**ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL**

# ANAEROBIC DIGESTION OF LIGNOCELLULOSIC WASTE USING PHYSICO-CHEMICAL PRETREATMENT METHODS INTERMS OF PERFORMANCE, MICROBIAL COMMUNITY, AND COST ANALYSIS

**M.Sc. THESIS**

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**SEPTEMBER 2024**

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**Thesis Advisor: Prof. Dr. Orhan İNCE**

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**İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ**

# FİZİKOKİMYASAL ÖN ARITIM YÖNTEMİ İLE LİGNOSELÜLOZİK ATIKLARIN ANAEROBİK ÇÜRÜTÜLMESİ OPTİMİZASYONU, MİKROBİYAL TOPLULUĞU VE MALİYET ANALİZİ

**YÜKSEK LİSANS TEZİ**

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**EYLÜL 2024**

İrem GÜVEN BEYAZ, a M.Sc. student of İTU Graduate School student ID 501211805, successfully defended the thesis entitled "ANAEROBIC DIGESTION OF LIGNOCELLULOSIC WASTE USING PHYSICO-CHEMICAL PRETREATMENT METHODS INTERMS OF PERFORMANCE, MICROBIAL COMMUNITY, AND COST ANALYSIS", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

| | | |
|---|---|---|
| **Thesis Advisor :** | **Prof. Dr. Orhan İNCE**<br>Istanbul Technical University | ..... |
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**Date of Submission : 24 May 2024**
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**To my beloved husband and precious family,**

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**ABBREVIATIONS**

**AD** : Anaerobic Digestion
**CAGR** : Compound Annual Growth Rate
**CE** : Coulombic Efficiency
**CHP** : Combined Heat and Power
**COD** : Chemical Oxygen Demand
**DGGE** : Denaturing Gradient Gel Electrophoresis
**DP** : Degree of Polymerization
**FDA** : The United States Food and Drug Administration
**FISH** : The Fluorescence in Situ Hybridization
**GHG** : Greenhouse Gas
**HRT** : Hydraulic Retention Time
**LCB** : Lignocellulosic Biomass
**MSW** : Municipal Solid Waste
**NGS** : Next-generation Sequencing
**OFMSW** : Organic Fraction of Municipal Solid Waste
**OLR** : Organic Loading Rate
**ONT** : Oxford Nanopore Technologies
**PD** : Power Density
**PHAs** : Polyhydroxyalkanoates
**Q-PCR** : Real-Time Polymerase Chain Reaction
**sCOD** : Soluble Chemical Oxygen Demand
**SRT** : Solids Retention Time
**TKN** : Total Kjeldahl Nitrogen
**TS** : Total Solids
**VFA** : Volatile Fatty Acid
**VS** : Volatile Solids
**WGS** : Whole-Genome Sequencing

**SYMBOLS**

| | |
|---|---|
| **°C** | **:** Celsius temperature scale |
| **pH** | **:** Measure of the acidity or basicity |
| **rpm** | **:** Revolutions per minute |
| **µL** | **:** Microliter |
| **%** | **:** Percentage |
| **C** | **:** Carbon |
| **$CH_4$** | **:** Methane |
| **$CO_2$** | **:** Carbon dioxide |
| **HCl** | **:** Hydrochloric Acid |
| **$H_2O_2$** | **:** Hydrogen Peroxide |
| **$H_2S$** | **:** Hydrogen sulfide |
| **N** | **:** Nitrogen |
| **P** | **:** Phosphorus |
| **m** | **:** Milimeter |
| **mL** | **:** Milliliter |
| **mg** | **:** Miligram |
| **L** | **:** Liter |
| **$NO_3^-$** | **:** Nitrate |
| **$SO_4^{-2}$** | **:** Sulfate |
| **$NH_3$** | **:** Ammonia |
| **$H_2$** | **:** Hydrogen |

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## ANAEROBIC DIGESTION OF LIGNOCELLULOSIC WASTE USING PHYSICO-CHEMICAL PRETREATMENT METHODS INTERMS OF PERFORMANCE, MICROBIAL COMMUNITY, AND COST ANALYSIS

## SUMMARY

Increasing population and energy demand emphasize the global need to use energy resources more effectively and responsibly. Specifically, reliance on fossil fuels and the repercussions of climate change have hastened the hunt for renewable energy. However, current sustainable energy supplies are insufficient to fulfill rising demand alone. In this context, transforming organic solid waste into a sustainable resource and energy solves the environmental pollution problem while also creating new resource prospects. Although anaerobic treatment is a well-known and widely utilized method, it remains a field for further improvement. However, the lignocellulosic structure of organic solid wastes makes hydrolysis, the first stage in anaerobic digestion, more difficult than with other forms of waste.

The use of lignocellulosic wastes in anaerobic digestion systems offers significant waste management potential. However, biodegradation of such wastes is challenging due to their complicated structure, hence several pre-treatment procedures have been devised. Physicochemical pretreatment procedures, particularly microwave and acid treatments, speed up the breakdown of lignin and cellulose structures, allowing microorganisms to operate more efficiently with the waste. As a result, the anaerobic process becomes more efficient, and important chemicals like methane and volatile fatty acids are produced in greater quantities. Sunflower waste is a major raw material source for anaerobic treatment procedures in Turkey and across the world. Turkey is one of the world's largest sunflower producers, with sunflowers growing across vast agricultural regions, particularly in the Thrace region. Stem, head, and other biomass wastes from sunflower cultivation are typically left on agricultural areas or disposed of using inefficient ways.

However, these wastes have a significant potential in biogas production thanks to their high cellulose and lignin content. Integrating these wastes into the circular economy makes significant contributions to both environmental sustainability and economic gains. Agricultural wastes have great potential for biomass energy production and can be converted into various products such as biogas, bioethanol, compost. In addition, by utilizing agricultural waste, farmers' income sources diversify and contribute to the strengthening of the rural economy. Products obtained by recycling agricultural wastes both ensure efficient use of resources within the scope of the circular economy model and contribute to meeting Turkey's energy needs with sustainable resources. As a result of these reasons, sunflower was chosen as organic waste in the study.

Even though pretreatment methods are basically divided into physical, chemical, biological and combined pretreatment, research has shown that combined pretreatment can be more successful. Combined pretreatments are preferred to ensure more effective breakdown of lignocellulosic wastes, because methods used alone often cannot

adequately break down the complex structure of the waste. Combined pretreatments are generally applied by combining physical, chemical or biological methods, whereby the advantages of each method create a synergistic effect, providing more efficient results. For example, the combination of microwave and acid-based pretreatment disrupts the structure of biomass both thermally and chemically, resulting in higher biogas and volatile fatty acid production. While these methods increase energy efficiency, they also provide sustainable solutions in terms of cost effectiveness. In this study, physico-chemical pretreatment was chosen to break down the lignocellulosic structure most successfully.

For a physicochemical process, microwave was used for physical pretreatment and hydrochloric acid was used for chemical pretreatment. In this way, a more effective hydrolysis process was achieved with combined pretreatment. The substrate was microwave pretreated in 0.8%, 1.2% and 1.6% HCl solutions for 30 minutes at 120oC and 140oC. After pre-treatment, sCOD values were examined to understand under which condition the efficiency was more successful. Compared to the control sample without pretreatment, it was observed that 47% higher sCOD was obtained in the sample that underwent combined pretreatment in 1.2% HCl solution at 120oC for 30 minutes. The sCOD value measured without pretreatment is 22395 mg/L sCOD, and with 1.2% HCl 120oC pretreatment, the sCOD value is 32908 mg/L. In this way, the most effective pretreatment method was obtained in the study.

At the same time, TS and VS values for seed sludge, control sample and the most efficient pretreatment were determined by standard methods. When comparing control samples and pretreated samples, TS degradation increased by 45% and VS by 52%. An increase in the TS and VS values of the solid material is a sign of effective deterioration. Thus, lignocellulosic wastes can be shown to decompose more efficiently, meaning that a more efficient hydrolysis will result in a larger VFA output.

In recent years, it has become quite common to suppress the presence of archaea in the anaerobic digestion process and to ensure the formation of volatile fatty acids. The main reason for this is that although methane is used as an energy source, it is an important source of greenhouse gases. At the same time, $CO_2$ is also formed as a byproduct in addition to methane ($CH_4$) in semi-anaerobic digestion. The most important motivation of this study is to obtain volatile fatty acids. In addition to the development of pre-treatment technologies, the production of volatile fatty acids, which have an important place in the circular economy and are used in many different sectors, has become very popular. In this study, sunflower used as lignocellulosic waste was kept at pH 5.5 after being exposed to combined pretreatment.

While archaea, that is, microorganisms that provide methane formation, cannot survive in this pH range, only the formation of volatile fatty acids is possible with acidogenesis microorganisms. At this pH value, microorganisms produce volatile fatty acids, but these volatile fatty acids do not turn into methane because the archaea cannot survive. Once the archaea were inhibited and the pH was checked every day, samples were taken on certain days.

The formation amount of volatile fatty acid types, which are used in various sectors and have different usage purposes, on determined days was measured in mg/L sCOD. Cumulative VFA formation was determined for acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid and isovaleric acid.

The 1st day sCOD value for the pretreated sample was 32908 mg/L; At the end of the 30th day, it was observed to be 19324 mg/L. It is showed that a success rate of volatile fatty acid formation of approximately 40%.

In summary, the acid types, from most produced to least produced, are as follows:

Propionic Acid: 3089 mg/L, Isovaleric Acid: 2993 mg/L, Isobutyric Acid: 2700 mg/L, Butyric Acid: 2137 mg/L, Isocaproic Acid: 2421 mg/L, Caproic Acid: 760 mg/L, Acetic Acid: 539 mg/L, Valeric Acid: 245 mg/L.

Another important aspect of the study is the generation of genomic sequences of microbial communities for taxonomy categorization. The aim is to fully characterize microbial species using Oxford Nanopore MinION technology in combination with fast and long-read approaches. After DNA isolation, PCR and sequencing were performed under appropriate conditions. 16S, 18S and archaeal microorganism communities were classified and compared as phylum, class and species. Genetic sequencing of microbial communities has revealed a diverse spectrum of microorganisms involved in the anaerobic digestion process, including many species of bacteria and archaea.

The most dominant species in microbial communities were observed as follows: *Armatimonadota, Caloramator sp. E03, Dysgonomonadaceae:, Stephanoeca, Prototheca ciferrii, Prototheca wickerhamii, Tetramitus dokdoensis, Methanosarcina vacuolata, Methanothrix soehngenii, Methanosarcina barkeri. These findings will provide resources for future studies on understanding the microbial* community of the anaerobic digestion process and improving system efficiency.

Finally, a cost analysis was performed using all collected data. The economic values according to the types of volatile fatty acids obtained from 1.5 grams of substrate are as follows:

Acetic Acid: $17, Propionic Acid: $250, Isobutyric Acid: $476, Butyric Acid: $247, Isovaleric Acid: $673, Valeric Acid: $24, Isocaproic Acid: $385, Caproic Acid: $122.

Microbial analysis is very important to check system success efficiency. Archaea were shown to persist in this manner. Anaerobic digestion is an extremely sensitive process. Although successful pretreatment was used and archaea were predicted to be absent, their continued presence indicated that a more precise process application could yield more efficient VFA synthesis.

At the end of this entire study, the following conclusions can be made:

Increasing population and energy demand increases the need to replace traditional energy sources with sustainable alternatives. In this context, the use of organic solid wastes, especially those with a lignocellulosic structure, in the production of biogas and volatile fatty acids is important. However, the complexity of the lignocellulosic structure makes biodegradation difficult. Therefore, physicochemical pretreatment methods such as microwave and HCl enable waste to be hydrolyzed more easily and increase biogas yield. Studies show that combined pretreatment methods disrupt the structural integrity of wastes, leading to higher methane and volatile fatty acid production. In countries where sunflower production is intense, such as Türkiye, these wastes offer a great energy potential. While volatile fatty acid production in anaerobic treatment stands out as a more environmentally friendly and economical option, effective taxonomic analysis of microbial communities has the potential to further increase process efficiency.

# FİZİKOKİMYASAL ÖN ARITIM YÖNTEMİ İLE LİGNOSELÜLOZİK ATIKLARIN ANAEROBİK ÇÜRÜTÜLMESİ OPTİMİZASYONU, MİKROBİYAL TOPLULUĞU VE MALİYET ANALİZİ

## ÖZET

Artan nüfus ve enerji talebi, dünya genelinde enerji kaynaklarının daha verimli ve sürdürülebilir şekilde kullanılması gerekliliğini ön plana çıkarmaktadır. Özellikle fosil yakıtlara bağımlılık ve iklim değişikliği etkileri, temiz enerji arayışını hızlandırmıştır. Ancak mevcut sürdürülebilir enerji kaynakları, artan talepleri tek başına karşılamakta yetersiz kalmaktadır. Bu bağlamda, organik katı atıkların sürdürülebilir bir kaynağa ve enerjiye dönüştürülmesi, hem çevre kirliliği sorununa bir çözüm sunmakta hem de yeni kaynak olanağı sağlamaktadır. Anaerobik arıtma bilinen ve halihazırda kullanılan bir teknoloji olmasına rağmen gelişime açık bir alandır. Bununla birlikte, organik katı atıkların lignoselülozik yapısı, diğer atık türlerine göre anaerobik çürütmenin ilk adımı olan hidrolizi zorlaştırmaktadır.

Lignoselülozik atıkların anaerobik sindirim süreçlerinde değerlendirilmesi ise atık yönetimi açısından büyük potansiyel taşır. Ancak bu tür atıkların kompleks yapısı, biyolojik ayrışmayı zorlaştırır, bu yüzden çeşitli ön arıtma yöntemleri geliştirilmiştir. Fizikokimyasal ön arıtma yöntemleri, özellikle mikrodalga ve asit uygulamaları gibi teknikler, lignin ve selüloz yapılarının parçalanmasını hızlandırarak mikroorganizmaların atık üzerinde daha verimli çalışmasını sağlar. Böylece anaerobik süreç daha verimli hale getirilir ve metan ile uçucu yağ asidi gibi değerli ürünlerin üretimi artırılır.. Ayçiçeği atıkları, hem Türkiye'de hem de dünyada anaerobik arıtma süreçlerinde önemli bir hammadde kaynağı olarak öne çıkmaktadır. Türkiye, dünyanın önde gelen ayçiçeği üreticilerinden biri olup, özellikle Trakya bölgesi başta olmak üzere geniş tarımsal alanlarda ayçiçeği yetiştirilmektedir. Ayçiçeği üretiminden sonra ortaya çıkan sap, baş ve diğer biyokütle atıkları, genellikle tarım arazilerinde bırakılmakta ya da düşük verimli yöntemlerle bertaraf edilmektedir. Ancak bu atıklar, yüksek selüloz ve lignin içeriği sayesinde biyogaz üretiminde önemli bir potansiyele sahiptir. Bu atıkların döngüsel ekonomiye entegre edilmesi, hem çevresel sürdürülebilirliğe hem de ekonomik kazançlara önemli katkılar sunar. Tarımsal atıklar, biyokütle enerjisi üretimi için büyük bir potansiyele sahiptir ve biyogaz, biyoetanol, kompost gibi çeşitli ürünlere dönüştürülebilir. Ayrıca, tarımsal atıkların değerlendirilmesi sayesinde çiftçilerin gelir kaynakları çeşitlenir ve kırsal ekonominin güçlenmesine katkı sağlar. Tarımsal atıkların geri dönüştürülmesi ile elde edilen ürünler, döngüsel ekonomi modeli kapsamında hem kaynakların verimli kullanılmasını sağlar hem de Türkiye'nin enerji ihtiyacının sürdürülebilir kaynaklarla karşılanmasına katkı sunar. Bu sebepler neticesinde çalışmada, organik atık olarak ayçiçeği seçildi.

Ön arıtma yöntemleri temel olarak fiziksel, kimyasal, biyolojik ve kombine ön arıtım olarak ayrılsa bile yapılan araştırmalar kombine ön arıtımların daha başarılı olabildiğini göstermiştir. Kombine ön arıtımlar, lignoselülozik atıkların daha etkin bir

şekilde parçalanmasını sağlamak için tercih edilmektedir, çünkü tek başına kullanılan yöntemler genellikle atığın kompleks yapısını yeterince parçalayamaz. Kombine ön arıtımlar, genellikle fiziksel, kimyasal veya biyolojik yöntemlerin bir araya getirilmesiyle uygulanır ve bu sayede her bir yöntemin avantajları sinerjik bir etki yaratarak daha verimli sonuçlar elde edilmesini sağlar. Örneğin, mikrodalga ile asit bazlı ön arıtma kombinasyonu, biyokütlenin yapısını hem termal hem de kimyasal olarak bozarak daha yüksek biyogaz ve uçucu yağ asidi üretimi sağlar. Bu yöntemler, enerji verimliliğini artırırken, maliyet etkinliği açısından da sürdürülebilir çözümler sunar. Bu çalışmada ise lignoselülozik yapının en başarılı şekilde parçalanması için fiziko-kimyasal ön arıtım seçildi.

Fizikokimyasal bir proses için fiziksel ön arıtma amacıyla mikrodalga, kimyasal ön arıtma için hidroklorik asit kullanıldı. Bu sayede kombine ön arıtım ile daha etkili bir hidroliz süreci sağlandı. Substrat, 0.8%, 1.2% ve 1.6% HCl çözeltileri içerisinde 30 dakika boyunca 120$^{o}$C ve 140$^{o}$C'de mikrodalga ön işleme maruz bırakıldı. Ön arıtımdan sonra verimliliğin hangi koşul için daha başarılı geçtiğini anlamak üzere sCOD değerlerine bakıldı. Ön arıtım uygulanmamış kontrol numunesi ile kıyaslandığında, 1.2% HCl çözeltisinde 120$^{o}$C'de 30 dakika kombine ön arıtıma uğrayan numunede 47% daha yüksek sCOD elde edildiği gözlemlendi. The sCOD value measured without pretreatment is 22395 mg/L sCOD, and with 1.2% HCl 120$^{o}$C pretreatment, the sCOD value is 32908 mg/L. Bu sayede çalışmada en etkili arıtım yöntemi elde edildi.

Aynı zamanda aşı çamuru, kontrol numunesi ve en verimli ön arıtmaya ait TS ve VS değerleri standart yöntemlerle belirlendi. Kontrol numuneleri ve ön işleme tabi tutulmuş numuneler karşılaştırıldığında TS bozulması %45, VS ise %52 oranında arttı. Katı malzemenin TS ve VS değerlerinin yükselmesi etkili bir bozulmanın işaretidir. Böylece lignoselülozik atıkların daha verimli bir şekilde ayrıştığı gösterilebilir, bu da daha verimli bir hidrolizin daha büyük bir VFA çıkışıyla sonuçlanacağı anlamına gelir.

Son yıllarda anaerobik arıtma sürecinde arkelerin varlığının baskılanarak, uçucu yağ asitlerinin oluşmasını sağlamak oldukça yaygınlaştı. Bunun temel sebebi metan her ne kadar enerji kaynağı olarak kullanılsa bile önemli bir sera gazı kaynağıdır. Aynı zamanda yan anaerobik çürütmede metan ($CH_4$) haricinde yan ürün olarak $CO_2$ de oluşur. Bu çalışmanın en önemli motivasyonu ise uçucu yağ asitlerinin elde edilmesidir. Ön arıtım teknolojilerinin geliştirilmesinin yanında, döngüsel ekonomide önemli bir yere sahip olan ve bir çok farklı sektörde kullanım amacı olan uçucu yağ asitlerinin üretilmesi oldukça popüler hale gelmiştir. Bu çalışmada lignoselülozik atık olarak kullanılan ayçiçeği kombine ön arıtıma maruz bırakıldıktan sonra pH 5.5'ta tutuldu. Bu pH aralığında arkeler yani metan oluşumunu sağlayan mikroorganizmalar varlığını sürdüremezken, sadece uçucu yağ asitleri oluşumu acidogenesis mikroorganizmalar ile mümkün olur. Bu pH değerinde, mikroorganizmalar uçucu yağ asitleri oluşur fakat bu uçucu yağ asitleri arkeler varlığını sürdüremediği için metana dönüşmez. Arkeler inhibe edildikten ve her gün pH kontrolü yapılırken, belirli günler numuneler alındı.

Çeşitli sektörlerde kullanılan ve farklı kullanım amaçlarına sahip olan uçucu yağ asidi çeşitlerinin, belirlenen günlerde oluşum miktarına mg/L sCOD cinsinden bakıldı. Acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid and isovaleric acid için kümülatif VFA oluşumu belirlendi.

Ön işleme tabi tutulan numunenin 1. gün sCOD değeri 32908 mg/L idi; 30. günün sonunda 19324 mg/L olduğu görüldü. Uçucu yağ asidi oluşumunda başarı oranının yaklaşık %40 olduğu gösterilmiştir.

Özetle asit çeşitleri en çok üretilenden en az üretilene doğru şu şekildedir:

Propiyonik Asit: 3089 mg/L, İzovalerik Asit: 2993 mg/L, İzobütirik Asit: 2700 mg/L, Bütirik Asit: 2137 mg/L, İzokaproik Asit: 2421 mg/L, Kaproik Asit: 760 mg/L, Asetik Asit : 539 mg/L, Valerik Asit: 245 mg/L.

Çalışmanın bir diğer önemli yönü, taksonomi kategorizasyonu için mikrobiyal toplulukların genomik dizilerinin oluşturulmasıdır. Amaç, Oxford Nanopore MinION teknolojisini hızlı ve uzun okunan yaklaşımlarla birlikte kullanarak mikrobiyal türleri tamamen karakterize etmektir. DNA izolasyonu yapıldıktan sonra, PCR ve sekanslama uygun koşullarda uygulandı. 16S, 18S ve arkeal mikroorganizma topluluğu phylum, class ve species olacak şekilde sınıflandırıldı ve kıyaslandı. Mikrobiyal toplulukların genetik dizilimi, birçok bakteri ve arke türü de dahil olmak üzere, anaerobik sindirim sürecinde yer alan çeşitli mikroorganizma spektrumunu ortaya çıkardı.

Mikrobiyal topluluklarda en baskın türler şu şekilde gözlenmiştir: *Armatimonadota, Caloramator sp. E03, Dysgonomonadaceae:, Stephanoeca, Prototheca ciferrii, Prototheca wickerhamii, Tetramitus dokdoensis, Methanosarcina vacuolata, Methanothrix soehngenii, Methanosarcina barkeri*. Bu bulgular anaerobik sindirim sürecinin mikrobiyal topluluğunun anlaşılmasında ve sistem verimliliğinin geliştirilmesinde, yapılacak olacak çalışmalara kaynak sağlayacaktır.

Son olarak toplanan tüm veriler kullanılarak bir maliyet analizi yapıldı. 1,5 gram substrattan elde edilen uçucu yağ asitlerinin türlerine göre ekonomik değerleri şu şekildedir:

Asetik Asit: 17 $, Propiyonik Asit: 250 $, İzobütirik Asit: 476 $, Bütirik Asit: 247 $, İzovalerik Asit: 673 $, Valerik Asit: 24 $, İzokaproik Asit: 385 $, Kaproik Asit: 122 $.

Sistemin başarı verimliliğini kontrol etmek için mikrobiyal analiz çok önemlidir. Archaea'nın bu şekilde varlığını sürdürdüğü gösterildi. Anaerobik sindirim son derece hassas bir süreçtir. Başarılı bir ön arıtma kullanılmış olmasına ve arkelerin bulunmadığının tahmin edilmesine rağmen, bunların devam eden varlığı, daha hassas bir işlem uygulamasının daha verimli VFA sentezi sağlayabileceğini gösterdi.

Tüm bu çalışmanın sonunda kısacası şu çıkarımlar yapılabilir;

Artan nüfus ve enerji talebi, geleneksel enerji kaynaklarının sürdürülebilir alternatiflerle yer değiştirilmesi ihtiyacını artırmaktadır. Organik katı atıkların, özellikle lignoselülozik yapıya sahip olanların, biyogaz ve uçucu yağ asitleri üretiminde kullanılması bu bağlamda önemlidir. Ancak, lignoselülozik yapının kompleksliği biyolojik ayrışmayı zorlaştırır. Bu nedenle, mikrodalga ve HCl gibi fizikokimyasal ön arıtma yöntemleri, atıkların daha kolay hidrolize uğramasını sağlar ve biyogaz verimini artırır. Yapılan çalışmalar, kombine ön arıtma yöntemlerinin atıkların yapısal bütünlüğünü bozarak daha yüksek metan ve uçucu yağ asidi üretimine yol açtığını göstermektedir. Türkiye gibi ayçiçeği üretiminin yoğun olduğu ülkelerde, bu atıklar büyük bir enerji potansiyeli sunmaktadır. Anaerobik arıtmada uçucu yağ asidi üretimi daha çevre dostu ve ekonomik bir seçenek olarak öne çıkarken, mikrobiyal toplulukların etkili taksonomik analizi süreç verimliliğini daha da artırma potansiyeline sahiptir.

## 1. INTRODUCTION

In response to the ever-increasing population growth, the energy crisis is also growing. While fossil energy resources are being depleted, their economic value is also increasing in response to increasing demand. It is very important for developed countries to have independent energy sources and to be a sustainable and clean energy source. Dependence on fossil fuels has generated both economic and environmental issues. The burning of fossil fuels, such as coal, oil, and natural gas, releases greenhouse gases (GHGs) that are bad for the environment and people's health and significantly contributes to global climate change. Future energy supply is a major worldwide concern (Vats, et al., 2020).

Several transformation processes may be used to change agricultural wastes into biological products that are sustainable, such energy and fertilizers. This change has important implications for human well-being, biodiversity, economic expansion, and food security worldwide (Gontard et al., 2018).

Given that Turkey ranks seventh globally in terms of agricultural production, a substantial amount of agricultural residue is to be expected. In terms of collected residues, the investigation reveals that sunflower head is one of the most accessible agricultural residue kinds, with the maximum availability in Turkey at 1 million tons annually (FAO, 2016). As a result, sunflower head and stalk residues with high lignocellulosic content, as well as discarded treats with a large quantity of starch and sugar, were chosen as substrates in this study.

A complex structure consisting of lignin, cellulose, and hemicellulose makes up around 90% of lignocellulosic biomass. Lignin is one of these components that is most resistant to enzymatic and microbiological (chemical) breakdown. As a result, it is now challenging to digest lignocellulosic biomass for the production of biogas. The main goals of the pretreatment procedure are to decrease the structural and compositional barriers to lignocellulosic biomass and increase the accessibility of cellulose and hemicellulose to microbial breakdown. This will raise the rate of biomass degradation and biogas generation. Several pretreatment techniques, such as biological, chemical,

or physical processes, alone or in combination, have been researched lately to achieve this aim (Zheng, et al., 2014).

According to the Food and Agriculture Organization (FAO) in 2013, the leftovers from sunflower, more especially the sunflower heads and stalks, are a form of lignocellulosic biomass that are generated globally, with an annual production volume ranging from 78 to 182 million tons. It is standard procedure to burn these leftovers in fields or dispose of them as rubbish, which pollutes the environment. Nonetheless, sunflower stalks' high cellulose and hemicellulose content makes them a suitable feedstock for the synthesis of renewable energy.

The goal is to use appropriate pretreatment techniques to handle lignocellulosic wastes, which are organic wastes of major relevance. Finding the ideal balance between energy efficiency and deterioration efficiency is the goal. The efficiency of forming volatile fatty acids will also improve when the maximum degradation efficiency is reached.

The main goals in this situation are to prevent the synthesis of methane and to boost the production of volatile fatty acids as efficiently as possible. The selection of sunflower stalks was based on their unsuitability for use with cattle and agriculture. The chosen chemical for the physico-chemical treatments will be hydrochloric acid (HCl). The intention is to carry out an information-based, low-cost, high-yielding study. We'll look closely at how the study's pretreatment strategy affected the acidification efficiency. Furthermore, DNA sequencing will be used to address the dominating microbial population at the conclusion of the study, which will aid in investigations on biological treatments.

# 2. LITERATURE REVIEW

## 2.1 Anaerobic Digestion Process

Anaerobic digestion is a biochemical process in which microorganisms break down organic matter in the absence of an oxygen environment. The four primary phases of anaerobic digestion are acetogenesis, methanogenesis, hydrolysis, and acidogenesis. Numerous substrates can be purified using the anaerobic digestion method. For example, municipal solid waste (MSW), agricultural waste, animal waste, and industrial organics. Biogas, which is a renewable energy source obtained as a result of anaerobic digestion, is an alternative to traditional energy sources because it is economical and environmentally friendly (Adekunle & Okolie, 2015). Anaerobic digestion is a convenient way to treat the organic fraction of municipal solid wastes (OFMSW) and other easily biodegradable solid wastes, like agrowastes, to lessen their environmental impact, recover energy and/or material, and save operating costs without using landfills or incineration (Bolzonella et al., 2005). Methane, carbon dioxide, plus trace amounts of other gases and components create biogas. It is a flammable gas that may be used in place of fossil fuels like coal, natural gas, and firewood. Nowadays, biogas is used in many countries to power fuel cells or automobiles. It is enhanced and fed into natural gas networks in addition to being used for combined heat and power (CHP) generation. (Jingura & Kamusoko, 2017).

One waste-to-energy method that has been proposed is anaerobic digestion, which may handle waste in an environmentally friendly way. The widely acknowledged and validated technique of biodegrading organic resources to generate sustainable energy (De Bere, 2000). Anaerobic digestion produces biogas, which has several advantages over other methods of producing bioenergy. Anaerobic digestion is becoming a more promising and appealing technique of waste management due to its ability to limit carbon dioxide and other gas emissions, reduce air and water pollution, and produce electricity from biomass.

Because of its high carbohydrate content, lignocellulosic biomass is regarded as an excellent supply of substrates for anaerobic digestion for biogas generation. Unfortunately, due to their recalcitrance, these materials are exceedingly difficult to biodegrade since their structure prevents the microbial hydrolysis stage of the process.

Advantages and disadvantages of anaerobic treatment are briefly noted in the Table 2.1 below:

**Table 2.1 :** Advantages and disadvantages of anaerobic treatment.

| | |
|---|---|
| Advantages | 1. Low generation of sludge<br>2. Low need for nutrients (phosphorus and nitrogen)<br>3. Minimal operational and capital expenses<br>4. The energy-producing process that produces methane<br>5. Creation of residuals, both liquid and solid, that might be used as soil conditioners |
| Disadvantages | 1. Prolonged retention and start-up periods<br>2. Needs high temperatures to function properly<br>3. Needs observation to ensure proper operation<br>4. The microbial equilibrium might be disturbed by shock and fluctuating load<br>5. Usually used as a phase prior to treatment (as pretreatment) |

The interaction with methanogenic bacteria is not well understood. There are two suggested pathways for sulfate reduction of methanogenesis. i. Sulfate-reducing organisms compete with methanogens mostly for hydrogen and acetate. ii. The presence of sulfate causes sulfate-reducing bacteria to generate sulfide, which is hazardous to methanogens in high concentrations. The competition may or may not result in inhibition, depending on the sulfate concentration.

Understanding the biochemistry and the optimal operating parameters for anaerobic therapy are essential components of effectiveness.

### 2.1.1 The anaerobic digesting process's biochemistry

Through the biological process of anaerobic digestion, complex organic molecules are broken down in the absence of oxygen to produce a mixture of methane and carbon dioxide. about 50–75% $CH_4$ and 25–50% $CO_2$) (Frigon & Guiot, 2010). It is considered that the digestive process is complex and involves a number of anaerobic metabolic activities. Anaerobic digestion provides biogas (methane) through the steps of hydrolysis, acidogenesis, acetogenesis, and methanogenesis as show in Figure 2.1.

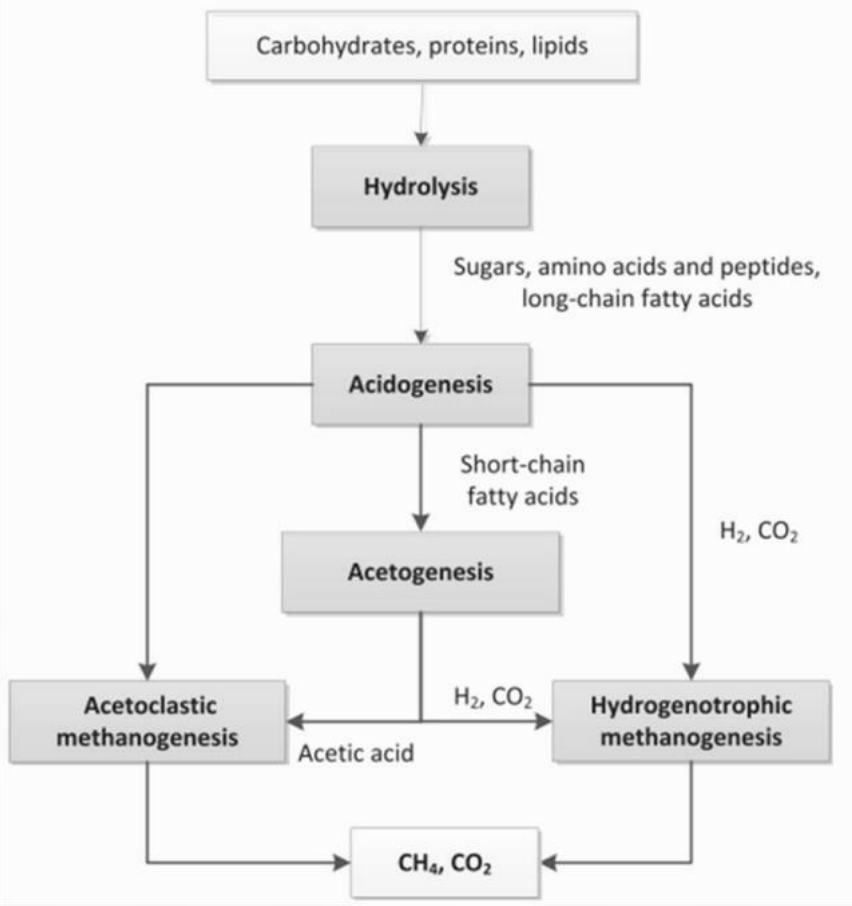



**Figure 2.1 :** Process flow during anaerobic digestion (Zheng, et al., 2014).

Anaerobic digestion produces methane, carbon dioxide, hydrogen sulfide, ammonia, and new bacterial biomass from organic waste that is breaking down (Veeken et al., 2000; Gallert et al., 2005).

An analytical chemical reaction can be used to describe the entire anaerobic digestion process as shown reaction formula 2.1. (Deublein and Steinhauser, 2008):

$$C_aH_bO_cN_dS_e + \frac{1}{4}(4a\text{-}b\text{-}2c+3d+2e)H_2O \rightarrow \frac{1}{8}(4a\text{-}b+2c+3d+2e)CO_2 + \frac{1}{8}(4a+b\text{-}2c\text{-}3d\text{-}2e)CH_4 + dNH_3 + eH_2S \quad (2.1)$$

If the waste's chemical makeup is known, this chemical process may be utilized to calculate the output of methane ($CH_4$) in theory. The organic substrate, often referred to as feedstock, is collected and finely shredded before being fed into a closed reactor in the generalized anaerobic digestion system that has been infected with active methanogenic bacteria. Conditions for operating the reactor are regulated (Arsova, 2010). Since methane is a major greenhouse gas, anaerobic digestion offers better control over methane output and lower emissions than landfilling and aerobic composting (Levis et al., 2010).

## 2.2 Anaerobic Digestion's Metabolic Pathways

### 2.2.1 Hydrolysis

The first step of anaerobic digestion is called hydrolysis, and it is the process by which microbes break down complex substrates like proteins, lipids, and carbohydrates into monosaccharides, fatty acids, and amino acids, in that order (Pesta, 2007).

It is probable that pretreatment, as opposed to only hydrolyzing, will be necessary for the cellulose and hemicellulose obtained from lignocellulosic biomass (Jørgensen & colleagues, 2007). To enhance hydrolysis, many pretreatment approaches are being researched, especially for lignocellulosic waste. Two essential pretreatment techniques are crystallinity and accessible surface area. Pretreatment techniques such as milling, irradiation, microwave, alkaline hydrolysis, steam explosion, ozonolysis, liquid hot-water pretreatment, wet oxidation, organosolv procedures, concentrated and diluted acid hydrolyses, and biological pretreatments can also be employed to increase the generation of biogas (Taherzadeh and Karimi, 2008).

### 2.2.2 Acidogenesis

This stage produces water-soluble organic end products from the hydrolysis process, such as fatty acids, alcohols, and carbon dioxide gas. Moreover, the same fermentative bacteria that cause hydrolysis are also in charge of these changes (Pesta, 2007). Acidogenic bacteria create $NH_3$, $CO_2$, $H_2S$, and various byproducts, including volatile fatty acids (VFAs). (Appels et al., 2008). Significant increases in VFA concentration can be harmful to methanogens in anaerobic digesters because they lower pH. Thus, process monitoring is crucial throughout these phases (Franke-Whittle et al., 2014).

### 2.2.3 Acetogenesis

Acetogenesis refers to the process by which water-soluble chemicals degrade and methane is produced. By this process, short-chained volatile fatty acids like $CO_2$ and acetate are produced from the byproducts of microbial metabolisms (Pesta, 2007). Methane-producing bacteria and acetogenic bacteria coexist together (Chandra et al., 2012).

### 2.2.4 Methanogenesis

The final stage of anaerobic digestion, known as methanogenesis, is when bacteria generate methane and carbon dioxide. Due to their stringent anaerobic nature, methanogenic bacteria can only exist in the absence of oxygen (Pesta, 2007). Methanogenic bacteria are highly sensitive to pH and may work best at a pH of 6.5 to 8, while fermentative microorganisms are less sensitive and may live in a wider pH range between 4.0 and 8.5 (Hwang et al., 2004; Boe, 2006).

## 2.3 Important Parameters of Anaerobic Digestion

### 2.3.1 Temperature

Temperature is regarded as one of the most crucial process factors because of its substantial impact on the microbiological activity taking place inside the reactor. Anaerobic digestion often occurs in two temperature ranges: 45–60°C (ideal 55°C) for thermophilic bacteria and 30–40°C (optimal 37°C) for mesophilic germs.

The temperature must be precisely controlled in accordance with the microorganisms utilized in the procedure.

The mesophilic spectrum of anaerobic bacteria is assumed to be more robust and capable of withstanding larger variations in environmental factors, such as temperature. The anaerobic system operates at mesophilic temperatures, thus it does not require any extra energy input. But thermophilic reactors provide smaller digester sizes, reduced capital costs, larger organic loading rates, and faster substrate breakdown rates (Mackie and Bryant, 1995). Mesophilic anaerobic reactors are more common in contemporary anaerobic digestion facilities because of their stability and lower initial cost, although producing less biogas and having a longer retention time than thermophilic reactors.

### 2.3.2 pH

In anaerobic processes, pH value indicates the efficacy and stability of the anaerobic digestion process. Changes in pH have a major effect on many aspects of complex microbial metabolism and are strongly related to the rate of volatile fatty acid (VFA) formation and the system's alkalinity. Even though acidogenesis requires a pH of 5.0 to sustain their enzymatic activity at a high rate, methanogenesis can only operate

correctly in the neutral pH range of 6.5 (Stronach et al., 1986; Lay et al., 1998). Because intermediate products, such volatile functional acids, don't accumulate, there isn't much pH variation in a healthy, balanced system. Therefore, in order to maintain a consistent pH in the anaerobic reactor, it is necessary to adjust the connection between the concentrations of VFA and bicarbonate (Liu et al., 2007).

### 2.3.3 Substrate characteristics

Features of solid waste, such biogas production and substrate degradability, affect how well anaerobic digestion works. The substrate's degradability and ibility to produce biogas are influenced by the quantity of lipids, proteins, and carbs it contains (Hartmann and Ahring, 2006). Because it produces more methane than other organic materials, high-lipid organic waste is an attractive substrate for the anaerobic digestion process (Nayono, 2009). Lipids, on the other hand, adsorb onto solid surfaces and can delay the hydrolysis process by making enzyme attack less accessible (Neves et al., 2008).

The relative quantities of organic carbon and nitrogen (C/N ratio) in the waste substrate are another important factor influencing process efficiency. A solid waste substrate with a high C/N ratio lacks enough nitrogen to support bacterial growth, which results in a low rate of gas generation. On the other hand, an extremely low C/N ratio causes ammonia to accumulate during the breakdown process, which is harmful to the bacteria (Hartmann and Ahring, 2006). Based on biodegradable organic carbon, a C/N ratio of 25–30 is thought to be ideal for the anaerobic digestion process (Kayhanian and Hardy, 1994). A typical recommendation for anaerobic substrate digestion is an average COD/N/P ratio of around 600/7/1 (Mata-Alvarez, 2003).

Anaerobic co-digestion of nutrient-rich wastes (e.g., food waste and animal manure) with organic wastes can successfully maintain optimal C/N ratios in the digester material (Zaher et al., 2007). Lastly, particle size is important because a smaller particle size gives an enzyme more surface area to attack, especially during hydrolysis (Palmowski and Müller, 2000; Hartmann and Ahring, 2006) and improves energy production and substrate degradation (Mshandete et al., 2006).

### 2.3.4 Moisture content

The water content of the organic waste to be digested is determined at the process selection stage of anaerobic digestion. It is common practice to ascertain a waste's water content by looking at its solid composition. The phrase "dry process" describes a number of anaerobic systems that deal with wastes that vary in water content (Rilling, 2005). In addition to microbial activity, water content affects anaerobic reactor capacity, mixing, and feeding type, among other design and operational parameters. Table 2.2 is showed comparison of wet and dry anaerobic digestion. In AD systems, water has been required to initiate and maintain bacterial activity. The water input relies on the selected anaerobic method of treatment and the properties of the organic waste to be decomposed.

**Table 2.2 :** Comparison of wet and dry anaerobic digestion (Rilling, 2005).

| | **Dry** | **Wet** |
|---|---|---|
| **Total Solids(TS) Content** | High, 25-45% | Low, 2-15% |
| **Reactor Volume** | Minimized | Increased |
| **Conveyance Technique** | Expensive | Simple |
| **Mixing** | Difficult | Easy |
| **Scumming** | Little risk | High risk |
| **Short Circuit Flow** | Little risk | High risk |
| **Solid-liquid Separation** | Simple | Expensive |
| **Variety of Waste Components** | Small | Great |

### 2.3.5 Reducing factors

At a certain threshold level, anaerobic digesters fail due to a number of toxicants (Zaher et al., 2007). The most common harmful chemicals are hydrogen sulfide, free ammonia, and volatile fatty acids; however, salts (salinity) and xenobiotics can infrequently cause anaerobic systems to fail (Lien, 2004). Ammonia, hydrogen sulfide, and volatile fatty acids are pH-dependent substances because only their unionized forms exhibit microbial toxicity because they are easily transported into the inner regions of microbial cells (Bernard, 2006).

Volatile Fatty Acids (VFAs): Produced during the acidogenesis stage of the anaerobic breakdown pathway, the toxicity threshold level of volatile fatty acids is determined by the pH and alkalinity potential of solid waste (Lien, 2004). Because of the lower

pH levels during anaerobic digestion, raising the initial hydrolysis rate over a threshold value prevents methanogenesis and subsequently hydrolysis. Additionally, because of VFA inhibition, a build-up of VFAs may reduce the rate of hydrolysis (Veeken et al., 2000).

Ammonia: Ammonium inhibits methanogens through a pH-dependent inhibitory mechanism. It is a nutrient that is necessary for anaerobic digestion (Lien 2004). Ammonium leaves a solution as free ammonia or ammonium ions, depending on the pH. It has been noted that the anaerobic digestion process is more significantly inhibited by free ammonia (Gallert et al., 1998; Eldem et al., 2004). Free ammonia limit concentrations have been reported to be 850 mg/L for thermophilic settings; yet, at neutral pH, it was shown to be an inhibiting substance at 800 mg/L for mesophilic conditions (Björnsson, 2000; Mata-Alvarez, 2003).

Hydrogen Sulfide: Ammonia and hydrogen sulfide are comparable in terms of toxicity. Hydrogen sulfide toxicity is also highly reliant on environmental factors such as pH and alkalinity (Mata-Alvarez, 2003). It is possible to withstand hydrogen sulfide concentrations up to 100 mg/L without the need for acclimatization (Zaher et al., 2007). Hydrogen sulfide concentrations up to 200 mg/L may also be tolerated by specialized microorganisms. Methanogens are more vulnerable to hydrogen sulfide under anaerobic conditions than acidogens. Compared to acetoclastic methanogens, hydrogen-oxidizing bacteria are thought to be more vulnerable (Zaher et al., 2007).

### 2.3.6 Toxicity

The process of anaerobic digestion should not include the use of certain dangerous materials or chemicals. Free oxygen elements and oxygen-containing compounds like $NO_3^-$, $H_2O_2$, and $SO_4^{-2}$ are undesired in anaerobic digesters. $H_2S$ may be produced and sulfate-reducing bacteria can grow on substrates containing sulfate (Bozan, 2018). Ammonium is another hazardous chemical that can be fatal in high concentrations. Anaerobic digestion is impeded by free ammonia nitrogen, which is generated from proteins and urea during biological substrate hydrolysis. Anaerobic digestion is harmed when the free ammonia nitrogen concentration rises over the threshold (Chen et al., 2008).

### 2.3.7 Macronutrients

The organic matter in the feedstock provides food for most of the organisms in the anaerobic system. All of the organic matter in the feedstock provides the necessary carbon supply, with the exception of the autotrophic methanogens that turn hydrogen into methane. But in order for the bacteria's cells to grow, they need a specific amount of inorganic nutrients. To guarantee optimal removal efficiency, those nutrients must be delivered into the system (Gerardi, 2003). Macronutrients are nutrients that are essential for the growth of bacterial cells. Examples of these nutrients are carbon, nitrogen, and phosphorus. Carbon is also a biodegradable component of organic materials. Anaerobic digestion of complex wastes follows a known macronutrient ratio that yields satisfactory results. C/N/P is the ratio, which is 250 (400-200) / 5 / 1.

It is well acknowledged that nitrogen is the most important macronutrient and that bacterial growth requires the highest concentration of nitrogen. Ammonia ($NH_3$) and a little amount of organic nitrogen are generated during waste degradation, but microorganisms do not use them. They are therefore recognized as the anaerobic system's main providers of nitrogen. Nitrogen from nitrate ($NO_3^-$) and nitrite ($NO_2^-$) is mostly lost under anaerobic conditions of conversion to nitrogen gas, preventing growth (Stronach, et al., 2012).

For the growth of microorganisms, phosphorus is equally important as nitrogen. It has been discovered that in anaerobic digestion, the microbial absorption of phosphorus is approximately one-fifth to one-seventh that of nitrogen. It makes about 2% of total biological solid waste, according to certain studies. The majority of research standards concern a ratio for low loaded processes on a COD basis, which is N:P / 1000:7:1 (less than 0.5 kg COD/kg VSS/day)

### 2.3.8 Micronutrients

Since they make up around 4% of the dry weight of the cell, additional nutrients must be present in the anaerobic system in addition to the macronutrients (carbon, phosphorus, and nitrogen). These elements are referred to as "micronutrients." Organic growth agents and trace metals are the most prevalent types of micronutrients.

Since the sulfide needed by methanogens precipitates these elements, it is challenging to determine the precise quantity of trace metals in the anaerobic system. It is therefore

suggested that at equilibrium, trace metal concentrations in the system should be relatively low. Na, K, Mg, Ca, Fe, S, Ni, Co, and Mo are these metals.

High volatile solids concentration has been demonstrated to be aided by nickel, iron, and cobalt. Methanogens transform acetate into methane by using metals including iron, cobalt, and nickel. Other elements that are thought to be necessary for methanogens include tungsten, molybdenum, and selenium.

Organic growth factors are thought to be extremely significant in stimulating methanogenic activity. They contain coenzyme-M, factor F420, acetate, 2-methyl butyric acid, vitamins, Nacetyl glucosamine, riboflavin, B12, and several other chemicals (Nizami, 2012)

### 2.3.9 Electron donor and acceptor

As an electron donor, the biodegradable COD gives the system's biomass activity energy. The kind of system determines the electron acceptor. Oxygen serves as the electron acceptor in aerobic processes, where it is subsequently reduced to water. However, nitrate and nitrite are converted into nitrogen gas in anoxic settings. Sulfate or $CO_2$ are used as electron acceptors in anaerobic systems. Sulfate is transformed into $H_2S$ and $CO_2$ into methane gas (Gerardi, 2003).

### 2.3.10 Mixing

An important part of increasing the rate of digestion is proper mixing, which facilitates the transfer of nutrients and food to cells while also getting rid of waste. Mixing mainly fulfills two functions. Initially, the food, or substrates, easily pass through the bulk solution's microbe cell wall. Secondly, the bulk solution is mixed with cell wall waste materials. Gas recirculation and mechanical mixing are the two mixing techniques. Gas recirculation is the process of infusing compressed digester gas once again. Pumping or "jettling" sludge mechanically is one way to mix it mechanically (Stronach, et al., 2012).

## 2.4 Volatile Fatty Acids (VFAs)

By using a combination of microorganisms to break down organic waste in the absence of oxygen, a process known as anaerobic digestion produces biogas as an intermediate product. VFA synthesis can be increased in an anaerobic digestion process by

decreasing the reaction time to avoid methanogenesis. Adjusting the pH between 6-8 can also increase VFA synthesis while inhibiting methanogen development. Methanogenic inhibitors may also be added in lab-scale studies to suppress methanogens and boost the synthesis of volatile fatty acids (VFAs) (Liu et al., 2018; Zhou et al., 2018).

One of the most significant obstacles to the circular economy is the potential of different biomasses for the production of environmentally friendly chemicals. Because volatile fatty acids (VFAs) have more diverse end applications than methane and biogas, they may be generated in reactors that are similar to those used for producing biogas, hence increasing the productivity of a digestion plant. VFAs are intermediates in the methane generation pathway of anaerobic digestion.

VFAs provide more diversified and beneficial uses than methane. VFAs may be used as green and renewable chemical products in a variety of sectors and as industrial feedstocks, including bioplastics and biofuels (Kleerebezem, Joosse, Rozendal, & Loosdrecht, 2015).

It is widely known that the characteristics of the substrate and the microbial inoculum have an impact on the fermentation processes for both biogas and VFA. Process variables including pH and loading rate, as well as the make-up of microbial populations, have an impact on both processes (van Aarle et al., 2015).

The most frequent VFAs are acetic (C2), propionic (C3), isobutyric, butyric (C4), isovaleric, valeric (C5), and caproic acids (C6) (Wainaina et al., 2019). The various ratios of VFAs generated depend on operating circumstances, substrate composition, and microbial population in the anaerobic digestion system (Lukitawesa et al., 2020).

The primary volatile fatty acids formed during anaerobic decomposition include caproic acid, butyric acid, valeric acid, propionic acid, and acetic acid. Their COD equivalents are presented in Table 2.3.

**Table 2.3 :** VFA and COD correlation as coefficient value.

| Volatile Fatty Acid (VFA) | Carbon Equivalent (%) | COD Equivalent |
|---|---|---|
| Acetic ($CH_3COOH$) | 40,00 | 1,066 |
| Propionic ($CH_3CH_2COOH$) | 48,64 | 1,512 |
| Butyric ($CH_3CH_2CH_2COOH$) | 54,53 | 1,816 |
| Valeric ($CH_3(CH_2)_3COOH$) | 58,80 | 1,816 |
| Caproic ($CH_3(CH_2)_4COOH$) | 62,04 | 2,204 |

Acetic and propionic acids are the most critical intermediates that contribute to the production of methane. Propionic acid is produced as an intermediary mostly by the fermentation of the carbohydrates and proteins present, and around 30% of the complex waste is transformed to this acid before being turned into methane gas.

Acetic acid is the most abundantly produced acid, created as an intermediary during the anaerobic treatment of practically all organic matter. Acetic acid accounts for around 72% of methane generated in complex wastes such as household sludge and, when combined with propionic acid, accounts for approximately 85% of total methane production. A major percentage of the remaining 15% is due to the breakdown of other acids like formic and butyric.

### 2.4.1 VFAs' characteristics

VFAs are classified into several categories, each with its own set of features and uses.

Table 2.4. summarizes the chemical structure, market size, uses, qualities, and manufacturing techniques for acetic, propionic, and butyric acids. In addition to its widespread application in the chemical, pharmaceutical, and food sectors, each form of VFA has desirable features that have been described.

In the food and beverage sector, acetic acid is a basic component of numerous products used as flavorings, acidity regulators, and preservatives. Paint, rubber, plastics, synthetic fibers, textile finishes, insecticides, and polymer emulsions are among the other products that are made using it.

The synthesis of calcium and sodium salts, emulsions, flavors, scents, herbicides, eco-friendly coating formulation solvents, fake fruit flavors, and modified synthetic cellulose fibers are only a few of its numerous applications. By preventing the formation of mold and other microbes, it may also be used to preserve food, especially bread and other baked goods as calcium or sodium salts, animal feed, and cereals.

Butyric acid is a valuable component of biodiesel. It is used in the animal feeding industry as an antibiotic as well as a supplement because of its anti-pathogenic properties and the regulations that control the use of antibiotics in animal feeding. As a result, it is recognized as the most efficient energy source for animals, particularly poultry and pigs. Furthermore, butyric acid is well known for its anticancer qualities as it encourages morphological and biochemical differentiation in a variety of cells

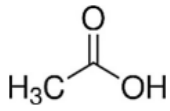
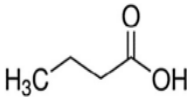
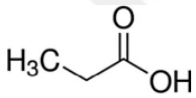
(Xu and Jiang, 2011; Zacharof and Lovitt, 2014). Since the US Food and Drug Administration approved bio-based butyric acid as a food flavoring component (a taste and scent additive), its manufacturing has increased (FDA).

Production techniques based on petrochemicals can economically outbid bio-based approaches in manufacturing. In general, the lesser efficiency of bio-based production methods and the lower manufacturing cost of petro-based production methods are the two key causes. However, switching from petroleum-based production to bio-based methods is required due to the negative effects on the environment caused by greenhouse gas emissions, the high energy and chemical requirements, the large amount of waste and wastewater produced during and after the production process, and the depletion of petroleum sources (Besselink et al., 2017; Mansouri et al., 2017; Wu et al., 2016). However, more recent research and analysis have recommended switching from current VFA manufacturing methods to cradle-to-cradle technologies (such bio-based processes) that use waste and wastewater as raw materials.

Bhatia and Yang (2017) characterized the value of bio-based VFA manufacturing techniques as renewability, degradability, and sustainability.

Efficiency, operating condition optimization, utilization of sustainable and renewable substrates, identification and evaluation of microbial populations and their interactions, and creation of new separation techniques are all areas where bio-based VFA manufacturing processes are starting to show improvement. Improving the operating conditions is one of the most important features of bio-based VFA manufacturing processes. As a result, the synthesis of VFAs is influenced by the operational parameters related to pH, temperature, substrate type, retention time, mixed microbial cultures, and reactor type.

**Table 2.4 :** Properties of VFAs including chemical formula, market size, market price, usage/application and production methods.

| VFAs | Chemical formula | Market size (kton/year) | Market price (€/ton) | Usage/application | Production methods | References |
|---|---|---|---|---|---|---|
| **Acetic acid** |  | 14000–17000 | 400–800 | Vinyl acetate monomer (polymers, adhesives, dyes), Food additive, Solvent, Vinegar, Ester production, Chemicals | Chemical synthesis (carboxylation of methanol) and microbial fermentation (oxidative and anaerobic) | Bhatia and Yang (2017) |
| **Butyric acid** |  | 90–105 | 1500–1650 | Animal and human food additive, Chemical intermediate, Solvent, Flavouring agent | Chemical synthesis (oxidation of butyraldehyde), Extraction from butter, microbial fermentation | Zigová and Šturdík (2000) |
| **Propionic acid** |  | 350–470 | 2000–2500 | Esters used food industry as aroma additive, Food additive, flavoring, Pharmaceuticals, Animal feed supplement, Fishing bait additive | Chemical Synthesis (ethylene hydro formylation, carboxylation of ethylene, direct oxidation of hydrocarbons), by product of acetic acid manufacturing, microbial fermentation | Cheryan ( 2009) |

### 2.4.2 Applications of VFAs

Various uses such as biogas production, biodegradable polymers (PHAs), hydrogen, biodiesel, and bioelectricity generation, and biological nutrient removal from wastewater have been made possible by the utilization of volatile fatty acids (VFAs) derived from waste and other lignocellulosic biomass.

#### 2.4.2.1 Biogas

Due to its high methane concentration (65-70% v/v), which is created anaerobically with VFA serving as a precursor, biogas is most frequently used in the generation of heat and power. Biogas is typically produced in the digester from volatile fatty acids (VFAs) produced during the acidogenesis stage by the breakdown of proteins, carbohydrates, and fats. Except for propionic acid and butyrate, which are first broken down by methanogens into acetic acid and then methane, acetic acid is transformed straight to methane. The process of producing biogas from volatile fatty acids in a single digester (one-phase AD) is more complicated since methanogens and acidogens

have distinct ideal conditions. As a result, the only way to address this problem is the two-phase AD, in which the neutral pH and long solids retention time (SRT) of the second digester enrich slow-growing methanogens, while the first digester maintains an acidic pH and short SRT to promote fast-growing acidogens (Lee et al., 2014).

#### 2.4.2.2 Bioplastics production

Microorganisms may produce biodegradable polymers called polyhydroxyalkanoates (PHAs) from renewable resources such as volatile fatty acids (VFAs). Because PHA produced by MMC doesn't need to be sterilized, it's a more economical way to produce VFAs. The chain length of the VFA has a major impact on the composition, and thus, the mechanical characteristics and application of the resultant PHA.

#### 2.4.2.3 Obtaining lipids from microorganism for biodiesel

Oleaginous microorganisms are considered an alternative raw material for the manufacturing of biodiesel since the fatty acid profiles of soybean oil and jatropha oil, which come from agricultural oil crops, are comparable. In environments where nitrogen is scarce, they may store 20% to 80% of their dry weight as lipids (triacylglycerols) (Annamalai et al., 2018). Oleaginous yeast may readily use VFAs as a carbon source since they need a shorter transformation route and have a higher theoretical lipid conversion efficiency (Chi et al., 2011; Lian et al., 2012)

#### 2.4.2.4 Hydrogen

Hydrogen ($H_2$) is produced as a byproduct of the anaerobic process that turns organic wastes into organic acids. Rhodobacter sphaeroides, a purple nonsulfur bacterium, can also make hydrogen from VFAs by photofermentation. In anaerobic environments, the nitrogenase-hydrogenase enzyme system converts VFAs into hydrogen.

As a two-step process that creates hydrogen, light fermentation is most commonly utilized in combination with dark fermentation since the latter generates both $H_2$ and VFAs, and the $H_2$ production may be increased overall by using the VFA-rich effluent that is produced. The kind of VFAs employed during photofermentation also has the greatest impact on $H_2$ creation; propionate and acetate are superior substrates for hydrogen synthesis than butyrate, leading to a fourfold increase in output (Levin et al., 2004; Uyar et al., 2009).

### 2.4.2.5 Biological nutrient removal

As a substantial carbon substrate that supports biological nitrogen and phosphorus removal from wastewater, VFAs have a useful use. Aerobic nitrification and anoxic denitrification are the processes used to remove biological nitrogen from wastewater. Anaerobic and aerobic conditions result in greater biological phosphorus elimination, which is how phosphorus is removed as well. Methanol, glucose, and acetate are examples of chemical carbon sources used in more expensive denitrification phases. The denitrification method uses VFA, which is created from waste streams, as an inexpensive carbon source. Additionally, VFAs as a carbon source result in greater removal efficiency and denitrification rate when compared to commercial chemicals (Kim et al., 2016; Liu et al., 2016). However, denitrification rate and efficiency are greatly influenced by the makeup of VFAs. Acetate is the first volatile fatty acid (VFA) to be used during nitrogen removal, followed by propionate, butyrate, and valerate. Denitrifying bacteria like longer-chain VFA, which explains why. The average specific denitrification rate with acetate is double that of propionate, indicating that increasing the acetate is necessary for effective denitrification (Elefsiniotis and Wareham, 2007). Schematic diagram showing VFA production and recovery is shown in Figure 2.2.

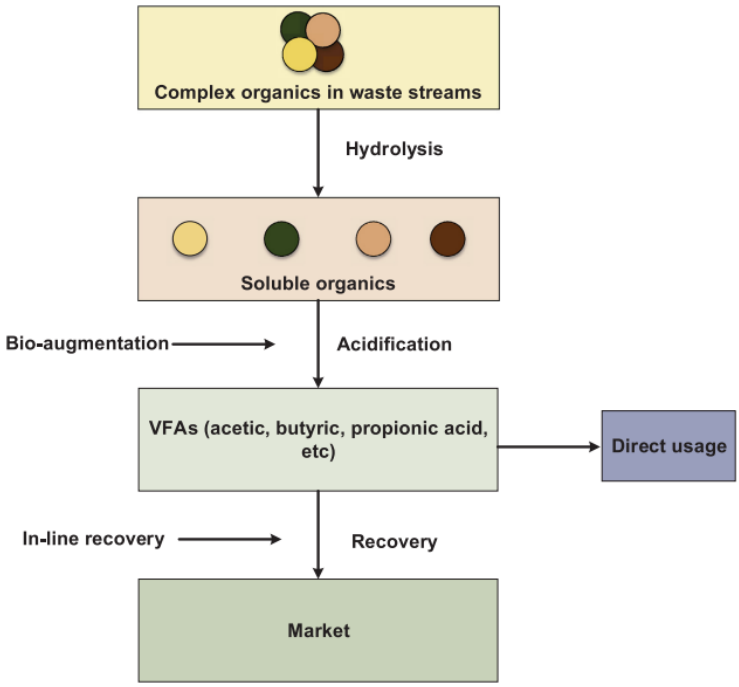



**Figure 2.2 :** Schematic diagram showing VFA production and recovery process from waste streams. Bioaugmentation and in-line recovery will enhance production and recovery of VFA respectively (Atasoy et al., 2018).

## 2.5 Lignocellulosic Structure

The main components of lignocellulosic biomass are cellulose, hemicellulose, and lignin, with trace amounts of extractives and ash. With several linear polysaccharide chains, cellulose comprises around 40% of the total dry weight of lignocellulose. It is strengthened by hydrogen bonds and incorporated in a structure that is resistant to hemicellulose. Lignin and acetyl molecules preserve the covalent connections that hold cellulose/hemicellulose chains and the macrostructure together. In order to break down the complex plant macrostructure, pretreatment techniques have been investigated when lignocellulosic biomass becomes resistant. To create monomeric sugars, exposed polysaccharides can then be hydrolyzed using an acid, alkali, or enzyme. Table 2.5 shows the percentage cellulose, hemicellulose and lignin content of different lignocellulosic raw materials.

**Table 2.5 :** Percentage cellulose, hemicellulose, and lignin content of some lignocellulosic feedstocks (Hassan SS et al., 2018).

| S/N | Materials | Cellulose (%) | Hemicellulose (%) | Lignin (%) |
|---|---|---|---|---|
| 1 | Pine | 45.60 | 24.00 | 24.00 |
| 2 | Groundnut shell | 37.00 | 18.70 | 28.00 |
| 3 | Rubber wood | 39.56 | 28.42 | 27.58 |
| 4 | Hardwood Eucalyptus | 44.90 | 28.90 | 26.20 |
| 5 | Softwood Spruce | 47.10 | 22.30 | 29.20 |
| 6 | Grasses Bamboo | 46.50 | 18.80 | 25.70 |
| 7 | Reed | 49.40 | 31.50 | 8.74 |
| 8 | Oak | 43.20 | 21.90 | 21.90 |
| 9 | Rye | 42.83 | 27.86 | 6.51 |
| 10 | Walnut shell | 23.30 | 20.40 | 53.50 |
| 11 | Sunfower | 34.06 | 5.18 | 7.72 |
| 12 | Japanese cedar | 52.70 | 13.80 | 33.50 |
| 13 | Silage | 39.27 | 25.96 | 9.02 |
| 14 | Szarvasi-1 | 37.85 | 27.33 | 9.65 |
| 15 | Hemp | 53.86 | 10.60 | 8.76 |
| 16 | Pine nut shell | 31.00 | 25.00 | 38.00 |
| 17 | Cotton stalk | 67.00 | 16.00 | 13.00 |
| 18 | Natural hay | 44.90 | 31.40 | 12.00 |
| 19 | Hemp stalk | 52.00 | 25.00 | 17.00 |
| 20 | Amur silver-grass | 42.00 | 30.15 | 7.00 |
| 21 | Coconut coir | 44.20 | 22.10 | 32.80 |
| 22 | Acacia pruning | 49.00 | 13.00 | 32.00 |
| 23 | Rice husk | 40.00 | 16.00 | 26.00 |
| 24 | Rice straw | 38.14 | 31.12 | 26.35 |
| 25 | Bamboo leaves | 34.14 | 25.55 | 35.03 |
| 26 | Extracted olive pomace | 19.00 | 22.00 | 40.00 |
| 27 | Palm oil frond | 37.32 | 31.89 | 26.05 |
| 28 | Sugarcane pee | 41.11 | 26.40 | 24.31 |
| 29 | Hazel branches | 30.80 | 15.90 | 19.90 |
| 30 | Barley straw | 35.40 | 28.70 | 13.10 |
| 31 | Corn stover | 43.97 | 28.94 | 21.82 |
| 32 | Pistachio shell | 15.20 | 38.20 | 29.40 |
| 33 | Cofee grounds | 33.10 | 30.03 | 24.52 |
| 34 | Almond shell | 27.00 | 30.00 | 36.00 |
| 35 | Hazelnut shell | 30.00 | 23.00 | 23.00 |

It is known that there is a strong correlation between lignocellulose recalcitrance and enzymatic saccharification and polysaccharide crystallinity. (Park, S. et al., 2010), the degree of polymerization (DP) (Puri, 1984), the lignin content (Ko, J.K. et al., 2015; Grabber et al., 2008) , and the surface areas (porosity) (Hendriks and Zeeman, 2019). Table 2.6 summarizes the short biomass structural and chemical features, as well as their refractory impacts on pretreatment and enzymatic hydrolysis. An efficient pretreatment strategy takes into account the traits and attributes of lignocellulosic materials in order to optimize the yields of cellulose and/or hemicellulose conversion.

Reducing cellulose crystallization and increasing its surface area for enzymatic digestion, solubilizing hemicellulose and/or lignin, preventing sugar loss, minimizing the formation of undesired lignocellulose-derived inhibitors, and lowering energy and capital costs are the main objectives of an efficient pretreatment.

**Table 2.6 :** Lignocellulosic biomass structural/chemical properties and their recalcitrant effects on pretreatment and enzymatic hydrolysis.

| Biomass Property | Effects on Pretreatment and Enzymatic Hydrolysis | Reference |
|---|---|---|
| Cellulose crystallinity | Chemical bonds that form between and within molecules, such as those seen in linear cellulose chains, are responsible for enzyme loading, feedstock recalcitrance, and severe pretreatment conditions. The feedstock becomes more recalcitrant due to the high cellulose crystallinity, which lowers the cellulose conversion. | (Kumar and Wyman, 2009; Jørgensen et al., 2007) |
| Degree of polymerization (DP) | The usual range for cellulose DP is 800-10,000 (up to 17,000). Because the high DP structure provides fewer reducing sugar endings, which might impact feedstock disobedience and enzyme catalysis, DP reduction is essential for successful cellulose conversion. | (Kim et al., 2015; Foston and Ragauskas, 2010) |
| Lignin | In lignocellulosic materials, lignin serves as a biological glue and secondary cell wall. The functions of lignin are harmful to pretreatment, enzyme usage, cellulose conversion, and total costs. Lignin content must be decreased or delignified using pretreatments, genetic/system engineering, and feedstock selection/modification in order to maximize the final conversion yield and productivity. | (Ko et al., 2015; Hendriks and Zeeman, 2009; Ximenes et al., 2011) |
| Hemicellulose | The most common hemicellulose found in plants, xyan, covalently links with lignin to protect plant cells and creates a coating layer with cellulose by hydrogen bonding. Solubilizing the hemicellulose components is the main objective of the pretreatment, which may also enhance the hydrolysis and digestibility of cellulose. | (Ishizawa et al., 2007; Selig et al., 2009) |

### 2.5.1 Degree of polymerization (DP) and cellulose crystallinity

One of the main ingredients for effective hydrolysis is assumed to be the crystalline element of cellulose, since its high degree of crystalline structure renders it less vulnerable to enzymatic activities than cellulose's amorphous component (Kumar and Wyman, 2009). The pretreatment method's crystallinity reduction has been considered a crucial element in determining the hydrolysis rate and cellulose conversion yield, particularly at low enzyme loadings. The accessible surface area of cellulose is significantly correlated with the kinetics of enzyme binding and the effectiveness of enzymatic digestion, according to additional research (Qin et al., 2016; Jørgensen et al., 2007). Cellulose has an enhanced exterior surface area due to its very small particle size, which may increase conversion yield by enabling more enzyme access to the surface. It also has an interior surface area connected to its narrow vein structure, or capillary tube. For example, a 50% increase in cellulose conversion to glucose was seen when the particle size of mixed hardwood subjected to liquid hot water pretreatment was reduced from 3 mm to 2 mm (Kim et al., 2015).

### 2.5.2 Lignin

Lignin acts as a solid adhesive to cellulose and hemicellulose in lignocellulosic feedstock, preserving the proximity and integrity of the lignocellulose structure. As a significant indocile molecule and one of the notable molecules that may generate a multitude of inhibitory byproducts throughout the pretreatment process, lignin is made up of many phenolic acids, such as p-coumaryl, coniferyl, guaiacyl, syringyl, and sinaphy (Hendriks and Zeeman, 2009; Ximenes et al., 2011; Ximenes et al., 2010). A delignification method could be necessary to increase cellulose conversion since compounds generated from lignin are among the most significant inhibitors of enzyme reactions and microbial fermentation (Chang and Holtzapple, 2000; Nakagame et al., 2011)

Lignin may also impede the catalysis of enzymes directly or indirectly. Enzyme activity is greatly decreased by non-productive bindings of cellulase/hemicellulase enzymes to lignin molecules, most likely as a result of hydrophobic interactions between the lignin and the enzyme (Ko et al., 2015; Nakagame et al., 2011). After 1.5 hours of incubation at 25°C, lignin from hardwood that has been prepared with liquid hot water may adsorb up to 60% of the cellulase enzyme (Ko et al., 2015).

### 2.5.3 Hemicellulose

Compared to cellulose, hemicellulose is more likely to be hydrolyzed because it includes five or six different carbon sugars, including xylose, arabinose, galactose, and mannose. It does, however, primarily protect the cellulose fiber against enzymatic damage. Pretreatment is mostly used to solubilize hemicellulose, since its removal indicates that the cellulose with larger pore volumes would be more accessible to enzymes (Ishizawa et al., 2007). Acetyl groups are widely bonded to hemicellulose polymers, which can prevent appropriate enzyme binding to cellulose. In general, hemicellulose with interlinkages in the xylan backbone has its acetyl group released by a pretreatment with acidic chemicals (Pan et al., 2006; Selig et al., 2009). A deacetylation process using acetic acid was suggested as a way to lessen acetyl group inhibitions from lignocellulose; however, more study was needed on enzyme inhibition (Jönsson et al., 2013; Chen et al., 2012; Pu et al., 2013).

## 2.6 Pretreatment of Lignocellulosic Materials

Microbial digestion is complicated by the complex structure of lignocellulosic materials, which is caused by the lignin load and crystallinity (Hendriks and Zeeman, 2009). In certain instances, the biogas production is as low as 10% of the possible methane yield due to the inadequate fermentation of microbes with lignocellulosic material (Yang et al., 2015). Many different kinds of pretreatments may be applied to get rid of this lignocellulosic recalcitrance and improve anaerobic digestion. The primary physicochemical properties of the material that are susceptible to variation based on the selected pretreatment technique are the size of molecules, surface accessibility, particle size, and distribution of pore sizes (Hendriks and Zeeman, 2009). Furthermore, cellulose and lignin may become more soluble as a result of some pretreatment options that alter the material's chemical composition (Johnson and Elander, 2009).

Enhancing cellulose availability for lignocellulosic substrate hydrolysis is the main objective of pretreatment. Therefore, a number of pretreatment methods aim to alter the biomass's chemical and physical characteristics and promote quicker hydrolysis rates. The lignin and hemicellulose are successfully removed, and the crystallinity of the cellulose increases (Chandra et al., 2012). It is therefore normal to expect a decrease in lignin and hemicellulose content as well as an increase in cellulose quantity

following pretreatment. The application of heat pretreatment increased the lignin content in a rice straw study from 16.3 to 17.3% (Wang et al., 2018). After the method, the hemicellulose content of the raw substrate, which was 28%, rose to 35% with the help of ultrasound-assisted lime pretreatment with areca nut husk (Sasmal et al., 2012). Although cellulose is becoming more readily available, by-product production might also be noticed. Prior to the cellulose being exposed to microbial degradation, pretreatment frequently experiences side reactions that produce byproducts produced from lignocellulose that hinder biological processes. When byproducts build up in the media, inhibition problems get worse (Jönsson and Martín, 2016). For best outcomes, pretreatments try to get rid of hemicellulose and/or lignin. These objectives, though, may influence other variables. For example, increased solubilization of lignin and/or hemicellulose causes the solubilized components to degrade because of the harsh circumstances they are exposed to (Jönsson and Martín, 2016).

Furans, organic acids, and phenolic compounds are the major byproducts formed during the processing of lignocellulosic substrates (Palmqvist and Hahn-Hägerdal, 2000). Furan formation occurs when monomeric sugars are present at low pH levels and high temperatures (Kabel et al., 2007). The microbial system is inhibited by furan production, which also results in significant losses in sugar yields (Pol et al., 2014). Acetyl and uronic acid groups are present in hemicellulose's composition because it has non-sugar portions in its structure. These acids may be released and turn into systemic inhibitors upon the hydrolysis of polymers in lignocellulosic substrates (Sun et al., 2004). The primary organic acids that inhibit include lactic acid, levulinic acid, formic acid, and acetic acid (Larsson et al., 1999). Pretreatment procedures result in the formation of numerous kinds of phenolic compounds. The hydrolysis process can be inhibited and microorganisms' cell structure can be destroyed by phenol, p-cresol, and possibly other phenolic compounds (Sun et al., 2004).

An extra upstream step to eliminate byproducts produced during fermentation could be introduced to lessen the inhibitory impact (Pol et al., 2014). Furthermore, the generated byproducts from pretreated lignocellulosic substrate can be eliminated by a pre-fermentation carried out using microorganisms that consume inhibitors (Koopman et al., 2010)

Ultimately, the pretreatment procedure's objective is to modify the substrate's composition in order to get around hydrolysis obstacles and improve the rate of

solubilization and yields of cellulose and hemicellulose (Taherzadeh and Karimi, 2008). An successful pretreatment should be economical, minimize the synthesis of inhibitory chemicals, stop the consumption of carbohydrates, and encourage the creation of sugar through microbial breakdown (Sun and Cheng, 2002).

A combined pretreatment approach consists of utilizing two or more pretreatment procedures at the same time. Biomass with lignin types, content, and characterization are critical elements in determining the best combination pretreatment application. The Table 2.7 provides information regarding the influence of pretreatment procedures on biogas output (Paul et al., 2018).

**Table 2.7 :** Effect on pretreatment methods on biogas production (Paul et al., 2018).

| | Physical | Thermal | Hydrothermal | Alkaline-Thermal | Alkaline-Hydrothermal |
|---|---|---|---|---|---|
| Woody | Lowest | Low | Medium | High | High |
| Non-Woody | Lowest | Low | Medium | High | Highest |

## 2.7 Microbiology of Anaerobic Digestion

When organic macromolecules like proteins, lipids, and carbohydrates are broken down into soluble organics by the AD process, an oxygen-free environment is created that is then converted by a variety of bacteria and archaea species into biogas (Hatti-kaul R. et al., 2016 ; Weiland P, 2010). These bacteria participate in four complex and interrelated metabolic reactions: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, which result in the breakdown of organic molecules and the formation of $H_2$, $CO_2$, and $CH_4$, as well as $H_2S$ in trace levels. However, significant microbiological and operational hurdles must be overcome in order to develop viable AD platforms for a variety of end products. The AD process's microbiological characteristics could be very different from those of other industrial processes. Whole-genome sequencing (WGS), next-generation sequencing (NGS), comparative analysis, microcosm studies, and omics approaches (genomics, metagenomics, transcriptomics, metatranscriptomics, proteomics, metaproteomics, metabolomics, or meta-metabolomics) may be more applicable and useful than sterility or pure cultures in certain situations (Kumar Awasthi M. Et al., 2020; Tabatabaei and Ghanavati , 2018).

Microorganisms with connected metabolisms perform the steps of hydrolysis, acidogenesis, acetogenesis, and methanogenesis during anaerobic digestion.

Therefore, as long as a balanced reaction rate is kept across the phases and microbial guilds, constant digestion may be accomplished (Venkiteshwaran et al., 2015).

These processes involve acetogens that produce and use hydrogen, hydrolytic fermentative bacteria, acidogenic microorganisms, and methanogenic organisms (Lettinga et al., 1996; Chernicharo, 2007).

Bacteria that are hydrolytic fermenters break down complex organic materials into monomers. Numerous factors, including diffusion, pH, and the amount of enzymes produced, influence the pace of hydrolysis. The bulk of the known species of hydrolytic bacteria belong to the two phyla Bacteroidetes and Firmicutes, notwithstanding their diversity (Venkiteshwaran et al., 2015). Species from these two phyla dominate the hydrolysis stage.

In acidogenesis, hydrolysis products are transformed into VFAs such as acetate, propionate, isobutyrate, butyrate, valerate, and isovalerate. In addition to VFAs, additional chemicals generated include alcohols, lactate, formate, $CO_2$, and $H_2$.

Methanogens are members of the Archaea domain and are susceptible to a variety of environmental factors, including variations in pH, VFA concentrations, ammonium ion, and free ammonia concentrations (Westerholm et al., 2012; Manyi-Loh et al., 2013). Methanogens are vital in anaerobic digestion because they create methane in the last stage. Methanogens are now divided into six categories: Methanopyrales, Methanobacteriales, Methanosarcinales, Methanococcales, Methanomicrobiales, and Methanocellales. Methanosarcinales consists of two families: Methanosarcinaceae and Methanosaetaceae. Even though they are both categorized as acetoclastic methanogens, the two families have different physiologies, growth environments, and biokinetics depending on the acetate content. Moreover, about half of the production of methane is attributed to acetate (Manyi-Loh et al., 2013). Table 2.8 shows some examples of microorganisms involved in the different stages of anaerobic digestion.

**Table 2.8 :** Microorganism involved in the various phases of the AD process (Insam et al., 2019; Khanal, 2011; Korres et al., 2013; Amin et al., 2021; Ahamed and Prasad, 2021; Shnürer, 2016).

| AD Phase | Microbial Domain | Microbial Genus | Examples of Identified Species |
|---|---|---|---|
| Hydrolysis and Fermentation | Bacteria | Acetivibrio, Aminobacterium, Aminomonas, Anaeromusa, Anaerosphaera Bacillus, Bacteroides, Bifidobacterium, Butyrivibrio, Caldanaerobacter, Caldicellulosiruptor, Campylobacter, Cellulomonas, Clostridium, Devosia Espiroquetas. Eubacterium Fervidobacterium, Fibrobacter, Fusobacterium Gelria, Gracilibacter Halocella Lactobacillus Paludibacter, Peptococcus, Peptoniphilus, Proteiniborus, Pseudomonas, Psychrobacter Ralstonia, Ruminoclostridium, Ruminococcus Selenomonas, Shewanella, Sporanaerobacter, Streptococcus, Streptomyces Thermanaerovibrio, Thermomonas, Thermomonospora, Thermotoga, Treponema, Trichococcus | Pseudomonas mendocina Bacillus halodurans Clostridium hastiforme Gracilibacter thermotolerans Thermomonas haemolytica |
| | Fungi | Aspergillus Humicola Penicillium Trichoderma | Trichoderma reesei |
| Acetogenesis | Bacteria | Acetobacterium Clostridium Desulfotignum Eubacterium Holophaga Moorella Ruminococcus Sporomusa Thermoanaerobacter, Treponema | Moorella thermoacetica Desulfotignum phosphitoxidans Holophaga foetida |
| Methanogenesis | Archaea | Methanobacterium, Methanobrevibacter, Methanococcus, Methanoculleus, Methanosaeta, Methanomicrobium, Methanosarcina, Methanospirillum, Methanothermobacter | Methanobrevibacter smithii Methanobrevibacter arboriphilus Methanococcus vannielii |

### 2.7.1 DNA Sequencing and microbial analyses in anaerobic digestion

Anaerobic digestion (AD) is a process that produces biogas from organic wastes and involves a variety of microorganisms (Venkiteshwaran et al., 2015). Life and ecological balance are thought to depend on the functionality, interaction, and dynamics of microbial communities. thought to depend on the functionality, interaction, and dynamics of microbial communities.

Numerous methods, including as genomic sequencing, metagenomics, denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH), and real-time polymerase chain reaction (Q-PCR), are available for identifying the microbial community in anaerobic digesters (Bozan, 2018).

### 2.7.2 Real-Time polymerase chain reaction (Q-PCR)

The quantitative polymerase chain reaction, or Q-PCR, is one technique to determine the amount of PCR product. It is also a very valuable technique for assessing gene expression and for improving functional genomics. Different types of Q-PCR exist.

For gene identification, the basic Q-PCR method is useful without establishing the expression level. By tracking the amount of PCR product over time and evaluating the effects of other parameters, including melting temperature, one may use the data gathered during the process. To determine expression, transcriptase reverse PCR is a commonly utilized technique. To achieve that, prior to the Q-PCR procedure, the RNA is extracted and transformed into cDNA (Maddocks & Jenkins, 2016)

### 2.7.3 Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) technology allows us to find out more about both culturable and unculturable microorganisms as well as identify specific groupings of bacteria. The microorganisms in the sample can be defined in a variety of ways, including by domain, family, genus, and species. Furthermore, certain concepts about the composition and roles of the intricate microbial community may be obtained using fluorescence in situ hybridization.

However, due to the unique properties of its cell wall, it is difficult to see using fluorescent microscopy (Dinova, et al., 2018).

### 2.7.4 Metagenomics

The investigation of complex microbial communities is a well-known use of the metagenomics approach. The two most popular techniques to do this for high throughput data are whole metagenomic shotgun sequencing and amplicon-based technologies. The majority of the species in the environmental sample may be identified using shotgun metagenomic analysis, which is classified into two categories: functional screens and sequence-based screens. The internal transcribed spacer, the 16S ribosomal RNA for bacteria, and the 18S region for fungi and eukaryotes are the components of the amplicon-based technique, respectively (Ghosh, et al., 2019).

The initial generation of automated DNA sequencers used fluorescent dye-terminator reagents and the Sanger technique to sequence DNA populations. Computers were added to these sequencers to enable the collection, archiving, and analysis of sequencing data.

Genomic research has evolved as a result of next-generation sequencing (NGS) technology; in the second generation, parallel sequencing dramatically expanded. Compared to second-generation sequencing technologies, which allow for the direct

sequencing of single DNA molecules, third-generation sequencing technologies allow for longer reads (Heather & Chain, 2016).

Nanopore sequencing is the most recent and much anticipated development in the field of genomic sequencing. This innovative technique may lead to the usage of nanopores for the detection and measurement of a broad spectrum of biological and chemical substances. This innovative technique may lead to the usage of nanopores for the detection and measurement of a broad spectrum of biological and chemical substances. Targeted amplicons and reference sequences for the bacterial genome may be produced using MinION, a tiny USB device the size of a cell phone. Owing to its compact design and quick charging periods, the MinION gadget is highly practical (Heather & Chain, 2016).

The first commercial nanopore sequencer, the MinION, was introduced by Oxford Nanopore Technologies (ONT) in 2014. One has to examine the differences in electrical conductivity caused by DNA strands flowing through biological pores in order to comprehend how the MinION functions. Once these alterations are measured, the DNA bases are determined by the MinION. Real-time applications might benefit from its swift data production, portability, and affordable price. The genomics community was enthusiastic and interested in pathogen surveillance and clinical diagnostic applications when the long-read sequencer MinION was introduced (Lu, et al., 2016). Table 2.9 briefly summarizes the comparison of 16S metagenomic analysis sequencing platforms using the Metabarcoding technique

**Table 2.9 :** A comparison of the 16S metagenomic analysis sequencing platforms employing the metabarcoding technique (Adres et al., 2020).

| Sequencing Platform | Read Length (bp) | Accuracy | Output | Sequencing Chemistry | Run Time | Advantages in Metabarcoding approaches |
|---|---|---|---|---|---|---|
| Sanger | 400–900 | 99.999% | 1.9–84 Kb | Dideoxy chain termination | 20 min – 3 h | Long read length, high quality |
| llumina MiSeq | 75–300 | 99.9% | 13.2– 20 Gb | Sequencing by Synthesis | 21–56 h | High Throughput, read quality |
| MinION | >200,000 | ~95% | ~50 Gb | Single Sequencing real time long read | 1–48 h | High Throughput, Long read length, portability |
| PacBio | 10–15 Kb | 99.999 | 5–10 Gb | Single Sequencing real time- long read | 4 h | High Throughput, Long read length, portability |

# 3. MATERIALS AND METHOD

## 3.1 Substrate and Inoculum Characterization

Sunflower residues, which are widespread in specific parts of Turkey, were used as a substrate in this study. These remains were harvested in the Thrace Region during the summer of 2023. The fundamental motivation behind picking this substrate was its total disposal status, leaving it obsolete for any future industrial or agricultural applications. As a result, the sunflower remains, which included stalks and heads, had fallen out of use following the harvesting procedure. Figure 3.1 shows the collected dried sunflowers.

**Figure 3.1 :** Dried sunflower head and stalks.

Before the experiment, dried sunflower waste was kept at Boğaziçi University's Microbial Ecology Laboratory in anaerobic conditions at +4 degrees Celsius.

The stored samples were homogeneously pulverized to 1-3 mm in size using a household blender in line with the experimental protocol.

After milling, the substrate was subjected to UV light for 30 minutes to kill any leftover microorganisms. The primary reason for this is that no mold or microbial variety remains, and a homogenous dry substrate is supplied.

### 3.1.1 Anaerobic seed sludge

The inoculum sludge employed in the anaerobic process was sourced from a fully operational wastewater treatment plant in Antalya, Turkey.

Following collection, the sludge was stored at a temperature of +4 degrees Celsius in anaerobic conditions until used in the experiments.

### 3.1.2 Analytical determinations

Standard Procedures were followed in order to quantify total solids (TS), volatile solids (VS), alkalinity, soluble Chemical Oxygen Demand (sCOD), and total Kjeldahl Nitrogen (TKN) (Federation & Association, 2005). Figure 3.2 is the image of the samples after burning in total solid and volatile solid measurements.

**Figure 3.2 :** The process of measuring TS, VS.

With the use of dried samples and an automated elemental analyzer (ECS 4010 CHNS-O Analyzer, COSTECH Analytical Technologies, INC., USA), the carbon to nitrogen (C: N) ratio was calculated.

With the Hach Pocket Pro+ pH meter, the pH was determined.

The titration technique using 0.1N $H_2SO_4$ was utilized to calculate the alkalinity based on the amount of sulfuric acid consumed. For chemical analysis, samples' soluble chemical oxygen demand (sCOD) was evaluated both before and after anaerobic digestion. Table 3.1 is given substrate and seed sludge of characterization.

**Table 3.1 :** Initial characterization of substrate and seed sludge.

| | pH | TS (%) | VS (%) | VS/TS (%) | Alkalinity (mg CaCO3/L) | TKN (mg/L) | C:N |
|---|---|---|---|---|---|---|---|
| **Substrate, as Sunflower** | 7,65 | 93.1 | 82.9 | 0,89 | 3200 | 336 | 17 |
| **Seed Sludge** | 7,80 | 1.28 | 0,87 | 0,72 | 5150 | 550 | 7 |

## 3.2 Experimental Procuders

### 3.2.1 Physico-Chemical pretreatment

In this study, physico-chemical pretreatment was applied to better break down lignocellulosic structures. Sunflower waste was pretreated using three different doses of HCl (hydrochloric acid) (0.8%, 1.2%, 1.6%) calculated according to the dry matter content of the substrates.

The inoculum sludge: substrate ratio was determined as 2:1. 0.86 grams of VS was measured for 100 mL of inoculum. Based on this, it was calculated that 0.52 g VS inoculum was required for 0.43 g substrate VS.

The samples were subjected to physicochemical pretreatment with three doses of HCl acid of different percentages, respectively, at 120 and 140 degrees Celsius for 30 minutes in a microwave (Cem Mars 6 microwave digestion system).

Samples to be physicochemical treated in HCl solutions of different concentrations in the microwave were prepared according to the procedure. For each measurement in the microwave, 20 mL of solution and 1 g of substrate had to be mixed. However, since the substrate value was 0.93 in the TS value measurements, it was calculated as 1/0.93 = 1.075 g. Therefore, 1.075 g of substrate was added to each 20 mL sample solution. While preparing HCl solutions, for example for 1.2% HCl solution; 0.24 g of HCl acid was required. Since the density of HCl is 1.19, so 0.24 g /1.19 = 0.201 mL HCl was calculated. 0.201 mL HCl was made up to 20 mL with deoinized water. Samples prepared for each condition were pre-treated with CEM MARS 6 microwave at 120 and 140 degrees for 30 minutes.

Exposure to microwave treatment in acid solution completed the physicochemical pretreatment of the samples. Additionally, a control sample was prepared using deionized water, denoted as 0% HCl. This sample was kept in deoinized aqueous

solution for only 30 minutes. Figure 3.3 is showed the microwave used in the pretreatment process.


**Figure 3.3 :** CEM MARS 6 microwave.

After the pretreatment process, all samples were filtered using coffee filters and filters with a pore size of 0.2 mm to help separate the liquid and solid fraction and it is showed in Figure 3.4.


**Figure 3.4 :** 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.

For VFA measurement, samples were collected from the anaerobic digestion system on the 0th, 1st, 2nd, 3rd, 6th, 10th, 15th, 20th, and 30th days after it had been operational for 30 days.

### 3.2.2 Setup of the anaerobic digestion process

Following separation, the solid portions were weighed and transferred to 100 mL vials, where they were blended with the inoculum sludge. In each digester, a specific amount of 3.3 gVS, equivalent to 165 mL of seed sludge, was combined with 1.65 gVS of each sample, resulting in a 1:2 inoculum-to-substrate ratio.

After the computations, the inoculum and corresponding samples were added to the bottles, and the pH was adjusted to 5.5 to guarantee the best circumstances for acidification. All of the bottles were closed securely and purged with $N_2$ gas for five minutes in order to preserve anaerobic conditions. Every experimental method was carried out in triplicate to increase dependability. Following this, the bottles were incubated for 30 days at 37 degrees Celsius in a room. A 50 mL sample from the inoculum was also taken out and stored at -20 degrees Celsius in preparation for further examinations. Figure 3.5 is showed end of anaerobic digestion setup.

**Figure 3.5 :** Anaerobic digestion setup.

### 3.2.3 Controlling of pH

To keep the acidification process running smoothly, pH measurements were taken on a daily basis with the Hach Pocket Pro+ pH meter. If a change in pH was observed, 1 M HCl or 1 M KOH solutions were added to the anaerobic bottles with a syringe to bring the pH to 5.5. Figure 3.6 is showed pH controlling and sampling in on specified days.

**Figure 3.6 :** pH controlling and sampling.

### 3.3 COD Analysis

To determine the effect of pretreatment on Chemical Oxygen Demand (COD) levels, tests were performed on the liquid fraction of the separated samples.

Soluble COD tests were performed on the filtrates collected after a 24-hour pretreatment period in order to evaluate the difference in COD levels between treated and untreated samples. 0% HCl solution was used to create a control sample. In order to separate soluble COD levels for testing, the filtrates were first run through filters with a size of 0.22 μm.

HACH Heating COD reactor and HACH DR/2010 portable datalogging spectrophotometer devices were used for sCOD measurement. 500 μL of sample was made up with 49.5 mL of ionized water. The aim is to ensure that COD values come to the range measurable by the device after physicochemical pretreatment. For this reason, a 1/100 dilution was made. Confidence was ensured for data acquisition with triplicate measurement. 2.5 mL of each diluted sample was taken into COD tubes and 1.5 mL digest solution and 3.5 mL sulfuric acid agent were added. COD tubes were heated in the heating reactor at 150$^{o}$C for 2 hours. After waiting for 15-20 minutes to cool, the values were read with a HACH DR/2010 portable datalogging spectrophotometer at 600 nm and the results were recorded. HACH DR/2010 portable datalogging spectrophotometer is showed in Figure 3.7.

For the sulfuric acid agent, a mixture of 10.1 g $Ag_2SO_4$ and 1 L $H_2SO4$ was left for 1-2 days.

While preparing the digest solution, 500 mL deonized water, 10,216 g/L $K_2Cr_2O_7$ (dried in an oven at 105$^{o}$C for 1 day) and 167 mL/L $H_2SO_4$ solution were prepared.

This solution was mixed in the mixer to dissolve. 33.3 g/L $HgSO_4$ was added to it. When it cooled, the solution was completed to 1 L with deonized water and left for 2 days.


**Figure 3.7 :** sCOD measurement with HACH DR/2010 portable datalogging spectrophotometer at 600 nm.

### 3.4 VFA Analysis

Throughout the trial, systematic sampling took place at predetermined intervals: days $0^{th}$, $1^{st}$, $2^{nd}$, $3^{rd}$, $6^{th}$, $10^{th}$, $15^{th}$, and $30^{th}$. A syringe was used to collect 0.9 ml of each sample, which was then mixed with 0.1 ml of 10N phosphoric acid. VFA was stabilized with phosphoric acid until the sample was measured and then stored at +4 degrees Celsius to fix all biological activity until VFA analyses were performed.

Samples were placed into a Shimadzu GC-2025 Gas Chromatograph, a VFA measurement device, following the separation procedure. Liquid seperated from anaerobic digestion sample is showed in Figure 3.8 and the VFAs' creation was observed.

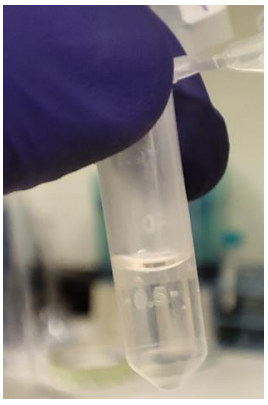


**Figure 3.8 :** Liquid seperated from anaerobic digestion samples.

Acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleic acid, valeic acid, isocaproic acid, caproic acid and heptanoic acid obtained in terms of area were determined. VFA measurement at Shimadzu GC-2025 Gas Chromatograph is showed in Figure 3.9. The values in area were converted to ppm with the coefficient formulas determined for each acid type. After the ppm value, all VFA values were converted to COD equations, that is, g COD/g acid. In this way, the value of each measurement in sCOD was found.

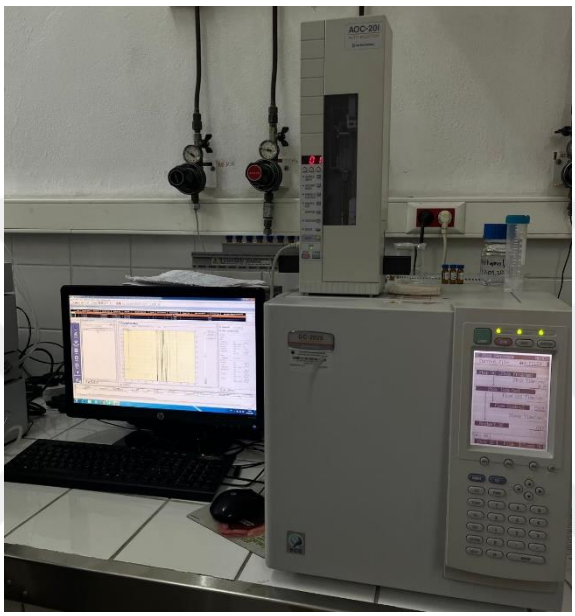


**Figure 3.9 :** VFA measurement at Shimadzu GC-2025 Gas Chromatograph.

### 3.5 Microbial Analysis

The microbial communities of inoculum sludge and the most effective pretreatment technique's microbial communities were compared in the study by looking at 16S, 18S, and archaea. Under the most effective pretreatment condition, the types and changes of the microbial community were identified. DNA extraction and the 16S and 18S methods were used for this, respectively.

#### 3.5.1 DNA extraction protocol

The Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (D6010, Zymo Research Corp, California) was used to extract genomic DNA, and the manufacturer's instructions were adhered to. 1 ml of the samples was taken ZR BashingBead™ Lysis Tube and the tube was closed in a controlled manner to prevent leakage. The tubes were fixed to a bead beater in a 2 ml tube holder assembly. The samples were centrifuged at 13,000

x g for 5 minutes and the supernatant was removed. One minute at 8,000 x g centrifugation was performed after transferring up to 400 µl of supernatant to a Zymo-SpinTM III-F Filter in a Collection Tube. 1,200 µl of Genomic Lysis Buffer was added to the filtrate in the Collection tube. 800 µl of this mixture was transferred to a new collection tube, Zymo-Spin™ IICR Column. Since the Zymo-Spin™ IICR Column has a maximum capacity of 800 µl, this amount of sample was taken and centrifuged at 10,000 x g for 1 minute. The centrifuged sample was transferred to a new collection tube and 200 µl of DNA Pre-Wash Buffer was added and centrifuged at 10,000 x g for 1 minute. 500 µl g-DNA Wash Buffer was added to the Zymo-Spin™ IICR Column and centrifuged 10,000 x g for 1 minute. Zymo-Spin™ IICR Column was transferred to a 1.5 ml clean microcentrifuge tube and added to 50 µl DNA Elution Buffer. To elute DNA centrifuged at 10,000 x g for 30 seconds. The Zymo-Spin™ III-HRC Filter was placed in a clean collection tube and 600 µl of Prep Solution was added. It is centrifuged at 8,000 x g for 3 minutes. After the DNA has been eluted, transfer it to a prepared Zymo-SpinTM III-HRC Filter in a sterile 1.5 ml microcentrifuge tube. Centrifuge for 3 minutes at precisely 16,000 xg. DNA extraction protocol is completed and was suitable for PCR and sequencing. Following isolation, DNA concentrations were determined using the iQuant dsDNA high sensitivity (N103) kit and the Qubit 4 device.

### 3.5.2 Polymerase chain reactions (PCR) and sequencing

All conditions were kept on ice during the experiment. Each sample was thawed and centrifuged. The following PCR reactions were set up. 2x HiFi Taq Master Mix was used as enzyme (HF115, MobiomX, Turkey). For each sample, mixtures amount were taken at the rates given in the Table 3.2.

**Table 3.2 :** Mixtures amount of sample and seed sludge for PCR.

| | For pretreated Sample-16S | For Seed Sluge – 16S | For pretreated Sample-18S | For Seed Sluge – 18S | For pretreated Sample Archaea | For Seed Sluge – Archaea |
|---|---|---|---|---|---|---|
| 2 x HiFi Taq Master Mix | 10 µL | 10 µL | 10 µL | 10 µL | 10 µL | 10 µL |
| Primer mix (10 µM each) | 2 µL | 2 µL | 2 µL | 2 µL | 2 µL | 2 µL |
| DNA | 4 µL | 3,5 µL | 4 µL | 3,5 µL | 4 µL | 3,5 µL |
| Water | 4 µL | 4,5 µL | 4 µL | 4,5 µL | 4 µL | 4,5 µL |

16S, 18S , archaea PCR conditions were created in accordance with the manufacturer's protocol report and showed in Table 3.3, Table 3.4, Table 3.5, respectively.

**Table 3.3 :** PCR conditions for 16S (V1-V9).

| | | | |
|---|---|---|---|
| Initial Denaturation | 95 °C | 3 minutes | 1 cycle |
| Amplification | 95 °C<br>58 °C<br>72 °C | 15 seconds<br>15 seconds<br>45 seconds | 30 cycle |
| Final Extension | 72 °C | 5 minutes | 1 cycle |
| Hold | 4 °C | hold | |

**Table 3.4 :** PCR conditions for 18S.

| | | | |
|---|---|---|---|
| Initial Denaturation | 94 °C | 30 seconds | 1 cycle |
| Amplification | 94 °C<br>57 °C<br>72 °C | 20 seconds<br>1 minute<br>1 minute | 35 cycle |
| Final Extension | 72 °C | 10 minutes | 1 cycle |
| Hold | 4 °C | hold | |

**Table 3.5 :** PCR conditions for archaea primary.

| | | | |
|---|---|---|---|
| Initial Denaturation | 94 °C | 30 seconds | 1 cycle |
| Amplification | 94 °C<br>56 °C<br>72 °C | 20 seconds<br>1 minute<br>1 minute | 35 cycle |
| Final Extension | 72 °C | 10 minutes | 1 cycle |
| Hold | 4 °C | hold | |

Th ideal PCR conditions were found for effective full-length 16S rRNA (V1 to V9), 18 rRNA and archaea gene amplicon sequencing, as given Table 3.6:

**Table 3.6 :** Primers used for 16S, 18S anc archaea gene full reading.

| Oligo name | 5' to 3' | Amplicon | References |
|---|---|---|---|
| 16S-27F | AGAGTTTGATCMTGGCTCAG | 16S | Cusco, 2020 |
| 16S-1492R | CGGTTACCTTGTTACGACTT | 16S | Cusco, 2020 |
| 18S-566F | CAGCAGCCGCGGTAATTCC | 18S | Davidov et al., 2020 |
| 18S-1289R | ACTAAGAACGGCCATGCACC | 18S | Davidov et al., 2020 |
| SSU1ArF | TCCGGTTGATCCYGCBRG | Archaea | Bahram et al., 2017 |
| 1000R | GGCCATGCACYWCYTCTC | Archaea | Bahram et al., 2017 |

After the PCRs were completed, 2 μL of all amplicons was taken and the gel was run in a 1% agarose gel at 120 V for 20 minutes. Marker used: DL5000 Plus DNA Marker (MD102-02 MobiomX, Turkey). The quality of PCR amplification was evaluated of this approach.

### 3.5.3 Library preparation and sequencing

Native Barcoding Kit 24 V14 (SQK-NBD114.24) was utilized for the rapid barcoding of PCR amplicons of every samples and for the priming and loading the flow cell, following the protocol (NBA_9168_v114_revO_15Sep2022) provided by Oxford Nanopore Technologies. Library preparation was carried out in accordance with the Nanopore amplicons by ligation protocol by taking 50 fmol from each amplicon. NEBNext Ultra II End Repair / dA-tailing Module (NEB, E7546) was used for the end-repair and dA-tailing phase. Used a thermal cycler, incubated at 20°C for 5 minutes and 65°C for 5 minutes. Then, the barcodes in the SQK-NBD114.24 kit and the NEB Blunt/TA Ligase Master Mix (NEB, M0367) enzyme were used to barcode the samples. Bardods are included in Table 3.7.

**Table 3.7 :** Native barcode sequences.

| Component | Forward sequence | Reverse sequence |
|---|---|---|
| NB01 | CACAAAGACACCGACAACTTTCTT | AAGAAAGTTGTCGGTGTCTTTGTG |
| NB02 | ACAGACGACTACAAACGGAATCGA | TCGATTCCGTTTGTAGTCGTCTGT |
| NB03 | CCTGGTAACTGGGACACAAGACTC | GAGTCTTGTGTCCCAGTTACCAGG |
| NB04 | TAGGGAAACACGATAGAATCCGAA | TTCGGATTCTATCGTGTTTCCCTA |
| NB05 | AAGGTTACACAAACCCTGGACAAG | CTTGTCCAGGGTTTGTGTAACCTT |
| NB06 | GACTACTTTCTGCCTTTGCGAGAA | TTCTCGCAAAGGCAGAAAGTAGTC |
| NB07 | AAGGATTCATTCCCACGGTAACAC | GTGTTACCGTGGGAATGAATCCTT |
| NB08 | ACGTAACTTGGTTTGTTCCCTGAA | TTCAGGGAACAAACCAAGTTACGT |
| NB09 | AACCAAGACTCGCTGTGCCTAGTT | AACTAGGCACAGCGAGTCTTGGTT |
| NB10 | GAGAGGACAAAGGTTTCAACGCTT | AAGCGTTGAAACCTTTGTCCTCTC |
| NB11 | TCCATTCCCTCCGATAGATGAAAC | GTTTCATCTATCGGAGGGAATGGA |
| NB12 | TCCGATTCTGCTTCTTTCTACCTG | CAGGTAGAAAGAAGCAGAATCGGA |
| NB13 | AGAACGACTTCCATACTCGTGTGA | TCACACGAGTATGGAAGTCGTTCT |
| NB14 | AACGAGTCTCTTGGGACCCATAGA | TCTATGGGTCCCAAGAGACTCGTT |
| NB15 | AGGTCTACCTCGCTAACACCACTG | CAGTGGTGTTAGCGAGGTAGACCT |
| NB16 | CGTCAACTGACAGTGGTTCGTACT | AGTACGAACCACTGTCAGTTGACG |
| NB17 | ACCCTCCAGGAAAGTACCTCTGAT | ATCAGAGGTACTTTCCTGGAGGGT |
| NB18 | CCAAACCCAACAACCTAGATAGGC | GCCTATCTAGGTTGTTGGGTTTGG |
| NB19 | GTTCCTCGTGCAGTGTCAAGAGAT | ATCTCTTGACACTGCACGAGGAAC |
| NB20 | TTGCGTCCTGTTACGAGAACTCAT | ATGAGTTCTCGTAACAGGACGCAA |
| NB21 | GAGCCTCTCATTGTCCGTTCTCTA | TAGAGAACGGACAATGAGAGGCTC |
| NB22 | ACCACTGCCATGTATCAAAGTACG | CGTACTTTGATACATGGCAGTGGT |
| NB23 | CTTACTACCCAGTGAACCTCCTCG | CGAGGAGGTTCACTGGGTAGTAAG |
| NB24 | GCATAGTTCTGCATGATGGGTTAG | CTAACCCATCATGCAGAACTATGC |

After the reaction was left at room temperature for 20 minutes, the reaction was stopped by adding EDTA and all samples were collected in a single tube. After the pretreatment stages, SQL-NBD114.24 adapter and Rapid T4 DNA ligase (RL02,

MobiomX) were added for adapter ligation and incubated at 30°C for 10 minutes. Before loading, the sample was purified again, SFB washing was performed and the concentration was measured. 50 fmol of library was loaded into a spot-on flow cell. When a minimum of 10,000 reads were obtained from each barcode, the sequence was stopped and analysis started. Utilizing the MinKNOW program, the sequencing run was initiated and continued for 72 hours to get enough data.

# 4. RESULTS AND DISCUSSION

The main purpose of this research and study is to increase the efficiency of sunflower substrate selected as agricultural waste by physicochemical pretreatment before anaerobic digestion. Since lignocellulosic wastes are difficult to decompose, the anaerobic treatment process was supported by subjecting them to a combined physical and chemical pretreatment process, which can be one of the most effective methods in the studies (Haghighi Mood, et al., 2013). The formation of volatile fatty acids was observed. In this way, the extraction and cost analysis of volatile fatty acids, which have an important contribution to the circular economy, were obtained. Microbial dominant species for the conditions where the highest VFA was obtained were obtained by DNA sequencing.

Pretreatment of lignocellulosic biomass is typically required prior to digestion. Biomass digestibility has been primarily based on the quantity of lignin, interlinkage between hemicellulose and cellulose association in the structure, surface field that is open for microorganisms, and crystallinity of the cellulose (Kainthola, et al., 2019; Sawatdeenarunat et al., 2015). However, lignocellulosic biomass is generated in three stages: pretreatment, enzyme hydrolysis, and fermentation. Thus, the most significant, influential, and costly pretreatment stage is required for lignocellulosic biomass. Furthermore, the optimum pretreatment procedure enables for a sustainable, inexpensive process to offer minimal cellulose crystallinity with a surprisingly low lignin concentration and inhibitory chemicals (Beig, et al., 2020). Absent any pretreatment on lignocellulosic biomass, the yield of total sugar was reduced by 20%, but it could be increased from 0 to 90% with various pretreatments (Alizadeh, et al., 2005). Recently conducted study has found that the removal of hemicellulose and lignin is closely connected to the removal of cellulose (Kumar, et al., 2009; Behera, et al., 2014). The pretreatment procedures generally classified into four categories; physical, chemical, biological and combined.

In this research, the use of microwave and acid pretreatment was recommended. There are several causes behind this, which may be stated as follows:

During the acid pretreatment, hemicellulose hydrolyzes into monosaccharides, whereas lignin condenses and precipitates (Hendriks and Zeeman, 2009; Salihu and Alam, 2016; Sousa et al., 2009). The technique is useful for substrates with high lignocellulosic content because it breaks down the lignin, as well as because the hydrolytic bacteria can acclimate to acidic environments (Salihu and Alam, 2016). Strong acidic pretreatment may produce inhibitory by-products such as furfural and hydroxymethylfurfural (HMF) (Sousa et al., 2009; Mussoline et al., 2013). To avoid the creation of such inhibitory compounds, it is usually suggested to utilize diluted acids in combination with heat techniques (Jain et al., 2015). With mesophilic anaerobic digestion of wheat plants, diluted sulfuric acid pretreatment at 121$^{o}$C enhanced methane output by 15.5% compared to untreated plants after 120 minutes (Taherdanak et al., 2016). Semi-continuous digestion studies at 35$^{o}$C with HCl at pH 2 resulted in a 14.3% increase in methane output over untreated substrates (Devlin et al., 2011).

The following acids are used in acid pretreatment: maleic acid, phosphoric acid ($H_3PO_4$), nitric acid ($HNO_3$), nitrous acid ($HNO_2$), sulfuric acid ($H_2SO_4$), and hydrochloric acid (HCl). The pretreatment of lignocellulosic biomass with diluted acids has been the subject of several investigations. Acid breaks down the glucosidic linkages in the hemicellulose fraction, making it easier for microorganisms to solubilize it into oligomers and monomers by enzymatic hydrolysis. This enhances the generation of biogas. The formation of various inhibitory compounds (furfural, 5-hydroxymethyl furfural, acetic acid, aldehyde, ketone, and phenolic acid), loss of dry matter, and corrosiveness hinder the process of methanogenesis, in addition to raising capital costs because reactor configuration requires expensive nonmetallic containers. This is true even though the use of concentrated acid was highly adapted for cellulose hydrolysis (Taherzadeh and Karimi, 2008). By rupturing the hydrogen bonds, Van der Waal forces, and covalent bonds that retain and give the biomass a hard structure, HCl primarily causes hemicellulose solubilization and cellulose degradation.

Recently, efforts have been made to optimize the pretreatment effect on biogas generation by integrating two or more pretreatment processes.

Particle size and crystallinity are decreased, system acidification is decreased, and blending capacity is increased by the physical pretreatment approach. The stability of lignocellulosic bonds is reduced and the biogas estimations are improved by the

application of temperature and chemicals in thermal and chemical pretreatment techniques. Biological agents, on the other hand, are essential to the creation of biogas and use lignin for energy.

While each pretreatment technique has the potential to significantly increase biogas output, there are a number of issues that are specific to each pretreatment technology. By using a variety of pretreatment techniques, the degradation of renewable but difficult-to-degrade lignocellulosic biomass can be enhanced (Akhtar et al., 2016). Combining pretreatment technologies can potentially improve cellulose's enzyme accessibility and ease the recovery of lignin and hemicellulose, which is necessary for the production of high-value products, while also mitigating the drawbacks of individual pretreatment technologies. By combining two pretreatment technologies, it is possible to improve the pretreatment effect and simultaneously make up for any shortcomings in each technology (Yu et al., 2019). Combining pretreatments results in shorter processing times, less inhibitor development, and an increase in sugar yield (Kumari and Singh 2018). Furthermore, certain combination pretreatment technologies, such as physiochemical pretreatment (Divyalakshmi et al., 2017) thermo-chemical pretreatment (Mlaik et al., 2018), ultrasonication assisted acid pretreatment (Rehman et al., 2014), electron beam irradiation combined with ionic liquid (Jusri et al., 2019), bioderived cholinium ionic liquids and ultrasound irradiation (Ninomiya et al. 2013), fungal pretreatment in combination with alkaline treatment (Alexandropoulou et al., 2017), extrusion combined with alkali pretreatment (Zhang et al., 2015), have been investigated to increase the effectiveness of lignocellulosic structure anaerobic digestion.

A combination pretreatment of HCl acid and microwave was used in the physicochemical process in light of all these scientific studies.

For physicochemical pretreatment, the substrates (as mixed sunflower head and stalk) were pretreated in 0.8%, 1.2% and 1.6% HCl solution at 120 and 140 degrees Celsius using CEM MARS 6 microwave for 30 minutes. Following the procedure, the materials were divided into liquid and solid components for further examination and the anaerobic digestion process.

An analysis of several variables was conducted to compare the pretreatment efficiency in order to determine the most ideal pretreatment condition with various parameters.

The following factors are under control: the substrate's chemical composition, the synthesis of volatile fatty acids, the soluble chemical oxygen requirement, and the removal of TS/VS. All of these assessments led to the major achievement of the most effective pretreatment condition.

DNA sequencing was done as a consequence of the investigations to identify the microbiological identity of "anaerobic digestion," a crucial biological activity. To further improve efficiency, it's critical to look at the bacterial and archaeal population for the most effective pretreatment. The biological pretreatment efficiency can be raised because of these microorganisms.

### 4.1 Effect of Pretreatment on Soluble COD of Samples

Following the completion of the pretreatment, the samples were divided into liquid and solid phases. Chemical oxygen demand (COD) was released in the form of sugars and other organic molecules throughout the pretreatment process, mostly as a result of the hydrolysis of cellulose and hemicelluloses.

The liquid phases of the control sample, which was not pretreated, and the ones that had were found to vary in color. In the sample, no pretreatment was applied, and the lightest colored liquid phase was seen. Because the pre-treated samples are darker than the control sample, this color difference indicates that the pre-treated samples have a greater COD level.

Testing for soluble COD was done on the filtrates that were obtained following pretreatment in order to measure the amount of COD released from the treated and untreated samples. Only deionized water was utilized in the control sample. A 1:100 ratio of deionized water was used to dilute the filtrates for the test.

By comparing the COD levels produced in the filtrates from treated and untreated straw samples, the sCOD analysis proved to be a useful tool for assessing pretreatment efficiency.

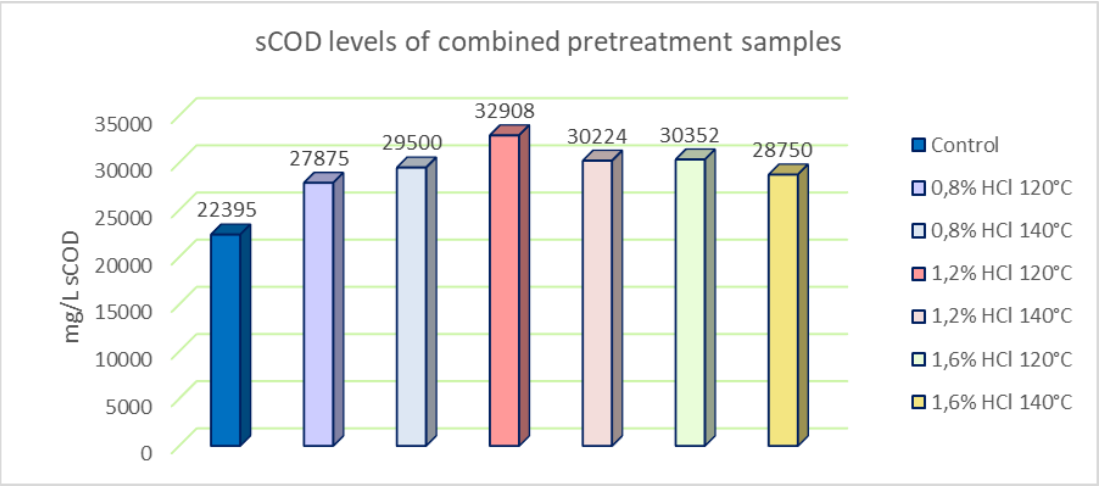



**Figure 4.1 :** sCOD values of pretreated samples.

In the Figure 4.1, sCOD values after pretreatment application are given for both samples without and with pretreatment. When pretreatment was applied under different conditions, it was observed that the highest sCOD value was achieved with pretreatment with 1.2% HCl acid at 120 degrees Celsius for 30 minutes. The sCOD value measured without pretreatment is 22395 mg/L sCOD, and with 1.2% HCl 120 degrees Celsius pretreatment, the sCOD value is 32908 mg/L. The substrates underwent improved hydrolysis with pretreatment, and as a result, the sCOD value rose, when the sCOD value of the sample without pretreatment was compared with the condition with the greatest sCOD value. A more efficient method is attained since pretreatment breaks down the cellulose structure. In the pretreatment with 1.2% HCl acid solution, it was observed that the sCOD value decreased again when the temperature of the microwave was 140 degrees Celsius. Considering the highest sCOD value, it has a 47% higher sCOD value than the sample without any pretreatment.

The improved solubilization of organic matter in the samples is responsible for the rise in sCOD that results from applying physicochemical pretreatment. Complex organic structures, such as lignocellulosic materials, can be effectively broken down by microwaves with acid pretreatment, producing soluble organic molecules that contribute to the sCOD. The pattern found is in line with other research findings that demonstrate the combined pretreatment of microwave and acid may greatly increase the solubilization and ensuing destruction of organic materials (Divyalakshmi et al., 2017; Mahajan et al., 2019)

The sCOD value reached its maximum level with 47% achieved in 1.2 HCl at 120 degrees Celsius. It has been observed that increasing the microwave temperature or

increasing the acid concentration under different conditions no longer increases the sCOD value. This may be the result of organic matter degrading or solubilizing too much, which might create inhibitory chemicals or cause the loss of important structural elements required for microbial digestion (Li et al., 2018). This result emphasizes the need of optimizing to strike a balance between the possible negative effects of over-treatment and the advantages of solubilization.

The best concentration for maximizing sCOD levels in the samples and improving organic matter solubilization is 1.2% HCl microwave pretreated at 120°C for 30 minutes, according to the data. This concentration is very appropriate for use in waste treatment and the production of bioenergy since it not only enhances the availability of soluble organics but may also boost the generation of volatile fatty acids (VFAs). Waste lignocellulosic material breaks down and has a superior hydrolysis stage, which yields more energy with greater efficiency. Beyond this threshold, though, there is a greater chance of negative consequences, thus vigilance is required while raising the acid concentration or temperature.

### 4.2 TS/VS Removal

Total Solids (TS) and Volatile Solids (VS) of the samples were evaluated in relation to various combination pretreatment concentrations and a control sample.

**Table 4.1 :** Value of TS and VS of seed sludge and samples, as percentage.

| | TS% | VS% |
|---|---|---|
| Seed Sludge | 1,28 | 0,87 |
| Control Sample, Day 30 | 1,245 | 0,852 |
| 1.2% HCl 120ºC, Day 30 | 1,23 | 0,843 |

The percentage changes of TS and VS of both pre-treated and non-pre-treated samples after anaerobic digestion are shown in Figure 4.2.

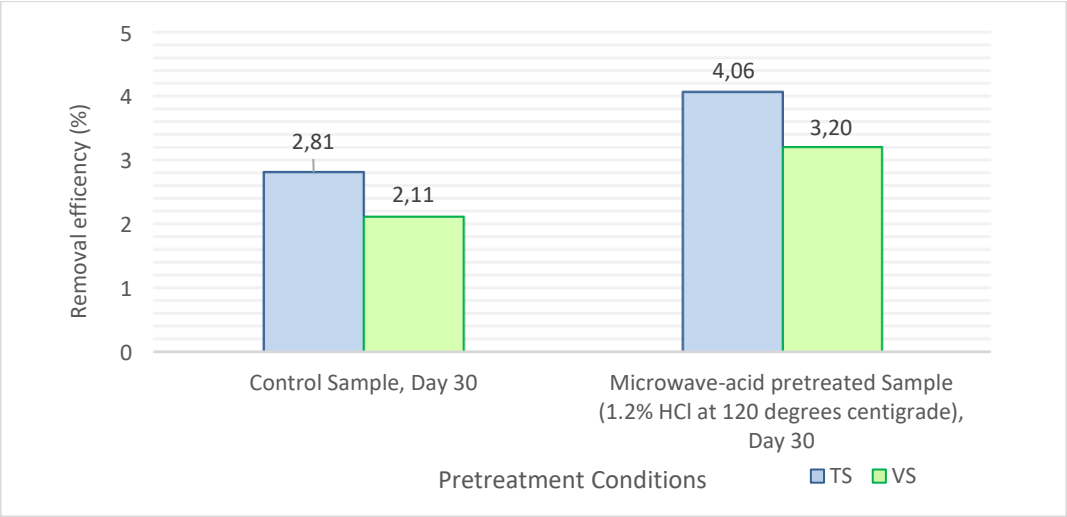



**Figure 4.2 :** Percentage changes in TS and VS of sunflower waste under pretreatment and pretreatment anaerobic digestion conditions.

As seen in the figure, the pretreatment condition with the most efficient sCOD value, that is, the sample pretreated with 1.2% HCl acid in the microwave for 30 minutes and at 120 degrees Celsius, also made a significant contribution to the TS and VS yield. VS increased from 2.11% to 3.20% , and TS increased from 2.81% to 4.06%. TS degradation has increased by 45% and VS by 52% as a result, when compared control samples and pretreated samples.

Increased generation of volatile fatty acids (VFAs), which are essential intermediates in the anaerobic digestion process and the biodegradability of agricultural waste, is directly correlated with improved removal efficiency (Chandra et al., 2012). The efficiency of lignocellulosic waste degradation is closely correlated with the production efficiency of VFA. This study's primary goal is to improve lignocellulosic wastes' hydrolysis efficiency by pretreatment. When the right circumstances are met, the VFA efficiency will rise in this manner. The rise in the solid material's TS and VS values is a sign of effective deterioration. Thus, it can be showed that lignocellulosic waste decomposes more efficiently, which means that a more efficient hydrolysis will result in a larger VFA output. In summary, this study suggests that 1.2% HCl acid at 120 degrees Celsius leads to a decent proportion of TS VS yield and is useful for VFA synthesis.

### 4.3 VFA Production During Acidification

In anaerobic digestion (AD), the rate of development and effectiveness of the acidogenesis phase are primarily determined by the cumulative synthesis of volatile fatty acids (VFAs). The breakdown of complex organic substrates by acidogenic bacteria results in the production of VFAs, such as acetic, propionic, butyric, and valeric acids, as intermediaries. Anaerobic process performance as a whole and system stability are both well-understood by using the cumulative measurement of VFAs.

Organic matter, including carbohydrates, proteins, and lipids, is degraded into simpler molecules in the early stages of anaerobic digestion. Fermentative microbes then transform these simpler components into volatile fatty acids (VFAs). With increased substrate degradation over time, the cumulative VFA concentration rises. Usually, this buildup is watched to make sure everything is working as it should. The composition of the substrate, pH, temperature, and the presence of inhibitory chemicals are some of the variables that affect the pace and degree of cumulative VFA synthesis. An imbalance in the system, such as a pH decrease or an excess of organic material, may be indicated by a rapid jump in volatile fatty acid levels, for example, since they might block methanogenic bacteria and slow down the conversion of VFAs to methane.

For the AD process to be optimized, cumulative VFA formation monitoring is essential since it facilitates the identification of ideal operating conditions and the early detection of any problems. Anaerobic digestion systems may be made more sustainable and efficient by improving process stability, increasing methane output, and comprehending and managing VFA dynamics.

So, during anaerobic digestion, samples were collected from digesters on days 0, 1, 2, 3, 6, 7, 8, 9,10, and 30, the total VFA concentrations were measured and calculated as the ppm and equivalent of total acetic acid.

In this experiment, the synthesis of volatile fatty acids (VFAs) over time is compared between a control group and a sample that was microwave-pretreated at 120°C and treated with 1,2% HCl. The measurements, which were labelled D0 through D30, were made at various intervals.

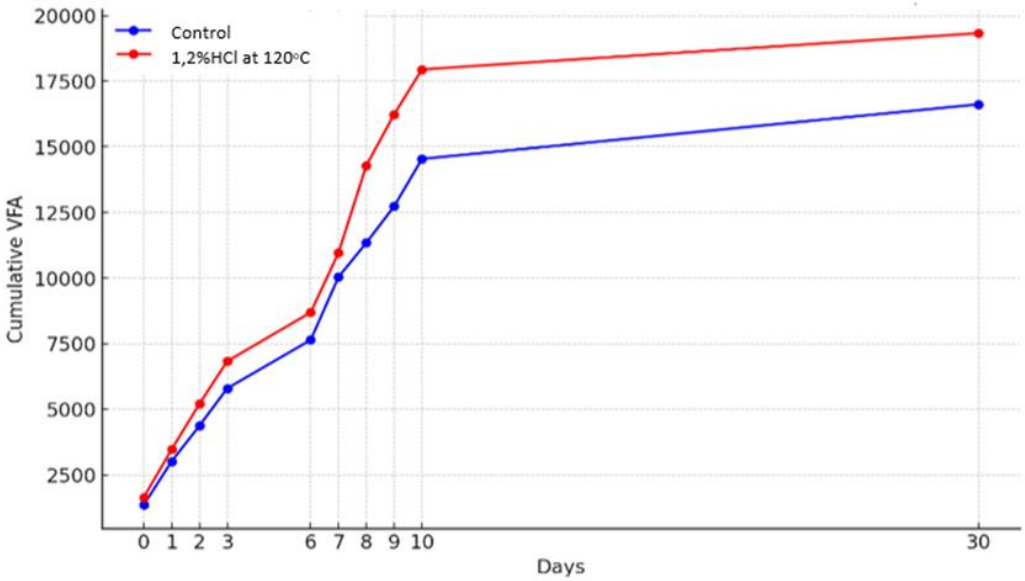



**Figure 4.3 :** Cumulative VFA results of control and 1.2% HCl acids with microwave pretreatment at 120°C.

**Table 4.2 :** Cumulative VFA results of control and 1.2% HCl acids with microwave pretreatment at 120°C, as value.

| | D0 | D1 | D2 | D3 | D6 | D7 | D8 | D9 | D10 | D30 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Control** | 1358 | 3016 | 4373 | 5798 | 7640 | 10040 | 11340 | 12730 | 14538 | 16616 |
| **1.2 HCl at 120°C** | 1645 | 3469 | 5207 | 6832 | 8688 | 10962 | 14284 | 16234 | 17946 | 19324 |

As seen in the Figure 4.3, the difference in cumulative VFA production between the control and combined pretreatment is seen in mg/L sCOD and Table 4.2 values are given in detail. The increase was observed more in the pre-treated sample from the first day. However, the difference increased especially on the 8th day. At this point, the cumulative VFA value in the control sample is 11340 mg/L sCOD; The cumulative VFA value of the pretreated sample is 14284. This difference was maintained after the eighth day. At the end of the 30th day, the final VFA values were 16616 for the control sample; The sCOD for the pretreated sample is 19324 mg/L.

The pretreated sample had a more pronounced rise than the control sample, which had no pretreatment. This is because pretreatment facilitates the hydrolysis process, which speeds up and improves the efficiency of the VFA synthesis stage. In contrast, the control sample shows a slower and less effective hydrolysis process in the anaerobic environment when microorganisms are present since it is not treated to any pretreatment.

The 30th-day combined pretreated sample is approximately , more efficient compared to the control sample. The effectiveness of combined pretreatment in improving the anaerobic digestion process is demonstrated by this outcome. Because the lignocellulosic structure of biomass is efficiently disrupted by combined pretreatment, microbial activity may more easily access cellulose and hemicellulose, leading to an increase in VFA synthesis.

The data may be summed up as follows to help with a more thorough interpretation:

**Control Group**: The VFA levels started at 1358 and steadily increased to 16616 by D30.

**1.2% HCl at 120°C**: The initial VFA level was higher at 1645.07, and it increased more rapidly, reaching 19323.83 by D30.

**Control Group Performance**: Despite the control group showing a steady increase in VFA production, the lack of pretreatment likely resulted in a slower hydrolysis rate of organic materials. The untreated materials might have required more time to break down, leading to a less efficient conversion into VFAs. This suggests that while the control group is capable of VFA production, it is less efficient in both the rate and total yield compared to the pretreated group.

**Pretreated Group Performance**: The HCl and microwave pretreatment likely enhanced the breakdown of complex organic materials, leading to faster hydrolysis and higher VFA production. The pretreatment likely caused more significant cell disruption and solubilization of materials, facilitating microbial access and, consequently, higher VFA yields. This suggests that the pretreatment process effectively accelerates the hydrolysis phase, leading to more efficient anaerobic digestion or similar bioconversion processes.

The results indicate that the 1.2% HCl pretreatment under microwave conditions is beneficial for increasing the efficiency of VFA production, making it a potentially valuable step in processes that rely on rapid and high-yield fermentation.

The findings of this study are supported by several other investigations. The following are a few of these;

Research indicates that microwave radiation (MWR) causes large-scale collisions and heat that speed up biological, chemical, and physical processes by polar molecular

vibration and ion mobility. The application of MWR-assisted biomass pretreatments, such as those involving MWR and water, MWR and alkali, MWR and acid, MWR and ionic liquid, MWR and salt, and additional combination pretreatments aided by MWR (Xu, 2015).

Acid pretreatment can function in two ways: in a low temperature and high acid concentration condition (concentrated-acid pretreatment) or in a high temperature and low acid concentration condition (dilute-acid pretreatment). Dilute $H_2SO_4$ pretreatment (1.2% $H_2SO_4$ and 30 min) combined with microwave irradiation increased the biogas output of the leftover herbal extraction process waste by more than 100% and 45%, respectively, when compared to raw and acid pretreated materials (Liu and Cheng, 2009). Methane generation may be inhibited by large quantities of inhibitors, such as furfural and HMF (more than 15 mM) produced during acid pretreatment and/or $H_2S$ generated as a result of sulphate reduction during AD (Antonopoulou et al., 2010; Barakat et al., 2012). As a result, it's critical to choose the right parameters (such as temperature, pH, and duration) for process optimization in order to stop the creation of inhibitors. Other acids, such as HCl and $H_3PO_4$, have also been studied. It has also been discovered that hydrochloric acid pretreatment for 30 days at room temperature, with an acid concentration of 1 M, increases the biogas output from bagasse and coconut fiber by 32% and 76%, respectively (Kivaisi and Eliapenda, 1994). By rupturing the hydrogen bonds, Van der Waal forces, and covalent bonds that retain and give the biomass a hard structure, HCl primarily causes hemicellulose solubilization and cellulose degradation.

When wheat straw was pretreated with MWR/dilute acid (0.5% $H_2SO_4$, w/v) at 160 °C for 10 min, the maximum yield of total sugars was obtained after enzymatic pretreatment. This was higher than the yield from MWR/alkali (0.1 g/g straw) at 160 °C for 10 min (604 mg total sugars/g straw) and MWR/water at 200°C for 10 min (544 mg/g straw) (Saha et al., 2008). Moreover, cellulose dissolves more quickly in ionic liquids when heated in a microwave (Zhu et al., 2006). By removing the hydrolysis stage, the hydrolysis and MWR processing of grass-type biomass into sugars might be completed in a single step, making the process more financially viable (Marx et al., 2014). The primary disadvantage of thermal pretreatment is that when the biomass was heated over 160°C, more soluble phenolic chemicals as well as hazardous derivatives such 5-hydroxymethyl 2-furfural and furfural were produced (Čater et al., 2014).

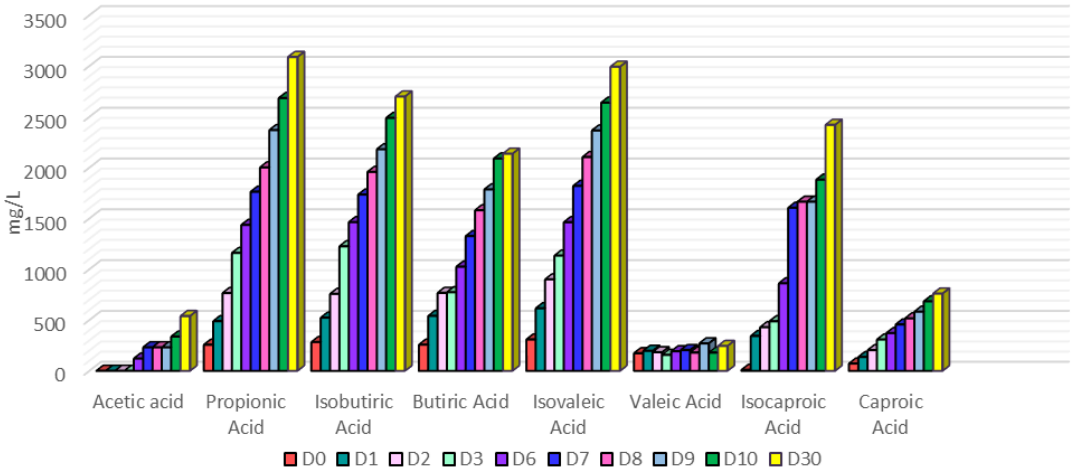



**Figure 4.4 :** Cumulative VFA production of pretreated samples, as acid types.

In the Figure 4.4, each acid shows substantial production changes over time, with various days exhibiting significant increases. The importance of these acids spans multiple industries, from food preservation to the production of chemicals, fragrances, and pharmaceuticals.

The importance of each VFA acid type and its increase amounts according to this graph can be briefly summarized as follows;

**Acetic acid:** Acetic acid is widely used in the production of vinegar and has applications in food preservation, as well as in the chemical and textile industries. Day 0 concentration is 0 and D30 concentration is 539 mg/L. From day 6 121 mg/L to day 7 232 mg/L the value nearly doubled.

**Propionic Acid**: Propionic acid is commonly used as a preservative in food, especially in baked goods, due to its antifungal properties. Day 0 concentration is 257 mg/L and day 30 concentration is 3089 mg/L. The most notable rise occurred between day 9 2369 mg/L and day 10 2685 mg/L. Another significant jump was from day 8 2002 mg/L to D9.

**Isobutyric Acid**: Isobutyric acid is used as a flavoring agent and in the production of plasticizers and varnishes. Day 0 concentration is 284 mg/L and day 30 concentration is 2700 mg/L. Between day 9 2181 mg/L and day 10 2490 mg/L , there was a significant rise.

**Butyric Acid**: Butyric acid is important in the production of pharmaceuticals and as a flavor additive in food, particularly for its characteristic rancid butter smell. Day 0

concentration is 257 mg/L and day 30 concentration is 2137 mg/L. The rise from day 7 1326 mg/L to day 8 1580 mg/L is notable.

**Isovaleric Acid**: Isovaleric acid is used in the synthesis of perfumes and flavoring agents, known for its pungent smell. Day 0 concentration is 309 mg/L and day 30 concentration is 2993 mg/L. A considerable jump happened between day 9 2365 mg/L and day 10 2640 mg/L.

**Valeric Acid**: Valeric acid is primarily used in the synthesis of esters for perfumes and food additives. Day 0 concentration is 173 mg/L and day 30 concentration is 245 mg/L. A sharp increase occurred from day 7 204 mg/L to day 9 270 mg/L.

**Isocaproic Acid**: Isocaproic acid is important in the flavor and fragrance industry, and is also used in animal feed. Day 0 concentration is 9 mg/L and day 30 concentration is 2421 mg/L. A massive rise occurred from day 7 1605 mg/L to day 8 1664 mg/L, and again between day 9 1664 mg/L and day 10 1882 mg/L.

**Caproic Acid**: Caproic acid is widely used in the manufacture of hexanoates, which are components in fragrances, lubricants, and rubber products. Day 0 concentration is 71 mg/L and day 30 concentration is 760 mg/L. The increase between day 8 516 mg/L and day 9 582 mg/L is significant.

In summary, the acid types, from most produced to least produced, are as follows:

Propionic Acid: 3089 mg/L, Isovaleric Acid: 2993 mg/L, Isobutyric Acid: 2700 mg/L, Butyric Acid: 2137 mg/L, Isocaproic Acid: 2421 mg/L, Caproic Acid: 760 mg/L, Acetic Acid: 539 mg/L, Valeric Acid: 245 mg/L.

Thus, Propionic Acid is the most produced acid, reaching a concentration of 3089 mg/L, while Valeric Acid has the lowest concentration at 245 mg/L. Every group had a notable uptick within itself. They play a significant role in the circular economy in a variety of industries and according to their intended purpose, as was previously indicated.

## 4.4 Microbial Analysis

### 4.4.1 16S Metagenomic analysis as microbial community

The microbial diversity, changes in seed sludge, and the best pretreatment state on day thirty are depicted in the pie graphics as phylum and class respectively.

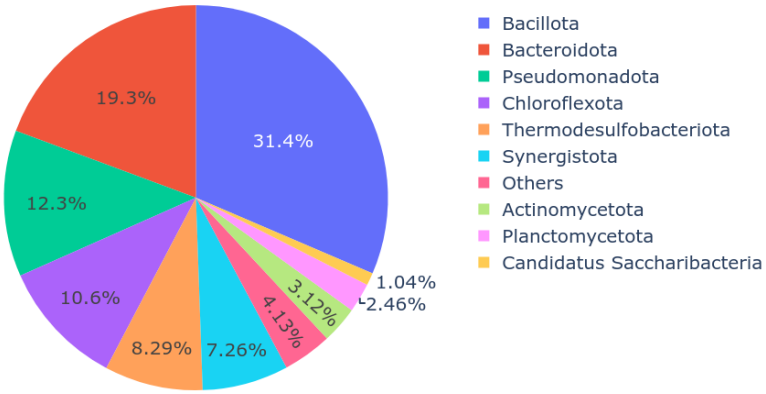



**Figure 4.5 :** Bacterial communities in the seed sludge as phylum level on the 0. Day.

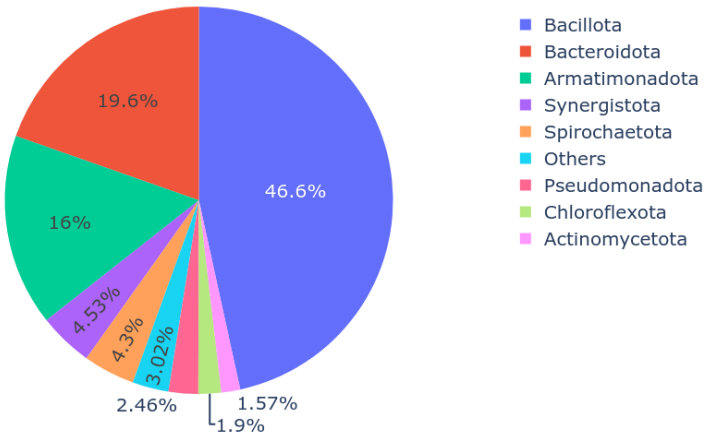



**Figure 4.6 :** Bacterial communities in the combined pretreated sample as phylum level on the 30. Day.

The seed sludge contains 31.4% Bacillota, 19.3% Bacteroidota, 12.3% Pseudomonadota, 10.6% Chloroflexota, 8.29% Thermodesulfobacteriota, and 7.26% Synergistota at the phylum level to be characterized in Figure 4.5.

Rod-shaped, endospore-forming, facultatively anaerobic, and Gram-positive *Bacillus* species are known for their ability to generate cultures that can eventually become Gram-negative. A vast array of physiological traits enable the genus's numerous species to coexist in every type of natural habitat. Each cell can only produce one endospore. Radiation, temperature, desiccation, and disinfectants cannot harm the spores. The necessity of oxygen for Bacillus anthracis sporulation carries significant implications for both control and epidemiology. It has been observed that significant microorganisms with the capacity to break down organic molecules in aquatic settings are members of the Cytophagia, Bacteroidia, and Flavobacteria groups (Cottrell and Kirchman, 2000; Lau et al., 2005; Liu et al., 2019).

The percentage order of the sample pre-treated with 1.2% HCl acid at 120 degrees Celsius for 30 minutes in the microwave is as follows in phylum level: 46.6% Bacillota, 19.6% Bacteroidota, 16% Armatimonadota, 4.53% Synergistota, as showed Figure 4.6.

That Bacillota's percentage share has risen is clear. In terms of proportion, Bacteroidota, which comes in second, also saw a rise. The material that was pre-treated shows evidence of a different phylum.

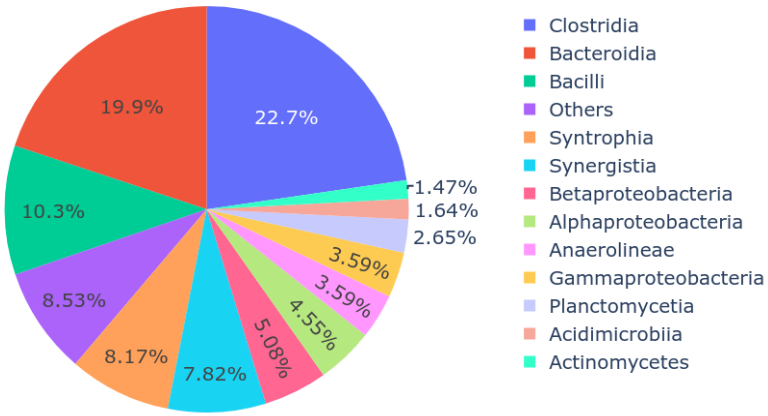



**Figure 4.7 :** Bacterial communities in the seed sludge as class level on the 0. Day.

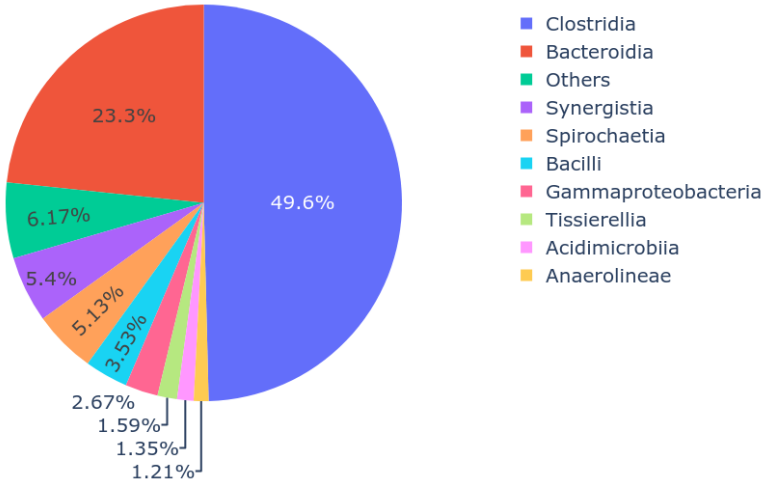



**Figure 4.8 :** Bacterial communities in the combined pretreated sample as class level on the 30. Day.

For the "class level" in biological classification, bacterial community percentages for seed sludge and pre-treated samples, respectively, are shown in Figure 4.7 and Figure 4.8.

Classes common to both are Clostridia, Bacteroidia, Bacili and Gammaproteobacteria. Closridia class became dominant after pretreatment and has 49.6%, compared to

22.7% for seed sludge. The Bacteroidia class also increased, although not as dominantly as the Clostridia class. While it is 19.9% for seed sluge; after pretreatment it was 23.3%.

### 4.4.2 18S Metagenomic analysis as microbial community

Significant changes in the makeup of the eukaryotic microbial community over the 30-day anaerobic digestion period are revealed by the 18S rRNA sequencing data for Days 0 and 30. The percentage distribution of eukaryotic microorganisms in inoculum sludge by phylum level is shown in Figure 4.9. Relatively close to a few eukaryotic phylum level percentages. It has a large fraction of 21.6% Chytridiomycota and 18.1% Ascomycota. Chytridiomycota, the most dominant phylum in the inoculum sludge, decreased in percentage to 14.4% after pretreatment as showed in Figure 4.10.

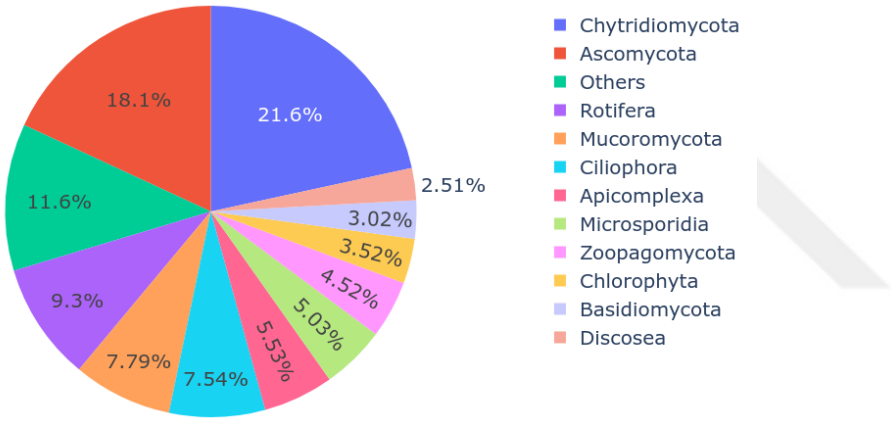



**Figure 4.9 :** Eukaryotic microbial communities in the seed sludge as phylum level on the 0. Day.

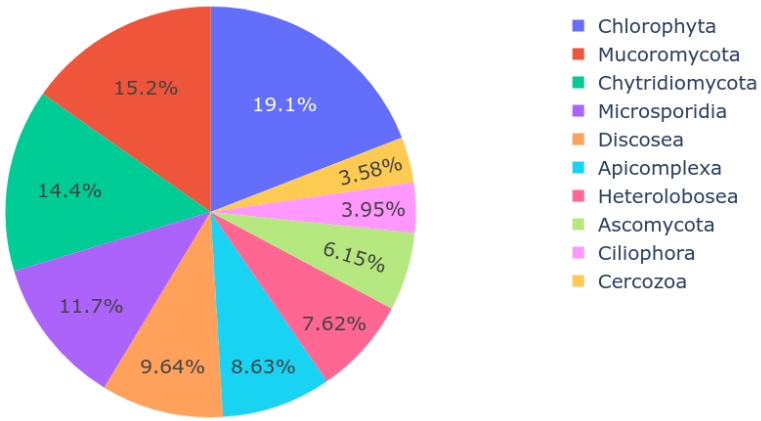



**Figure 4.10 :** Eukaryotic microbial communities in the combined pretreated sample as phylum level on the 30. Day.

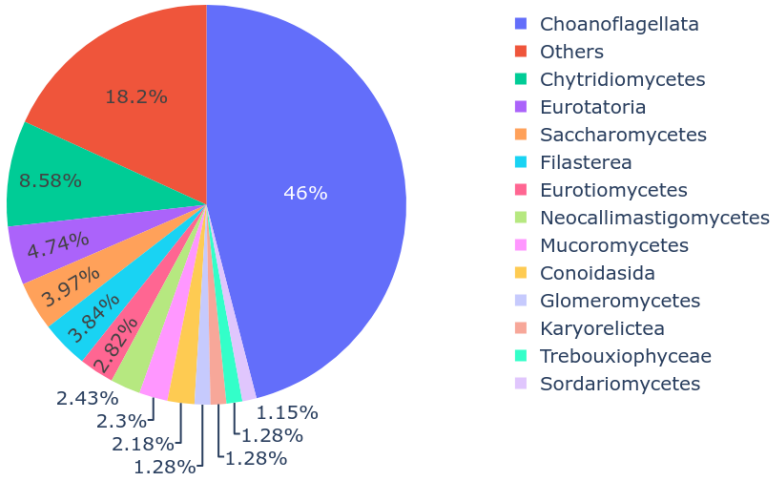



**Figure 4.11 :** Eukaryotic microbial communities in the seed sludge as class level on the 0. Day.

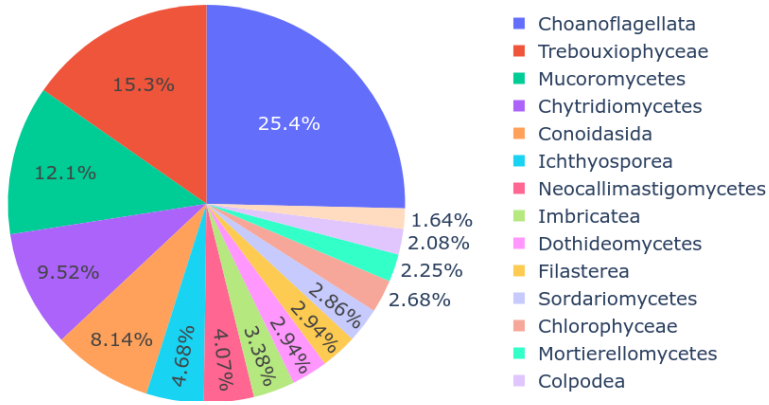



**Figure 4.12 :** Eukaryotic microbial communities in the combined pretreated sample as class level on the 30. Day.

Considering the eukaryotic microorganism community in terms of classification level, it can be seen in Figure 4.11 that the dominant class in the inoculum sludge is Choanoflagellata, with a large fraction of 46%. After pretreated, this class still had the highest fraction, with a percentage value of 25.4% as showed in Figure 4.12.

### 4.4.3 Archaea analysis and microbial community

Percentage distributions of archaea by phylum level are shown in Figure 4.13 and Figure 4.14 for both the inoculum and the pre-treated sample, respectively. Common to both conditions, the first noticeable Euryarchaeota phylum level has the highest fraction in percentage. While this rate is 89.6% for seed sludge, it is 81.1% for the pretreated sample. Bacillota phylum, which has the second highest percentage fraction in the inoculum sludge (as 5.87%), is observed to increase as a percentage fraction to 7.42% at the end of the 30th day in the pretreated sample.

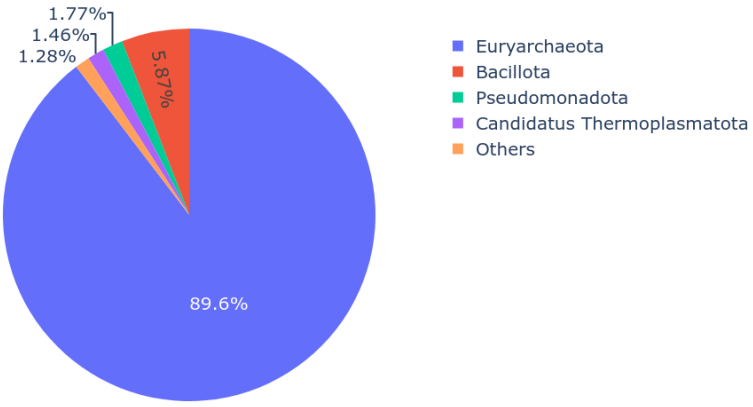



**Figure 4.13 :** Archaea communities in the seed sludge as phylum level on the 0. Day.

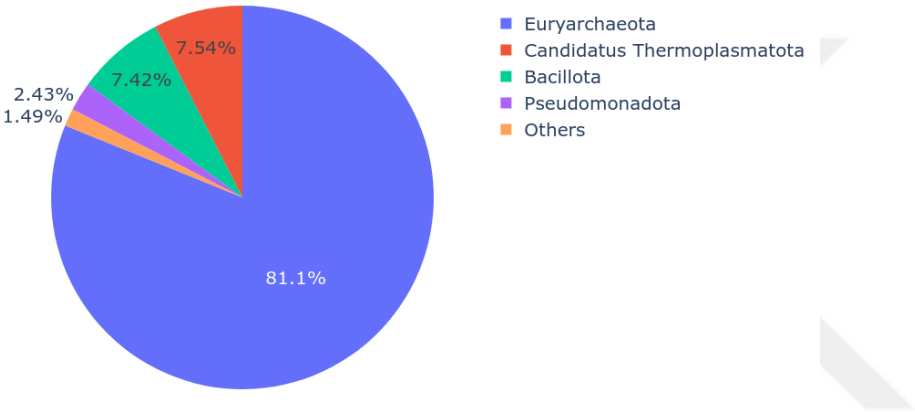



**Figure 4.14 :** Archaea communities in the combined pretreated sample as phylum level on the 30. Day.

The percentage distribution of archaea by class shows similarities to those found at the phylum level. Methanomicrobia is the class with the largest percentage fraction, 88.8% for the inoculum sludge and 81.1% for the pretreatment sample, in both cases as showed in Figure 4.15 and Figure 4.16.

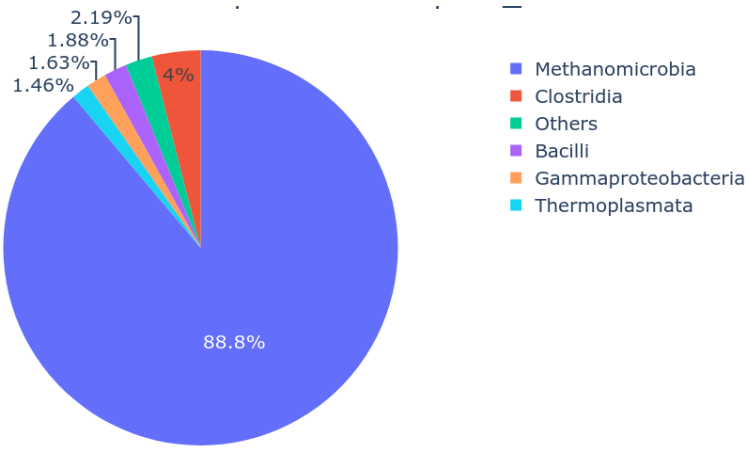



**Figure 4.15 :** Archaea communities in the seed sludge as class level on the 0. Day.

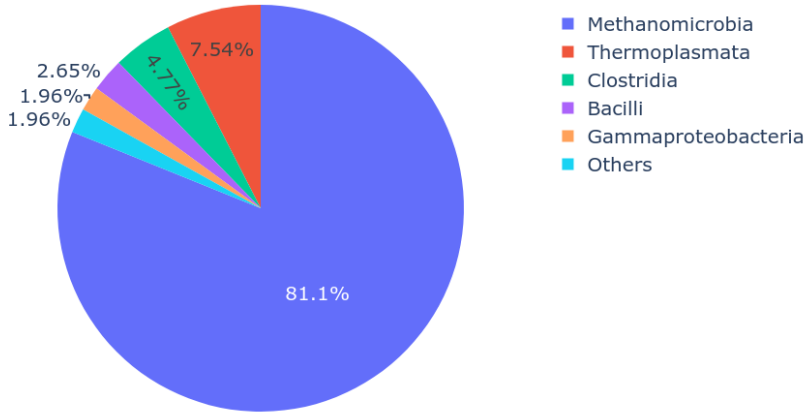



**Figure 4.16 :** Archaea communities in the combined pretreated sample as class level on the 30. Day.

The percentage distributions and names of each species are presented in the Table 4.3 to provide more specific information about the microbial communities whose distributions are given as phylum and class for pretreated sample.

The microbial result of 16S, 18S and archaea with a fraction greater than 1 percent of species is summarized:

**Table 4.3 :** Percentage distribution of 16s, 18s and archaea.

| SPECIES | 16S, as % |
|---|---|
| Armatimonadota bacterium | 16,026 |
| Caloramator sp. E03 | 8,094 |
| Dysgonomonadaceae bacterium zrk40 | 6,15 |
| Romboutsia sp. 13368 | 4,992 |
| Bacteroidales bacterium CF | 4,052 |
| uncultured Paludibacter sp. | 4,043 |
| Paraclostridium bifermentans | 3,102 |
| uncultured Sphaerochaeta sp. | 3,021 |
| Synergistaceae bacterium | 2,948 |
| Romboutsia sp. 13432 | 2,749 |
| Oscillospiraceae bacterium | 2,523 |
| Tenuifilaceae bacterium CYCD | 1,899 |
| Clostridium sp. OS1-26 | 1,682 |
| Clostridium perfringens | 1,574 |
| Intestinibacter bartlettii | 1,474 |
| Bacteroidales bacterium | 1,239 |
| Tissierella sp. MB52-C2 | 1,094 |
| Clostridium isatidis | 1,058 |

**Table 4.3 (continued) :** Percentage distribution of 16s, 18s and archaea.

| SPECIES | 18S, as % |
|---|---|
| Stephanoeca diplocostata | 16,832 |
| Prototheca ciferrii | 5,964 |
| Prototheca wickerhamii | 5,765 |
| Tetramitus dokdoensis | 5,5 |
| Acanthamoeba divionensis | 5,302 |
| Catenomyces persicinus | 4,639 |
| Mucor amphibiorum | 3,645 |
| Anurofeca richardsi | 3,579 |
| Syncystis mirabilis | 3,38 |
| Neocallimastix frontalis | 3,115 |
| Vittaforma corneae | 2,783 |
| Entophlyctis helioformis | 2,651 |
| Spongomonas minima | 2,584 |
| Colpodella tetrahymenae | 2,584 |
| Mucor mucedo | 2,319 |
| Cladosporium cladosporioides | 2,253 |
| Pigoraptor vietnamica | 2,253 |
| Parascedosporium tectonae | 2,187 |
| Desmodesmus baconii | 2,054 |
| Helmichia lacustris | 1,988 |
| Rhizopus stolonifer | 1,789 |
| Mortierella calciphila | 1,723 |
| Cryptosporidium andersoni | 1,657 |
| Acanthamoeba comandoni | 1,657 |
| Hartaetosiga balthica | 1,59 |
| Colpoda aspera | 1,59 |
| Blakeslea trispora | 1,524 |
| Sporanauta perivermis | 1,325 |
| Hazardia milleri | 1,259 |
| Loxodes striatus | 1,259 |
| Cryptosporidium struthionis | 1,193 |
| Ovavesicula popilliae | 1,06 |
| **SPECIES** | **Archaea, as %** |
| Methanosarcina vacuolata | 34,992 |
| Methanothrix soehngenii | 22,256 |
| Methanosarcina barkeri | 21,258 |
| Methanomassiliicoccus luminyensis | 8,491 |
| Methanosphaerula palustris | 3,566 |
| Methanosarcina horonobensis | 2,609 |
| Ruminococcus albus | 1,893 |
| Methanospirillum hungatei | 1,668 |

*Armatimonadota* is found all around the world under anoxic and hypoxic conditions, and it predominates in deep-sea sediments. Using the intermediary substances hydroxylamine and sulfite, armatimonadota may be involved in the cycling of sulfur and nitrogen (Carlton et al., 2023)

*Caloramator sp. E03 :* All rod-shaped, sporogenic, obligately anaerobic organisms that ferment a variety of substrates at moderately thermophilic temperatures are grouped together under the genus Caloramator, which belongs to the Clostridiaceae family (Collins et al., 1994)

*Dysgonomonadaceae:* Within the family Dysgonomonadaceae, Dysgonomonas is a facultatively anaerobic, gram-negative bacterium that has been isolated from human sources.

*Stephanoeca* are well-known for their capacity to break down complex organic compounds in anaerobic settings by feeding on bacteria and organic waste (Nguyen et al., 2015)

*Prototheca ciferrii* Small, colorless achlorophylic algae belonging to the Chlorellaceae family make up the species in this genus. They are found in nature in large quantities and are sometimes found in clinical specimens (Watts, 1990)

*Prototheca wickerhamii:* An algae called Prototheca wickerhamii is rarely harmful to humans, however cases of it among those with weakened immune systems are growing (Bandaranayake et al., 2015)

*Tetramitus dokdoensis:* Within the Heterolobosea, Tetramitus is a representative amoeboflagellate group that presently has more than a dozen species.

*Methanosarcina vacuolata* are a unique class of methanogens that are distinguished by their physical characteristics and range of substrate use. It's possible that *Methanosarcina* is the only anaerobic methanogen that uses all three of the established methanogenesis metabolic pathways to create methane. The waste treatment sector depends on methanogenesis, and biologically generated methane is a significant substitute fuel. The majority of methanogens use hydrogen gas and carbon dioxide to create methane. Acetate is used by others in the acetoclastic pathway. Methanotrophic methanogenesis is a further mechanism by which Methanosarcina species can metabolize methylated one-carbon molecules. Methanol, methyl thiols, and methylamines are examples of these one-carbon molecules (Galagan et al., 2002).

Methanosarcina species are the only ones that can use at least nine methanogenic substrates, including acetate, and have access to all three of the known methanogenesis routes.

*Methanothrix soehngenii* One species of methanogenic archaea is *Methanothrix* soehngenii. Its rod-shaped, non-motile, non-spore-forming cells are often joined end to end in lengthy filaments encircled by a structure resembling a sheath. Methanothrix soehngenii is not able to convert carbon dioxide with hydrogen in order to make methane, in contrast to other methanogenic archaea. Acetate is its only energy source (Jetten, 1992)

*Methanosarcina barkeri* The distinctive properties of Methanosarcina barkeri, an anaerobic methanogen capable of fermenting a wide range of carbon sources, may have far-reaching effects for environmental and biotechnology research in the future (Balch, 1979). *Methanosarcina barkeri* is categorized as an extreme anaerobe because it lives in the rumen of cows, an environment with extremely low oxygen levels. M. Barkeri is a severe anaerobe that lives in the rumen of cows, an environment with extremely low oxygen levels. Moreover, *Methanosarcina barkeri* produced methane gas in cows may contribute to the generation of greenhouse gases. Nevertheless, M. barkeri may be used in low pH environments to successfully neutralize the acidity environment and make it suitable for other methanogens since it can survive in harsh circumstances and create methane (Hook and McBride, 2010)

### 4.5 Cost Analsysis

The Table 4.4 shows how many dollars can be obtained from 1.5 grams of our substrate, considering the market values of VFA types. mg/L values were converted into units per ton and the amounts obtained with costs per ton were found. Even if microwaves are suitable for laboratory scale operation, the potential profit can be adapted to industrial scale with the development and adaptation of technology.

**Table 4.4 :** Pricing per ton of VFA acids obtained.

| | Substrate Production ,as mg/L | Substrate Production (grams/ in 100 mL) | 1 ton VFA production/1 ton substrate | Price per Ton (USD) | Total Amount (USD) |
|---|---|---|---|---|---|
| Acetic Acid | 539 | 0,054 | 0,035 | 500 $ | 17 $ |
| Propionic Acid | 3089 | 0,31 | 0,2 | 1250 $ | 250 $ |
| Isobutyric Acid | 2700 | 0,27 | 0,174 | 2750 $ | 476 $ |
| Butyric Acid | 2137 | 0,214 | 0,137 | 1800 $ | 247 $ |
| Isovaleric Acid | 2993 | 0,3 | 0,193 | 3500 $ | 673 $ |
| Valeric Acid | 245 | 0,025 | 0,0158 | 1500 $ | 24 $ |
| Isocaproic Acid | 2421 | 0,24 | 0,154 | 2500 $ | 385 $ |
| Caproic Acid | 760 | 0,076 | 0,049 | 2500 $ | 122 $ |

## 5. CONCLUSION AND RECOMMENDATIONS

In this study, sunflower was used as the substrate and physicochemical pretreatment was applied under different conditions. It was observed that the sample, which was pre-treated in 1.2% HCl acid solution in the microwave at 120 °C for 30 minutes, had a sCOD value of 32908 mg/L after pretreatment and was the most efficient condition. It has 47% higher sCOD value than the sample without pretreatment. After this stage, the study continued with this pretreatment sample, which was the most efficient. A higher sCOD value indicates a more efficient hydrolysis step, thus higher VFA formation was expected.

Changes in the TS and VS values of the sample before and after pretreatment were examined. TS degradation has increased by 45% and VS degradation by 52% as a result, when compared control samples and pretreated samples.

The increase in these values was consistent with the sCOD value and showed good degradation. Finally, cumulative VFA formation values were observed as follows: Propionic Acid: 3089 mg/L, Isovaleric Acid: 2993 mg/L, Isobutyric Acid: 2700 mg/L, Butyric Acid: 2137 mg/L, Isocaproic Acid: 2421 mg/L, Caproic Acid: 760 mg/L, Acetic Acid: 539 mg/L, Valeric Acid: 245 mg/L.

At the end of the 30th day, the dominant microorganism communities are as follows; Armatimonadota, Caloramator sp. E03, Dysgonomonadaceae, Stephanoeca, Prototheca wickerhamii, Prototheca ciferrii, Tetramitus dokdoensis, Methanosarcina vacuolata, Methanothrix soehngenii, Methanosarcina barkeri. Microbial analysis is very important. Methanogens were shown to persist in this manner. Anaerobic digestion is an extremely sensitive process. Although excellent pretreatment was used and methanogens were predicted to be absent, their continuing presence demonstrated that a more sensitive process execution may provide even more efficient VFA synthesis.

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## PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Güven, I.** and Ince, O. 2024: Anaerobic Digestion of Lignocellulosic Waste Using Physico-Chemical Pretreatment Methods Interms Of Performance, Microbial Community, and Cost Analysis, International Graduate Research Symposium – IGRS'24, May 8-10, 2024, Turkey. (Poster Presentation)