also holds considerable industrial value. It is a key precursor for the synthesis of butyrate esters, which are used as flavoring agents and solvents [43]. Additionally, butyric acid is of interest for its potential in the production of renewable biofuels [44]. The significant

increase in butyric acid observed in this study suggests that the KOH pretreatment effectively supports its production, providing a promising approach for industrial applications.

Isovaleric acid, the third most abundant VFA produced, has applications in the flavor and fragrance industries due to its distinctive odor [45]. The substantial increase in its concentration, particularly in the latter half of the study, indicates that isovaleric acid production may be favored during the later stages of fermentation, potentially offering opportunities for selective production strategies.

Acetic acid, although starting from a lower initial concentration, exhibited a significant increase by Day 30. Acetic acid is one of the most versatile VFAs, with applications ranging from its use as a solvent and chemical intermediate to its role in vinegar production [46]. The substantial increase observed in this study

suggests that, even though acetic acid was not the most dominant VFA, its production was still enhanced by the KOH pretreatment, making it a valuable co-product in the overall VFA profile.

In contrast to the dominant VFAs, isocaproic acid, caproic acid, and heptanoic acid exhibited more moderate increases. These acids, while less abundant, still play a role in the VFA profile and have specific industrial applications. For example, caproic acid is a key intermediate in the production of esters used in the fragrance and flavor industries [47]. The steady increase in these acids suggests that the KOH pretreatment also facilitates their production, albeit to a lesser extent.

Overall, the findings from this study highlight the importance of propionic, butyric, and isovaleric acids as the primary VFAs produced under KOH pretreatment conditions. Acetic acid also plays a significant role, albeit at lower concentrations. The potential for optimizing this process to enhance the production of these key VFAs could have substantial industrial implications, particularly in the fields of bioplastics, biofuels, and specialty chemicals.

4.4 Bacterial Community Analysis

The aim of this analysis was to investigate changes in bacterial community over a 30-day anaerobic digestion period, focusing on volatile fatty acid (VFA) production rather than methanogenesis. The results obtained from DNA sequencing data on days 0 and

30 indicate significant shifts in microbial composition, reflecting changes in metabolic activity favoring VFA accumulation.

The Shannon and Simpson indices were used to measure the diversity of microbial communities, with the Shannon index assessing species richness and evenness, and the Simpson index highlighting species dominance as shown in the Table 4.2. below.

Table 4.2 : Shannon and Simpson index comparison.

	Shannon Index		Simpson Index	
	Day 0	**Day 30**	**Day 0**	**Day 30**
16S	6.512	4.684	0.973	0.882
18S	4.738	4.583	0.828	0.921
Archaea	1.722	2.925	0.396	0.744

4.4.1 16S RNA sequencing

The analysis of the 16S rRNA gene sequencing data from days 0 and 30 revealed significant changes in the bacterial community structure. As it can be seen in Figure 4.7. at day 0, the bacterial population was diverse, with *Bacteroidales bacterium* (8.9%), *Smithellaceae bacterium* (6.07%), *Chloroflexota bacterium* (5.9%), and *Synergistaceae bacterium* (5.54%) being the most abundant species.

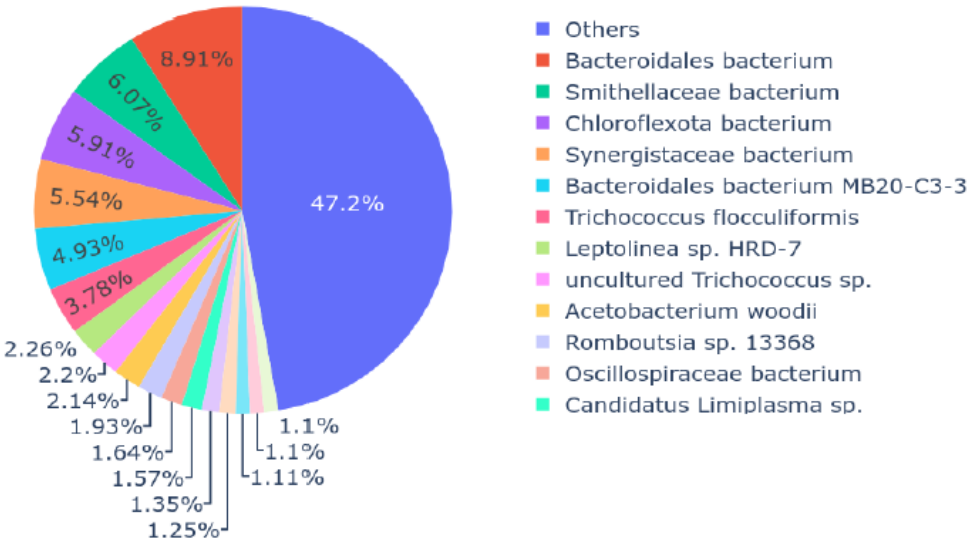

Figure 4.7 : 16S Species level taxonomic comparison of seed sludge (Day 0).

By day 30, as in the Figure 4.8. the community composition had shifted dramatically. *Romboutsia sp.* became the most dominant genus, making up 30.26% of the total bacterial abundance. Other species such as *Intestinibacter bartlettii* (6.09%) and

Coprothermobacter proteolyticus (7.04%) also saw significant increases. In contrast, the previously dominant species, *Bacteroidales bacterium* and *Smithellaceae bacterium*, showed marked decreases in their relative abundances to below 1%.

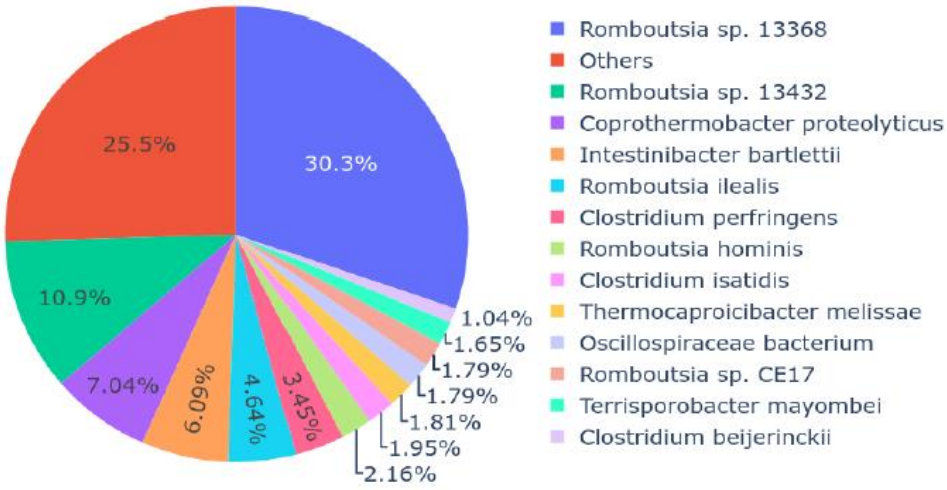

Figure 4.8 : 16S Species level taxonomic comparison of sample (Day 30).

The Shannon diversity index of the analysis decreased from 6.512 on day 0 to 4.684 on day 30, indicating a reduction in species evenness over time. Similarly, the Simpson index suggested a shift towards dominance by a few species as the overall community diversity decreased.

On day 0, the microbial community was characterized by a diverse range of bacteria involved in hydrolytic and acidogenic processes. The high abundance of species such as *Bacteroidales bacterium* and *Synergistaceae bacterium* suggests that the initial stages of anaerobic digestion, particularly the breakdown of complex organic matter into simpler molecules, were actively taking place.

By day 30, the dominance of *Romboutsia sp.* indicated a clear shift towards fermentative processes. The conditions in the reactor, which were set to inhibit methanogenesis, likely created an environment conducive to the accumulation of VFAs, favoring fermentative bacteria such as *Romboutsia* and *Coprothermobacter proteolyticus*. These bacteria are known to thrive in environments with high concentrations of VFAs, where they ferment organic compounds into acetate, butyrate, and other short-chain fatty acids.

The decrease in diversity over time, as indicated by the Shannon and Simpson indices, aligns with the notion that high-VFA concentrations create selective pressures that reduce species richness and evenness. Zhang et al. (2016) observed similar patterns in

anaerobic digestion systems, where only a few species adapted to the acidic conditions dominated the microbial community [48]. This shift in dominance likely reflects the reduction in substrate availability and the accumulation of VFAs, which inhibit the growth of many acid-sensitive hydrolytic and acidogenic bacteria.

4.4.2 18S RNA sequencing

The 18S rRNA sequencing results for Day 0 and Day 30 show significant shifts in the eukaryotic microbial community composition over the 30-day anaerobic digestion period.

The microbial community at Day 0 as it can be seen in Figure 4.9. was dominated by *Stephanoeca diplocostata* (40.32%), which accounted for a large portion of the total abundance. Other notable species included *Catenomyces persicinus* (5.92%), *Adineta vaga* (4.44%), and *Pigoraptor vietnamica* (3.58%). Additionally, various species, such as *Neocallimastix frontalis* (2.34%) and *Hartaetosiga balthica* (1.97%), were present in smaller proportions, contributing to the overall diversity. Species with lower abundances, such as *Tetramitus dokdoensis* (0.62%) and *Mucor amphibiorum* (0.62%), were also part of the community but made up a minor percentage of the total population.

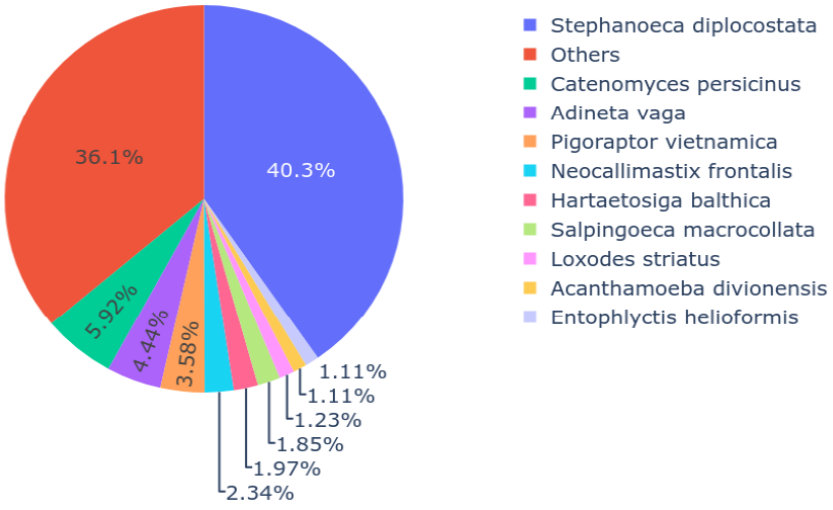

Figure 4.9 : 18S Species level taxonomic comparison of seed sludge (Day 0).

By Day 30, the composition of the microbial community had shifted, as it can bee seen in Figure 4.10. *Tetramitus dokdoensis* emerging as the dominant species, comprising 23.1% of the total abundance. Other prominent species included *Parascedosporium tectonae* (9.33%) and *Stephanoeca diplocostata* (6.50%), which saw a decrease

compared to its initial dominance on Day 0. Species such as *Aspergillus nomiae* (4.81%) and *Catenomyces persicinus* (3.63%) remained prevalent but at lower percentages. Additionally, *Prototheca ciferrii* (2.79%) and *Cryptosporidium struthionis* (2.07%) were part of the community at lower abundance levels.

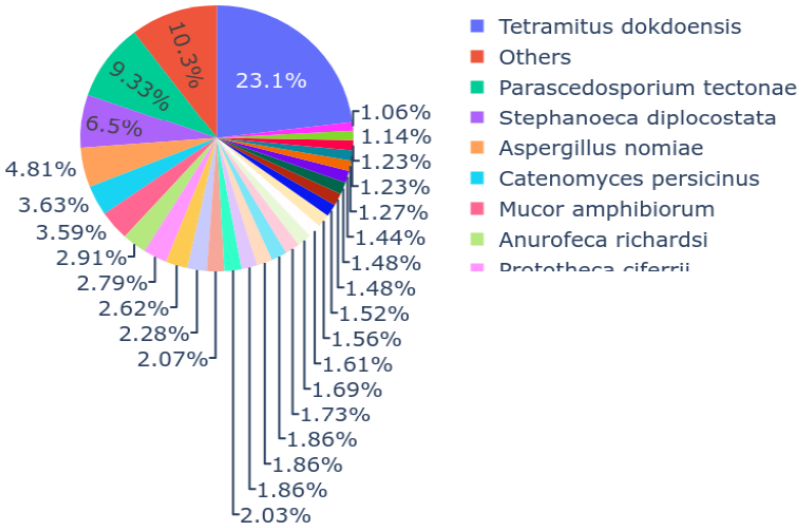

Figure 4.10 : 18S Species level taxonomic comparison of sample (Day 30).

On Day 0, the dominance of *Stephanoeca diplocostata* suggests its key role in the early stages of organic matter degradation. Protists like *Stephanoeca* are known for their ability to feed on bacteria and organic matter in anaerobic environments, facilitating the breakdown of complex organic compounds [49]. The high abundance of other species, such as *Catenomyces persicinus* and *Adineta vaga*, further indicates a diverse microbial community capable of handling a wide range of substrates at the beginning of the digestion process.

By Day 30, the rise of *Tetramitus dokdoensis* as the most dominant species signifies a major shift in the microbial ecosystem. *Tetramitus* species are often associated with environments where nutrient levels are low or where the microbial ecosystem faces stress due to environmental changes, such as increased VFA concentrations. This shift aligns with findings from Bardon et al. (2018), who reported that certain protists thrive under stress conditions, including high acidity and low nutrient availability, making them well-suited for environments that favor VFA accumulation [50]. The increased presence of *Parascedosporium tectonae* and *Aspergillus nomiae* points to the growing role of fungi in the later stages of anaerobic digestion. Fungi are known for their ability to degrade complex organic molecules, such as lignin and cellulose, making them key players in anaerobic environments where more recalcitrant substrates remain [51].

The reduction in diversity over time, as indicated by the Shannon and Simpson indices, suggests that the selective pressures imposed by VFA accumulation favored certain acid-tolerant species while inhibiting others. The decline in species richness is consistent with observations by Van Lier et al. (2001), who noted that microbial communities in anaerobic digesters become more specialized as the process progresses, especially in systems aimed at VFA production rather than methanogenesis [52].

4.4.3 Archea analysis

The analysis of the archaeal community composition of set up from Day 0 to Day 30 shows the development of a diverse microbial ecosystem throughout the anaerobic digestion process. This community structure highlights the ongoing breakdown of complex substrates and the adaptation of the microbial community to support VFA production. The presence and dynamics of various microbial groups reflect the system's ability to maintain balanced fermentation and acidogenic activities.

At Day 0, as can be seen in Figure 4.11. the archaeal community was dominated by *Methanothrix soehngenii* (77.35%). Other species included *Methanosphaerula palustris* (6.83%), *Methanosarcina vacuolata* (2.01%), *Ruminococcus albus* (1.80%), and *Methanomassiliicoccus luminyensis* (1.46%). These species contributed to the overall community composition at lower abundances.

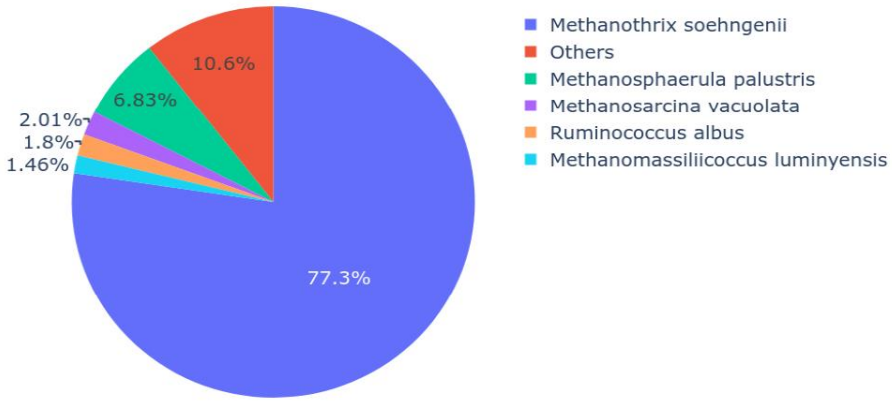

Figure 4.11 : Archeal community species level taxonomic comparison of seed sludge (Day 0).

By Day 30, in the figure 4.12. the archaeal community shifted, with *Methanosarcina vacuolata* (37.78%) and *Methanosarcina barkeri* (32.33%) becoming dominant. Other

species such as *Methanomassiliicoccus luminyensis* (6.37%), *Methanothrix soehngenii* (5.44%), and *Methanoculleus palmolei* (1.69%) were present at varying levels.

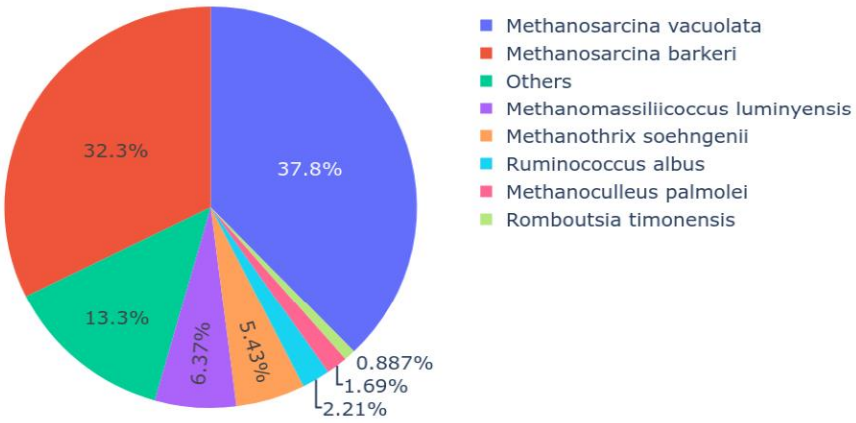

Figure 4.12 : Archeal community species level taxonomic comparison of seed sludge (Day 30).

The Shannon diversity index increased from 1.72 at Day 0 to 2.93 at Day 30, while the Simpson index rose from 0.396 to 0.744.

The archaeal community shifts observed over the 30-day period indicate that the system successfully fostered a balanced environment conducive to VFA production. The detected methanogens, while present, did not dominate the microbial community, ensuring that VFA pathways remained active throughout the digestion process. This microbial diversity and adaptability helped stabilize the system, supporting the intended goal of maximizing VFA accumulation.

4.5 Cost Analysis

Cost analysis of volatile fatty acid (VFA) production demonstrates that the use of KOH pretreatment is highly effective in enhancing the breakdown of biomass. This treatment improves the accessibility of organic material to microbial digestion, significantly increasing the yield of certain valuable VFAs such as isovaleric acid and isobutyric acid. These high-value VFAs, produced through the anaerobic digestion process, are integral to a variety of industrial applications, including biofuels and bioplastics. The economic benefits of focusing on these acids are substantial, especially when scaled from lab-scale experiments to commercial production.

The following Table 4.3. provides an analysis of VFA production from lab scale and calculated with one ton to estimate real scale , with corresponding market prices and

potential profitability [55]. From the lab-scale experiments using 1.5 grams of substrate, scaling up to 1 ton of substrate allows for a quantitative assessment of VFA production yields and potential profits. Isovaleric acid produces 104.87 kg per ton and is priced at $3,500 per ton, leading to a total profit of $366.05. Propionic acid which is the most produced resulted 106.73 kg and $134 profit. In contrast, acetic acid, which is produced in smaller quantities (27.73 kg per ton), generates $13.87 in profit due to its lower amount and lower market price of $500 per ton.

Table 4.3 : Estimated VFA revenue based on current market prices.

VFA Type	Substrate Production (Lab) (grams)	Substrate Production (Real) (kg)	Price per Ton (USD)	Total Profit (USD)
Acetic Acid	0.0416	27.73	$500	$13.87
Propionic Acid	0.1601	106.73	$1,250	$133.41
Isobutyric Acid	0.1418	94.53	$2,750	$260.96
Butyric Acid	0.1514	100.93	$1,800	$181.68
Isovaleric Acid	0.1573	104.87	$3,500	$366.05
Valeric Acid	0.1057	70.47	$1,500	$105.71
Isocaproic Acid	0.0534	35.60	$2,500	$89.00
Caproic Acid	0.0644	42.93	$2,500	$107.33
Heptanoic Acid	0.0434	28.93	$2,500	$72.33

Economically, the focus should be on producing these high-value VFAs, as they offer the best return on investment. By optimizing the anaerobic digestion process to increase the production of isovaleric and isobutyric acids, industries can enhance profitability while also supporting environmental sustainability. Producing VFAs from organic waste reduces landfill use and minimizes greenhouse gas emissions, aligning with circular economy principles. This method not only generates revenue but also addresses environmental challenges.ating the economic and environmental benefits of VFA production.

5. CONCLUSION AND RECOMMENDATIONS

The study focused on the impact of KOH pretreatment on agricultural waste, specifically sunflower heads and stalks, to enhance VFA (Volatile Fatty Acid) production during anaerobic digestion. Through multiple pretreatment dosages (0%, 6%, 8%, and 10% KOH), the key findings can be summarized as soluble COD (sCOD) production The highest sCOD was observed in the 8% KOH pretreated sample, with a peak value of 22200 mg/L. This represents a significant increase compared to the control (13200 mg/L). This result indicates that the 8% KOH dosage was optimal for releasing soluble organic compounds, enhancing the substrate's availability for further degradation. TS/VS removal resulted with significant improvement in total solids (TS) and volatile solids (VS) removal was noted with the 8% KOH treatment. TS and VS removal efficiencies increased by 28% and 29%, respectively, indicating enhanced breakdown of the organic matter in the sample. This further contributed to the higher production of VFAs and overall biodegradability of the waste material. The cumulative VFA production in the 8% KOH pretreated sample reached a final concentration of 15,738 mg/L, significantly higher than the control (7396 mg/L). Propionic acid was the most abundant VFA produced, with an increase from 168.69 mg/L to 1601.55 mg/L over the 30-day anaerobic digestion period. Other VFAs such as butyric and acetic acids also exhibited substantial increases, confirming that KOH pretreatment enhances the efficiency of organic matter conversion into VFAs. The microbial community shifted significantly by Day 30, with Romboutsia sp. becoming the most dominant species, increasing from 0% to 30.26%. The reduction in microbial diversity over time suggests selective pressures induced by the elevated VFA concentrations, favoring species that thrive in such acidic environments.

In conclusion, the application of 8% KOH was found to be the most effective pretreatment dosage, optimizing sCOD release, VFA production, and microbial community structure. This pretreatment method has potential applications in the enhancement of biogas production and other bioenergy-related processes, making it a promising approach for agricultural waste management and resource recovery.

However, caution must be taken to avoid excessive KOH concentrations, which may result in diminishing returns or adverse effects on microbial activity.

Future research should focus on refining process parameters, particularly in optimizing pretreatment variables, to further enhance VFA production efficiency. Additionally, exploring the industrial application potential of this process at a larger scale is crucial, as integrating it into existing waste management systems could maximize both environmental and economic benefits. Monitoring microbial community dynamics after pretreatment is equally important to ensure the long-term stability and sustained performance of the anaerobic digestion process over time. These steps will contribute to the practical viability and effectiveness of the method in real-world applications.

REFERENCES

[1] **Chandra, R., Takeuchi, H., & Hasegawa, T.** (2012). Methane production from lignocellulosic agricultural crop wastes: A review in context to second generation of biofuel production. *Renewable and sustainable energy reviews*, *16*(3), 1462-1476.

[2] **Kumari, D., & Singh, R.** (2018). Pretreatment of lignocellulosic wastes for biofuel production: a critical review. *Renewable and Sustainable Energy Reviews*, *90*, 877-891.

[3] **Blasi, A., Verardi, A., Lopresto, C. G., Siciliano, S., & Sangiorgio, P. (**2023). Lignocellulosic agricultural waste valorization to obtain valuable products: An overview. *Recycling*, *8*(4), 61.

[4] **Amin, F. R., Khalid, H., Zhang, H., Rahman, S. U., Zhang, R., Liu, G., & Chen, C.** (2017). Pretreatment methods of lignocellulosic biomass for anaerobic digestion. *Amb Express*, *7*, 1-12.

[5] **Hesami, S. M., Zilouei, H., Karimi, K., & Asadinezhad, A.** (2015). Enhanced biogas production from sunflower stalks using hydrothermal and organosolv pretreatment. *Industrial Crops and Products*, *76*, 449-455.

[6] **Rani, G. M., Pathania, D., Umapathi, R., Rustagi, S., Huh, Y. S., Gupta, V. K., ... & Chaudhary, V.** (2023). Agro-waste to sustainable energy: A green strategy of converting agricultural waste to nano-enabled energy applications. *Science of The Total Environment*, *875*, 162667.

[7] **Mao, C., Feng, Y., Wang, X., & Ren, G.** (2015). Review on research achievements of biogas from anaerobic digestion. *Renewable and sustainable energy reviews*, *45*, 540-555.

[8] **Angelidaki, I., Treu, L., Tsapekos, P., Luo, G., Campanaro, S., Wenzel, H., & Kougias, P. G.** (2018). Biogas upgrading and utilization: Current status and perspectives. *Biotechnology advances*, *36*(2), 452-466.

[9] **Zheng, Y., Zhao, J., Xu, F., & Li, Y.** (2014). Pretreatment of lignocellulosic biomass for enhanced biogas production. *Progress in energy and combustion science*, *42*, 35-53.

[10] **Nargotra, P., Sharma, V., Gupta, M., Kour, S., & Bajaj, B. K. (2018). Application of ionic liquid and alkali** pretreatment for enhancing saccharification of sunflower stalk biomass for potential biofuel-ethanol production. *Bioresource technology*, *267*, 560-568.

[11] **Varjani, S. J., Gnansounou, E., Gurunathan, B., Pant, D., & Zakaria, Z. A.** (Eds.). (2017). *Waste bioremediation*. Springer.

[12] **Toerien, D. F., & Hattingh, W. H. J.** (1969). Anaerobic digestion I. The microbiology of anaerobic digestion. *Water Research*, *3*(6), 385-416.

[13] **Öter, M.,** (2015). Anaerobic Mono-Digestion and Co-Digestion of Agricultural Wastes and Comparison of Valorization Potentials

[14] **Appels, L., Baeyens, J., Degrève, J., & Dewil, R.** (2008). Principles and potential of the anaerobic digestion of waste-activated sludge. *Progress in energy and combustion science*, *34*(6), 755-781.

[15] **Mao, C., Feng, Y., Wang, X., & Ren, G.** (2015). Review on research achievements of biogas from anaerobic digestion. Renewable and sustainable energy reviews, 45, 540-555.

[16] **Zhang, C., Su, H., Baeyens, J., & Tan, T.** (2014). Reviewing the anaerobic digestion of food waste for biogas production. Renewable and Sustainable Energy Reviews, 38, 383-392.

[17] **Mao, C., Feng, Y., Wang, X., & Ren, G.** (2015). Review on research achievements of biogas from anaerobic digestion. Renewable and sustainable energy reviews, 45, 540-555.

[18] **Gerardi, M. H.** (2003). The microbiology of anaerobic digesters. John Wiley & Sons.

[19] **Kim, N. J., Park, G. W., Kang, J., Kim, Y. C., & Chang, H. N.** (2013). Volatile fatty acid production from lignocellulosic biomass by lime pretreatment and its applications to industrial biotechnology. Biotechnology and bioprocess engineering, 18, 1163-1168.

[20] **Park, G. W., Seo, C., Jung, K., Chang, H. N., Kim, W., & Kim, Y. C.** (2015). A comprehensive study on volatile fatty acids production from rice straw coupled with microbial community analysis. Bioprocess and biosystems engineering, 38, 1157-1166.

[21] **Parchami, M., Wainaina, S., Mahboubi, A., I'Ons, D., & Taherzadeh, M. J. (**2020). MBR-assisted VFAs production from excess sewage sludge and food waste slurry for sustainable wastewater treatment. Applied Sciences, 10(8), 2921.

[22] **Cai, J., He, Y., Yu, X., Banks, S. W., Yang, Y., Zhang, X., ... & Bridgwater, A. V**. (2017). Review of physicochemical properties and analytical characterization of lignocellulosic biomass. Renewable and sustainable energy reviews, 76, 309-322.

[23] **Dutta, N., Usman, M., Ashraf, M. A., Luo, G., Gamal El-Din, M., & Zhang, S.** (2023). Methods to convert lignocellulosic waste into biohydrogen, biogas, bioethanol, biodiesel and value-added chemicals: a review. Environmental Chemistry Letters, 21(2), 803-820.

[24] **Agbor, V. B., Cicek, N., Sparling, R., Berlin, A., & Levin, D. B.** (2011). Biomass pretreatment: fundamentals toward application. Biotechnology advances, 29(6), 675-685.

[25] **Cai, J., He, Y., Yu, X., Banks, S. W., Yang, Y., Zhang, X., ... & Bridgwater, A. V**. (2017). Review of physicochemical properties and analytical characterization of lignocellulosic biomass. Renewable and sustainable energy reviews, 76, 309-322.

[26] **Taherzadeh, M. J., & Karimi, K**. (2008). Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. International journal of molecular sciences, 9(9), 1621-1651.

[27] **Mankar, A. R., Pandey, A., Modak, A., & Pant, K. K.** (2021). Pretreatment of lignocellulosic biomass: A review on recent advances. Bioresource Technology, 334, 125235.

[28] **Ashokkumar, V., Venkatkarthick, R., Jayashree, S., Chuetor, S., Dharmaraj, S., Kumar, G., ... & Ngamcharussrivichai, C.** (2022). Recent advances in lignocellulosic biomass for biofuels and value-added bioproducts-A critical review. Bioresource technology, 344, 126195.

[29] **Srivastava, N., Srivastava, M., Mishra, P. K., & Gupta, V. K. (Eds.).** (2020). Substrate analysis for effective biofuels production. Springer Nature.

[30] **Nargotra, P., Sharma, V., Gupta, M., Kour, S., & Bajaj, B. K**. (2018). Application of ionic liquid and alkali pretreatment for enhancing saccharification of sunflower stalk biomass for potential biofuel-ethanol production. Bioresource technology, 267, 560-568.

[31] **Monlau, F., Trably, E., Barakat, A., Hamelin, J., Steyer, J. P., & Carrere, H.** (2013). Two-stage alkaline–enzymatic pretreatments to enhance biohydrogen production from sunflower stalks. Environmental science & technology, 47(21), 12591-12599.

[32] **Massive Bioinformatics. (n.d.).** DNA Marker Ladder (DL2-5-15-100) (v1.2). Erişim tarihi: [01.09.2024].

[33] **Oxford Nanopore Technologies.** (2024). Ligation sequencing amplicons - Native Barcoding Kit 24 V14 (SQK-NBD114.24) (Version NBA_9168_v114_revO_15Sep2022). Oxford Nanopore Technologies. https://nanoporetech.com

[34] **Xu, H., Li, B., & Mu, X.** (2016). Review of alkali-based pretreatment to enhance enzymatic saccharification for lignocellulosic biomass conversion. Industrial & Engineering Chemistry Research, 55(32), 8691-8705.

[35] **Chen, Y., Liu, R., & Meng, X.** (2015). Effects of alkaline pretreatment on the structure of lignocellulosic biomass and enzymatic hydrolysis. Bioresource Technology, 192, 53-59.

[36] **Lee, S. H., Choi, W., & Kim, J.** (2017). Optimization of alkaline pretreatment conditions for enhanced enzymatic saccharification of biomass. Renewable Energy, 113, 1259-1266.

[37] **Li, H., Gao, W., & Zhao, Y.** (2018). The role of alkaline treatment in improving the efficiency of anaerobic digestion of lignocellulosic materials. Journal of Environmental Management, 22Mao, C., Feng, Y., Wang, X., & Ren, G. (2017). Review on research achievements of biogas from anaerobic digestion. Renewable and Sustainable Energy Reviews, 45, 540-555.3, 848-857.

[38] **Bhatia, S. K., & Yang, Y. H.** (2017). Microbial production of volatile fatty acids: current status and future perspectives. Reviews in Environmental Science and Bio/Technology, 16, 327-345.

[39] **Wang, Q., Jiang, J., Zhang, Y., & Li, K.** (2015). Effect of initial total solids concentration on volatile fatty acid production from food waste during anaerobic acidification. Environmental technology, 36(15), 1884-1891.

[40] **Liew, Y. X., Chong, G. H., Ng, K. S., & Tham, M. K.** (2016). A review on the pretreatment of lignocellulosic biomass for enhanced biogas production. Chemical Engineering Communications, 203(11), 1435-1448.

[41] **Liew, Y. X., Chong, G. H., Ng, K. S., & Tham, M. K.** (2016). A review on the pretreatment of lignocellulosic biomass for enhanced biogas production. Chemical Engineering Communications, 203(11), 1435-1448.

[42] **Wainaina, S., Awasthi, M. K., Sarsaiya, S., Chen, H., Singh, E., Kumar, A., & Wong, J. W. C.** (2019). Bioengineering of anaerobic digestion for volatile fatty acids production: Recent advances and future perspectives. Bioresource Technology, 292, 122566.

[43] **Dwidar, M., Park, J. Y., Mitchell, R. J., & Sang, B. I.** (2012). The future of butyric acid in industry. The Scientific World Journal, 2012.

[44] **Huang, W., Zhai, X., & Jia, B.** (2015). Butyric acid production from food waste by fermentation: Engineering strategies. Critical Reviews in Biotechnology, 35(1), 68-78.

[45] **González-Fernández, C., Sialve, B., Bernet, N., & Steyer, J. P.** (2015). Impact of microalgal biomass composition on the production of volatile fatty acids during anaerobic digestion. Chemosphere, 119, 1005-1008.

[46] **Zhang, W., & Mohan, S. V.** (2020). Acetic acid production from lignocellulosic biomass: The role of pretreatment, enzymes, and fermentation. Bioresource Technology, 310, 123383.

[47] **Jang, H., Lee, J., & Kim, H.** (2019). Alkaline pretreatment of biomass for enhanced anaerobic digestion: Mechanistic insights and operational optimization. Renewable and Sustainable Energy Reviews, 113, 109278. DOI: 10.1016/j.rser.2019.109278

[48] **Zhang, D., Fu, X., Jia, S., Dai, L., Wu, B., & Dai, X.** (2016). Excess sludge and herbaceous plant co-digestion for volatile fatty acids generation improved by protein and cellulose conversion enhancement. Environmental Science and Pollution Research, 23, 1492-1504.

[49] **Nguyen, T. M., Kim, J., & Kim, W.** (2015). Protist predation on bacteria in anaerobic ecosystems. Microbiology and Molecular Biology Reviews, 79(4), 449-468. https://doi.org/10.1128/MMBR.00046-15

[50] **Bardon, C., Piutti, S., Aragno, M., & Greub, G.** (2018). Protists in wastewater treatment systems: Stress tolerance and adaptation. Journal of Eukaryotic Microbiology, 65(4), 519-530. https://doi.org/10.1111/jeu.12482

[51] **Baldrian, P.** (2009). Microbial degradation of lignin. FEMS Microbiology Reviews, 33(6), 929-952. https://doi.org/10.1111/j.1574-6976.2009.00177.x

[52] **van Lier, J. B., Lemos, P. C., & Mahony, T.** (2001). Microbial community structure in anaerobic digesters: Diversity and specialization. Water Science and Technology, 44(8), 181-188. https://doi.org/10.2166/wst.2001.0512

[53] **Sun, J., Zhang, L., & Loh, K. C.** (2021). Review and perspectives of enhanced volatile fatty acids production from acidogenic fermentation of lignocellulosic biomass wastes. Bioresources and Bioprocessing, 8(1), 68.

[54] **Bhatia, S. K., & Yang, Y. H.** (2017). Microbial production of volatile fatty acids: current status and future perspectives. Reviews in Environmental Science and Bio/Technology, 16, 327-345.

[55] **Expert Market Research.** (2024). Propionic Acid Market Size & Share | Report Growth 2032. Expert Market Research. Retrieved from https://www.expertmarketresearch.com/reports/propionic-acid-market

CURRICULUM VITAE

Name Surname **:** Canberk KAZANCI

EDUCATION:

- **B.Sc.** **:** 2020, Eskisehir Technical University, Department of Environmental Engineering

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **KAZANCI, C.** and Ince, O. Y. 2024: Anaerobic Digestion of Lignocellulosic Waste Using Alkali Pretreatment Method Interms Of Performance, Microbial Community, and Cost Analysis, International Graduate Research Symposium – IGRS'24, May 8-10, 2024, Turkey. (Poster Presentation)