DETECTION AND IDENTIFICATION OF ANAEROBIC MICROORGANISMS BIOCHEMICALLY DEGRADING LIGNIN IN İĞNEADA FLOODPLAIN FOREST

by

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B.Sc. in Molecular Biology and Genetics, İstanbul Technical University, 2018

Submitted to the Institute of Environmental Sciences

in partial fulfillment of the requirements for the degree of

Master of Science

in

Environmental Sciences

Boğaziçi University

2022

ACKNOWLEDGEMENTS

Trying to do laboratory work during COVID-19 era was utmost crazy and challenging. There were times when I believed this thesis would never be finished, but here we are. There were some lovely people that made this experience less painful as much as possible and kept me going.

First and foremost, I would like to give my sincere gratitude to Prof. Dr. Bahar İnce. From the day we met, she was always positive, thoughtful and caring. Her approach and the freedom she gave taught me how to handle a laboratory and project, which was one of the most vital experiences for me on the way to becoming a scientist. This thesis would never be completed without her.

I would like to thank İbrahim Miraloğlu for being an indispensable partner for this project. The fact that he never gave up like me always gave me strength and his unique perspective opened my horizons.

My gratitude is extended to Prof. Dr. Orhan İnce for his support. Thanks to him and Prof. Dr. Bahar İnce, I had a clue about what it means to be part of a large research group which was invaluable. I would also like to thank Ömer Uzun, who opened my perceptions with our brainstorms, which he never avoided. Thanks to him, I will never be able to look at ovens the same way. I would like to express my gratitude Assoc. Prof. Dr. Gözde Özbayram as she is always ready to share her expertise with me and İbrahim. I also want to thank my group mates, Muhammed, Anıl and Esra for their help and Yağmur, who endured the pain of postgraduate education with me. I would like to express my appreciation to Filiz Ayılmaz, who never hesitated to help me whenever I needed it. Finally, I also would like to thank the members of the Institute of Environmental Sciences, especially Asu Ziylan Yavaş and Assoc. Prof. Dr. Ulaş Tezel, for helping me become a better person and scientist.

I am absolutely pleased to have Abdullah Said Okuşluk, Mr. Atabey Ünlü, Bora "The lad" Güneş and Tolhas Can Arslan as my dearest allies. They were my rock during this journey.

Last but definitely not the least, I would like to express my sincere thanks and gratitude to my family, my mother Fatma, my sister İpek and my brother Eyüp Can. Even though they are miles away, they always make me feel that they are by my side and have my back. This one is for you, mom. And I would to like appreciate and honor my uncle Mehmet Aydıner and Prof. Dr. Dang Duong Bang for being a role model for me. I owe them to be the person I am today.

ABSTRACT

DETECTION AND IDENTIFICATION OF ANAEROBIC MICROORGANISMS BIOCHEMICALLY DEGRADING LIGNIN IN İĞNEADA FLOODPLAIN FOREST

Lignocellulosic biomass, which is found in large amounts in nature and a significant part of it is considered as waste, is seen as one of the most promising source for green and renewable energy for various reasons. However, lignin prevents the lignocellulosic material from reaching its potential due to its recalcitrant structure. The anaerobic digestion of lignin by microorganisms seems to be the most viable option to overcome this obstacle.

The aim of this thesis is to discover anaerobic microorganisms that can digest lignin from sediment and dune samples taken from two different regions (Hamam Lake and Bulanık Stream) of the İğneada floodplain forest in northwest Turkiye. Samples were enriched with alkaline lignin as a sole carbon source at 37^{o}C with three incubation periods lasting a total of 150 days. Then, DNA of the samples was isolated and sequenced metagenomically by MinION. In addition, various physicochemical analyzes including biogas production, pH, gas composition, VFA production, VS removal, determination of lignin content were carried out to detect how the microbial community operates during the experiments.

As a result of the thesis, the degradability capacity of lignin in an oxygen-free environment has been demonstrated by various parameters for both stations. According to metagenomic data, it was determined that some microbial phyla and species, in which the microbial community changed as the transfers progressed, became dominant. Consequently, microbial community members that may be responsible for lignin digestion have also been identified.

ÖZET

İĞNEADA LONGOZ ORMANLARINDA LİGNİNİ SİNDİREBİLEN ANAEROBİK MİKROORGANİZMALARIN TESPİTİ VE KARAKTERİZASYONU

Doğada oldukça fazla miktarda bulunan ve önemli bir kısmı atık olarak değerlendirilen lignoselülozik biyokütle, çeşitli nedenlerden ötürü yeşil ve yenilenebilir enerji için en umut verici kaynaklardan bir tanesi olarak görülmektedir. Ancak lignin, dirençli yapısı nedeniyle lignoselülozik materyallerin potansiyeline ulaşmasını engellemektedir. Ligninin mikroorganizmalar tarafından anaerobik olarak parçalanması, bu engelin üstesinden gelmek için en uygun seçenek gibi görünmektedir.

Bu tezin amacı, Türkiye'nin kuzeybatısında bulunan İğneada longoz ormanının iki farklı bölgesinden (Hamam Gölü ve Bulanık Deresi) alınan sediman ve kumul örneklerinden lignini sindirebilen anaerobik mikroorganizmaları keşfetmektir. Örnekler, alkali lignin tek karbon kaynağı olarak kullanılarak, toplamda 150 gün süren üç inkübasyon periyodu ile 37ºC'de zenginleştirildi. Daha sonra örneklerin DNA'sı izole edildi ve MinION ile metagenomik olarak dizilendi. Ayrıca deneyler sırasında mikrobiyal topluluğun nasıl çalıştığını tespit etmek için düzenli olarak biyogaz üretimi, pH, gaz kompozisyonu, VFA üretimi, VS giderimi ve lignin içeriğinin belirlenmesi gibi çeşitli fizikokimyasal analizler yapılmıştır.

Tez sonucunda ligninin oksijensiz ortamda parçalanabilme kapasitesi her iki istasyon için de çeşitli parametrelerle ortaya konmuştur. Metagenomik verilere göre, transferler ilerledikçe mikrobiyal topluluğun değiştiği, bazı mikrobiyal filum ve türlerin baskın hâle geldiği belirlendi. Sonuç olarak, ligninin oksijensiz parçalanmasından sorumlu olabilecek mikrobiyal topluluk üyeleri tespit edilmiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Unit
$Ca(OH)_2$)	Calcium Hydroxide	
CH_3COOH	Acetic Acid	
CH_4	Methane	
cm	Centimeter	
CO_2	Carbon Dioxide	
H_2	Hydrogen	
HCl	Hydrochloric Acid	
H_2O	Water	
H_2SO_4	Sulfiric Acid	
KOH	Potassium Hydroxide	
kPa	Kilopascal	
ml	Mililiter	
mM	Micromolar	
mm	Milimeter	
NaOH	Sodium Hydroxide	
Na_2S	Sodium Sulfide	
NH_4OH	Ammonium Hydroxide	
OH	Hydroxyl	
µg	Microgram	
µL	Microliter	
µm	Micrometer	

Abbreviation	Explanation
1D	Single Stranded
2D	Double Stranded
ANOVA	One-way-analysis of Variance
AD	Anaerobic Digestion
B20cm	Bulanık 20cm
bp	Basepair
β-O-4	β-aryl Ether
C	Molar Volume of Ideal Gas

C — Molar Volume of Ideal Gas — $L\ mol^{-1}$

CELF	Co-solvent Enhanced Lignocellulosic Fractionation	
CHP	Combined Heat and Power	
COD	Chemical Oxygen Demand	
COVID-19	Coronavirus Disease of 2019	
ddNTPs	Dideoxynucleotides	
DESs	Deep Eutectic Solvents	
dNTPs	Deoxyribonucleotide Triphosphate	
DyP	Dye-decolorizing Peroxidase	
DNA	Deoxyribonucleic Acid	
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen	
GHG	Greenhouse Gas	
G	Guaiacyl	
H20cm	Hamam 20cm	
HGP	Human Genome Project	
I:S	Inoculum to Substrate Ratio	
IEA	International Energy Agency	
LCC	Lignin Carbohydrate Complex	
LiP	Lignin Peroxidase	
MnP	Manganese Peroxidase	
NGS	Next-generation Sequencing	
N.C.	Negative Control	
ONT	Oxford Nanopore Technologies	
OTU	Operational Taxonomic Unit	
PacBio	Pacific Biosciences	
PCA	Principal Component Analysis	
PCoA	Principal Coordinate Analysis	
PCR	Polymerase Chain Reaction	
R	Universal Gas Constant	$mbar\ cm^3\ mol^{-1}\ K^{-1}$
RNA	Ribonucleic Acid	
S	Syringl	
SCFs	Supercritical Fluids	
SMS	Single Molecule Sequencing	
T	Temperature	Kelvin
TS	Total Solids	mg/L
V_{biogas}	Volume of Biogas	mL

VP	Versatile Peroxidase	
VFA	Volatile Fatty Acid	mL
VS	Volatile Solids	mg/L
ZMWs	Zero-mode Waveguide	
ΔP	Pressure Difference	kPa

1. INTRODUCTION

Energy has always been one of the most fundamental instruments for humanity. Energy dependence has increased significantly since the beginning of the Industrial Revolution and has become an indispensable part of daily life as well as industry. Most of the energy is produced by burning fossil fuels such as coal and natural gas. Although fossil fuels have been the subject of much controversy over the past few decades, they continue to be widely used even today. For example, more than 60% of global energy production in 2018 comes from coal and natural gas alone (Leonard et al., 2020). However, it is clearly known that the use of fossil fuels disrupts the ecological balance of the Earth by increasing the rate of greenhouse gases that cause various negative effects on nature (Bajpai, 2020). Accordingly, the demand for alternative energy also increases and leads to new searches in energy production (Bilgen et al., 2008; Wolfson and Schneider, 2002). As a result, developed countries attach great importance to alternative energy sources, also called renewable, in order to reduce their greenhouse gas (GHG) emissions.

Renewable energy is based on wind, solar, water, thermal and biomass. The latter has the greatest potential because it is inexpensive and readily available. Various types of biomass including energy crops, agricultural wastes, domestic wastes, industrial wastes, etc. can be used in biofuel production. Among all wastes, lignocellulosic structures have received more attention recently as they are one of the most abundant organic resources worldwide. In addition, its low-cost structure and high yield rate make lignocellulosic biomass an excellent candidate (Wu and He, 2013).

Although there are many technological options such as combustion, co-firing, gasification, pyrolysis, CHP (combined heat and power) in the conversion of biomass to energy, all these methods offer advantages as well as some disadvantages. Anaerobic digestion (AD) seems to be the best technique considering all aspects as it is efficient in terms of energy input/output and also cost effective (Ahmed et al., 2019). In addition, AD prevents greenhouse gas emissions from the natural breakdown of biomass, which releases methane, one of the most harmful greenhouse gases to the environment, and converts this chemical into biofuel (Zheng et al., 2014). The emergence of fertilizer as a byproduct from AD may be another beneficial environmental effect (Millati et al., 2020). AD is usually managed by a group of microorganisms through their syntrophic relationships. It consists of four different stages, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis. Hydrolysis is a rate-limiting step for agricultural products and woody biomass due to the relatively difficult digestion of its structural elements (Hendriks and Zeeman, 2009). Lignocellulosic components, which

contain cellulose and hemicellulose along with lignin, are the most important barriers to reducing the speed and efficiency of converting biomass from forest and agricultural wastes to energy in anaerobic digesters. However, lignin is more resistant than cellulose and hemicellulose due to its internal structure that prevents enzymatic attack (Agbor et al., 2011). Since lignin binds cellulose to hemicellulose, it must be broken down by cleavage of aromatic rings. In this way, other lignocellulotic structures are exposed to microbial enzymes such as cellulases and hemicellulases. As mentioned earlier, lignin cannot be effectively broken down by microorganisms during hydrolysis, resulting in lower product yields. Therefore, lignin remains the most important reaction-limiting step for enzymes targeted for lignocellulotic substrates (Mansfield et al., 1999).

Pretreatment techniques, which can be divided into four groups as physical, chemical, physicochemical and biological, are used to increase the efficiency of AD. However, factors such as cost and harmful by-products make these methods undesirable (Zheng et al., 2014). Biological pretreatment, in which white-rot fungi are generally used, does not contain these disadvantages, but is not at the desired level due to its low efficiency (Rouches et. al, 2016). Therefore, there is still a need for an organism or system to effectively digest lignin within the AD system to maximize the valorization of lignocellulosic biomass.

For a long time, it was believed that the degradation of lignin, unlike cellulose and hemicellulose, is dependent on the presence of oxygen. Although there are some bacterial and fungal enzymes that can degrade lignin, such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase, these enzymes work under aerobic conditions and can be applied as pretreatment to anaerobic systems. But there is no commercial enzyme that breaks down lignin anaerobically. (Paudel et al., 2017). However, some recent research has suggested that it may be possible to break down lignin anaerobically. Several species of bacteria and fungi that degrade lignin have been described before, but almost all of them live inside another organism through mutualist association. However, there are some exceptions: two lignin-digesting anaerobic microbial communities have been discovered, one in the rainforest and the other in the mangrove forest (DeAngelis et al., 2011; Y. R. Wu & He, 2013). In addition, a few species have been identified recently that have been shown to be able to degrade lignin without oxygen.

Shrestrea et al. (2017) state that swamps, swamps, mangroves, and paddy fields share an environment comparable to tropical forests and can therefore host microorganisms that digest lignin anaerobically. Floodplain forests provide conditions quite similar to mangroves. Floodplains are one of the unique ecosystems on earth, like mangroves. The enormous microbial diversity, thought to

harbor a large previously unidentified genetic and biological pool, enables the discovery of new biomolecular genes for a variety of valuable products and metabolic pathways (Whitman et al., 1988). Another important factor is the height of the water table because it is directly dependent on the biodiversity of microbes in the soil and therefore their metabolism. This has critical consequences for organic matter decomposition, accumulation, and transformation (Zhang et al. 2018). Last but not least, the dynamic interface between aerobic and anaerobic layers in the soil due to constant nutrient availability, certain water level and salinity, changing water regimes also makes these forests extremely rare. (Marcial Gomes et al., 2008). Considering these findings, it seems quite possible to find a new fungal enzyme that breaks down lignin anaerobically in a floodplain forest.

In this study, sediments, and dune samples from two stations in the İğneada floodplain forest, namely Hamam Lake and Bulanık Stream were collected. Collected samples were exposed to alkaline lignin as sole carbon source at 37^{o}C and enriched for a total of 150 days. Interest of microorganisms or microbial committee were obtained selectively while other microorganisms were eliminated by this way. The microorganism or microbial community responsible for lignin digestion was identified by metagenomic sequencing.

2. LITERATURE REVIEW

2.1. Current Energy Outlook Around the World

Energy, as a fundamental need of human beings, has always been an integral part of life, but has become indispensable in recent years. Energy production and demand are increasing day by day. Even though the decrease in the global energy market in 2020 due to strict measures including quarantine related to the COVID-19 pandemic, energy demand in 2021 exceeds not just 2020 but also 2019 by boosting 4.6% compared to the previous year according to International Energy Agency (International Energy Agency, 2021). Most of the energy is produced by burning fossil fuels such as coal and natural gas. Although fossil fuels have been the subject of much controversy over the past few decades, they continue to be widely used even today. For example, more than 60% of global energy production in 2018 comes from coal and natural gas alone (Leonard et al., 2020). As a result, energy-related CO_2 emissions rise by approximately 5% in 2021 which makes it the second-highest annual increment of all time (International Energy Agency, 2021).

It is clearly known that the use of fossil fuels disrupts the ecological balance of the Earth by increasing the rate of greenhouse gases that cause various negative effects on nature (Bajpai, 2020). Despite the long-standing debate about the greater use of renewable resources instead of fossil fuels, governments still do not put enough effort to make this happen. However, most of the developed countries committed to lower CO_2 emissions through the reduction of energy demand by 2050 at the Paris Climate Conference (Platzer & Sarigul-Klijn, 2021). Accordingly, the increase in the demand for alternative energy leads to new searches in energy production. The shift from fossil fuels to renewables needs to accelerate soon, as every 1% increase in renewable energy consumption results in a reduction in CO_2 emissions of around 0.2% (Mohsin et al., 2021). The recent energy crises and war between Ukraine and Russia have also shown once again that reliable and sustainable energy alternatives should be adopted as soon as possible.

2.2. Renewable Sources as a Sustainable Energy Source

In direct proportion to the ever-increasing world population, the need for energy is increasing day by day. Even if the world's energy needs are met as a result of the intense use of fossil fuels, this has brought about negative consequences such as the depletion of these resources, higher levels of greenhouse gas emissions than ever before, and fluctuations in fuel prices. For this reason, factors such as sustainable development, energy security and the preservation of nature's balance have gained

interest in recent years (Amjith & Bavanish, 2022). Renewable energy, which naturally replenish itself without being depleted on earth, emerges as a sustainable alternative in terms of meeting the world's future energy needs, reducing carbon emissions and being cost-effective in general (Rosen, 2009). Renewable energy is based on wind, solar, water, thermal and biomass. Current renewable energy status in the world can be seen in Figure 1.1. Two-thirds of the energy produced from renewable sources is solar and wind. Likewise, the ratio of these two sources in electricity generated in 2021 reached almost 30%, reaching an all-time high (International Energy Agency, 2021).

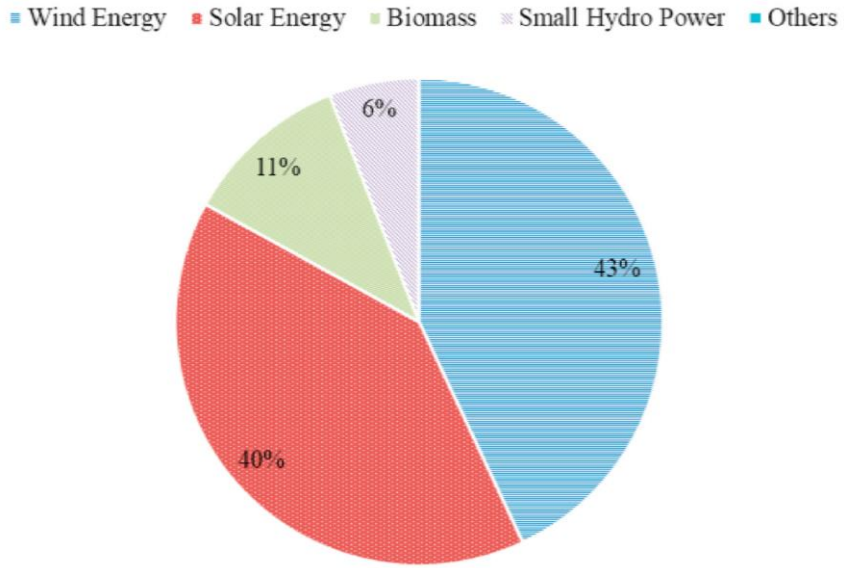

Figure 2.1 Current renewable energy status in the world.

Although solar and wind resources stand out among the diverse types of renewable energy, biomass has the highest potential. Biomass is more advantageous than other renewable energy alternatives in terms of geography and ecology because it can be obtained and stored from various sources throughout the year (Ozturk et al., 2017). Besides, the high oxygen/carbon ratio in lignocellulosic materials, a type of biomass, allows the production of chemicals that are expensive to produce from raw materials, which appear as another benefit of biomass (op de Beeck et al., 2015). Last but not least, the possibility of increasing the capacity of biomass energy by combining non-toxic waste materials that are expensive to dispose of but are not suitable for recycling is another significant advantage over fossil fuels as well as other renewable energy sources (Dumanli et al., 2007).

Several types of biomass including dedicated energy crops, agricultural and forestry residues, domestic wastes, industrial wastes etc. could be used in biofuel generation. The physicochemical properties of biofuels, which have diverse types such as biogas, syngas, bioethanol, biodiesel, vary

according to the biomass type and technique used (Verma et al., 2012). In fact, biofuels are divided into distinct groups as first, second, third and fourth generation biofuels according to the raw material and production method. First generation biofuels are fuels that are produced from edible biomass such as starch and sugar and can compete with food products. Second-generation biofuels are produced using lignocellulosic compounds, while third and fourth generation are based on wild or genetically modified microorganisms, usually microalgae (Alalwan et al., 2019). Having said that, lignocellulosic biomass, including agricultural waste, forest waste, domestic and industrial waste, has been receiving increasing attention in the literature recently, as its abundance, low cost, resource utilization, non-competition with edible crops makes it an excellent candidate for potential bioenergy source (Martínez-Gutiérrez, 2018).

2.3. Lignocellulosic Biomass

It is estimated that more than 180 billion tons of lignocellulosic biomass is produced in the world every year, and about 8 billion of them are used mostly by direct combustion (Dahmen et al., 2019). As a result, harmful substances such as CO_2 and persistent organic pollutants emerge, and the potential of biomass is wasted. Therefore, it is essential to evaluate these materials in much more effective and innovative approaches. In this way, lignocellulosic materials such as agricultural or forestry residues, which are seen as waste and can contribute to the greenhouse effect by taking up space in landfills and releasing methane, can be converted into energy (Liu & Yu, 2021). However, the amount and properties of lignocellulosic material are strictly dependent on local climatic conditions, differences in farming and livestock practices, and crop type. Therefore, which type of lignocellulosic material is more suitable for energy conversion varies from country to country. Currently, many governments regulate their incentives and laws in this regard, taking into account their own resources. Due to the high rate of agricultural production in Turkey, agricultural residues, which are the post-harvest part of the crop, come to the fore (Dumanli et al., 2007). On the other hand, since 27% of Turkey is covered with forests, biomass in forests can become another valuable asset in this respect compared to other sources. The conversion of forest wastes to methane gas with anaerobic digesters has an important energy source potential (Ates et al., 2007). Wheat and barley residues, which have the highest annual production rate among plant-derived biomass, also show a high potential in terms of energy due to their high carbon content together with woody materials.

Lignocellulosic materials are consisting of three components which are cellulose, hemicellulose and lignin in varying proportions depending on the type of source.

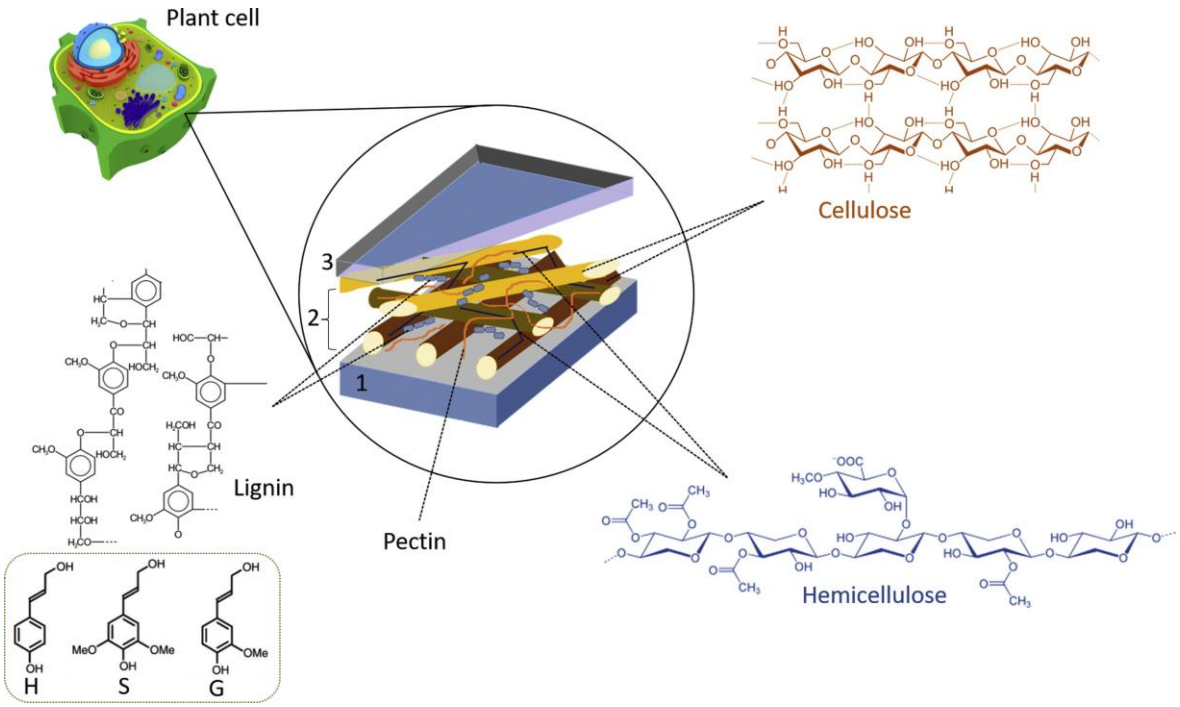

Figure 2.2. Structure of lignocellulosic biomass (Rodionova et al., 2022a).

2.3.1. Cellulose

Cellulose is the most abundant molecule in lignocellulosic biomass. It is a polysaccharide composed of cellobiose, glucose disaccharide, covalently linked by β-(1, 4) glycosidic bonds. Each cellulose unit is interlinked together by hydrogen bonds and van der Waals forces via hydroxylic groups, forming cellulose fibrils, also known as microfibrils (Kumar et al., 2018). The microfibrils are embedded in the lignocellulosic compound, bound together by hemicellulose and/or pectin and covered with lignin. Cellulose can be found as amorphous and crystalline structures with low and high crystallinity formation, respectively. Amorphous cellulose has loose hydrogen bonds compared to crystalline one and thus, it is more susceptible to enzymatic attacks. In addition to crystallinity, long chains with more hydrogen bonding networks reduce the biodegradation efficiency of cellulose (Zoghlami & Paës, 2019a).

2.3.2. Hemicellulose

Hemicellulose is highly branched biopolymer which consists of pentoses, hexoses, saccharides, acids and acetyl groups. It connects with cellulose fibrils non-covalently and form complex structure with lignin through covalent bonds including diferulic acid bridges and lignin-glucuronic acid ester links. However, amorphous and branched structure of hemicellulose as well as low degree of polymerization makes it more degradable compared with cellulose. Dilute acids and bases along with hemicellulase enzyme may easily hydrolyze hemicellulose (Ashokkumar et al., 2022).

2.3.3. Lignin

Lignin, the third most abundant polymer in nature, is the non-carbohydrate portion of lignocellulosic biomass, accounting for 15–40% of dry weight. While it is currently one of the most common biopolymers worldwide, it is estimated that more than 100 billion tons of lignin is produced annually, 50 billion tons of which is waste (Zakzeski et al., 2010). It is a long-chain, highly branched amorphous heteropolymer containing various functional components such as polyphenolic groups and phenylpropane without clear pattern of unit or bonding (Zoghlami & Paës, 2019b).

Table 2.1 Characteristics of lignin in lignocellulosic biomass and their correlations with biomass conversion (Yoo et al., 2020a).

Characteristics	**Description**	**Correlations with Biomass Conversion**
Lignin Content	Weight percentage over dry weight of biomass	Negative
Lignin Composition	Relative contents of S, G, and H units over total lignin subunits	Variable
Hydroxycinnamates	Relative contents of ferulate, *p*-coumarate over total lignin subunits	Negative
Interunit Linkages	Relative contents of C–O, C–C bonds (e.g., β-*O*-4, β-β, β-5) over total lignin subunits	Positive (β-*O*-4), Negative (β-5)
Hydroxyl Group Contents	Aliphatic OH, phenolic OH (C5 substituted OH, guaiacyl OH, *p*-hydroxyphenyl OH), and carboxylic OH	Positive (Aliphatic/Total OH), Negative (Phenolic OH)
Pseudo Lignin	Acidic condensation of fragmented carbohydrates	Negative
Molecular Weights	Weight-average molecular weight, number-average molecular weight, polydispersity index	Variable

Lignin is mainly formed through oxidative linkage joined by ether (O-4′, 4-O-5′) and carbon-carbon (5′, β-5′, β-β′, β-1′) linkages of three main compounds, namely, p-hydroxyphenyl (H), guaiacyl

(G), and syringyl (S) subunits which are derived from the synthesis of monolignols (p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol). β-aryl ether (β-O-4), β−β (resinol), and β-5 (phenylcoumaran) are the major interunit linkages between lignin subunits. β-O-4 is at the forefront of the bonds that need to be broken in order to biochemical degradation of lignin.

Lignin is known as one of the most recalcitrant structure in the nature since it provides hydrophobicity as well as rigidity to plant cell wall (C. Li et al., 2015). Numerous parameters including total lignin content, lignin composition/structure, hydroxyl group contents, interunit linkages etc. affect availability of lignocellulosic biomass to chemical or biological treatments. Table 2.1. shows correlations of these factors with biomass conversion.

The structure in which lignin forms covalent bonds with carbohydrates in the lignocellulosic matrix, especially with hemicellulose, is called lignin carbohydrate complex (LCC). Figure 2.3. shows the three-dimensional structure of the LCC in the cell wall. LCC is formed during lignin biosynthesis, when covalent bonds with a predominance of benzyl ether, benzyl ester, glycosidic or phenyl glycosidic cross-link lignin and carbohydrate (Tarasov et al., 2018). Its presence in the plant cell wall is critical since it is an important parameter in determining the physical, chemical, and biological properties of lignocellulosic biomass (Nishimura et al., 2018). Therefore, the nature and amount of LCC linkages as well as lignin substructures are factors to be considered in the valorization of lignin and lignocellulosic material, as it affects hydrolysis efficiency and digestibility.

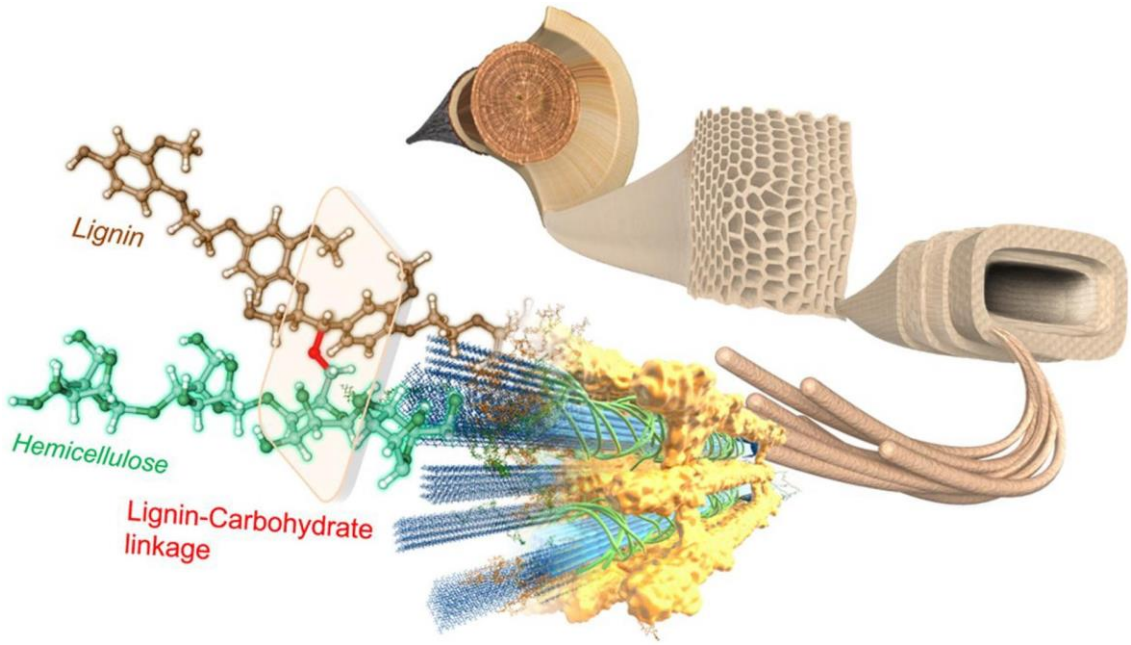

Figure 2.3. A three-dimensional view of lignin carbohydrate complex (Nishimura et al., 2018)

It is well-known that lignin is the most dominant factor impeding the efficient transformation of lignocellulosic biomass into valuable materials such as chemicals or fuels. Although there is limited information about the internal structure of lignin and the bonds it forms in the lignocellulosic matrix, it is estimated that lignin exerts resistance to degradation in two specific ways (Yoo et al., 2020b). Firstly, the nonproductive binding of lignin on enzymes through hydrophobic, electrostatic, and hydrogen bond interactions provides significant inhibition of degradation. It is suggested that this occurs through two different pathways: either by binding to the hydrophobic surface of cellulose or by interacting on the active side of cellulase, which serves to recognize cellulose (dos Santos et al., 2019). On the other hand, lignin can bind to the cellulose moieties that cellulase selectively attacks, thereby significantly restricting the cellulose-enzyme relationship. While it is still debated which pathway lignin prefers primarily, it is suggested that this is most likely largely dependent on the nature of the enzyme and the substrate (Vermaas et al., 2015). From this point of view, it is possible to emphasize that lignin not only inhibits the enzyme's activity through steric hindrance on cellulose, but also acts as a physical barrier, reducing the degradation efficiency of cellulose and hemicellulose in the LCC. Hence, the resistance of lignin to degradation is considered to be a crucial obstacle to the valorization of lignocellulosic biomass.

2.4. Conversion Techniques for Lignocellulosic Biomass

Lignocellulosic material can be converted into renewable fuels including bio-oil, syngas, bioethanol, biogas etc. by physical, thermochemical and biochemical techniques. Figure 2.4. illustrates diversified conversion methods along with final products. The technique to be chosen is directly related to the structure of the biomass, but also significantly determines the quality of the final product. However, the majority of these options have significant drawbacks such as low energy efficiency, harmful by-products and high capital investment (Rao et al., 2010).

Anaerobic digestion (AD) seems to be the best technique in all respects, as it is efficient in terms of energy input/output and at the same time cost-effective (Ahmed et al., 2019). In addition, AD not only prevents the greenhouse gas effect by releasing methane from the natural degradation of biomass, but also provides its conversion into bioenergy (Zheng et al., 2014). The emergence of fertilizer as a byproduct from AD may be another beneficial environmental effect (Millati et al., 2020).

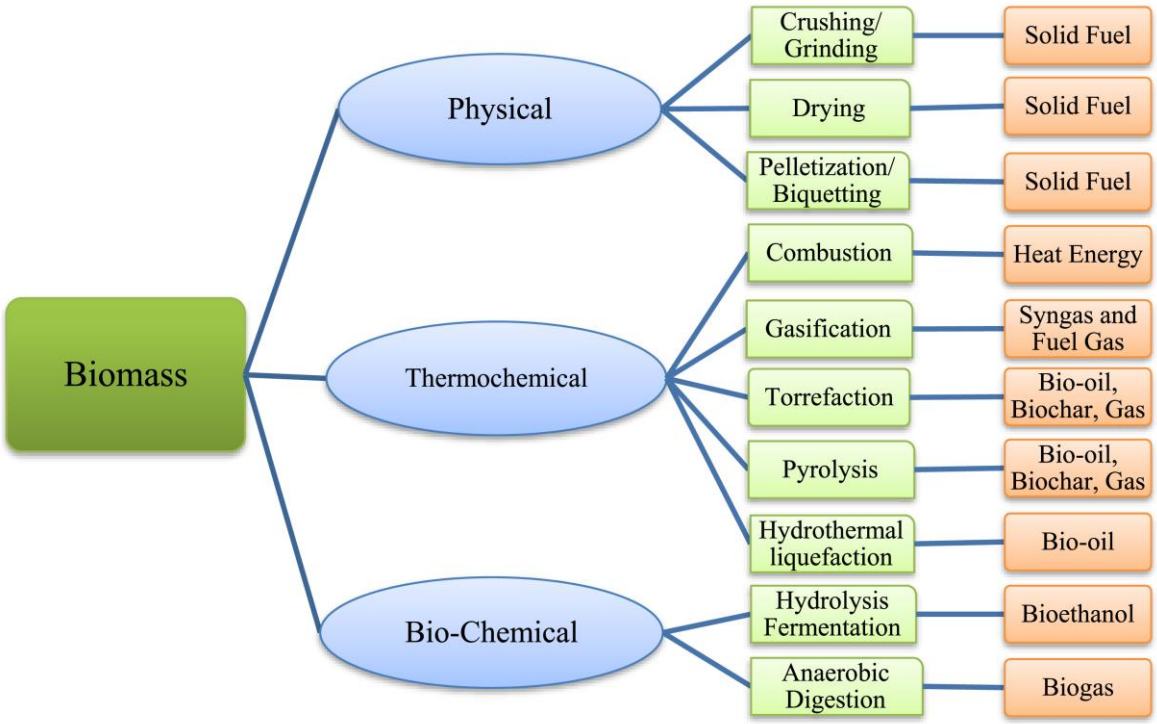

Figure 2.4 Conversion techniques for lignocellulosic biomass (Ong et al., 2019).

2.5. Anaerobic Digestion

Anaerobic digestion, in the simplest terms, is the conversion of macromolecules into smaller building blocks by biodegrading in an oxygen-free environment. The substrate is broken down and volatile fatty acids (VFA) such as acetic acid and propionic acid come out as intermediates, while biogas containing methane, carbon dioxide (50-70% and 25-50% respectively) along with trace amounts of water vapor, hydrogen, hydrogen sulfide, carbon monoxide, and nitrogen is generated as the final product at the end (Hendriks & Zeeman, 2009). Anaerobic digestion is utilized in various fields of industry such as waste management including urban and industrial wastes, energy generation, grid injection and fertilizer production.

Anaerobic digestion occurs in nature in ponds, swamps, paddy fields, lakes, intestinal tracts of humans and animals. This biochemical process consists of sequential phases performed together by microbiological communities including bacteria and archaea, thus it is affected by various parameters such as temperature, pH, raw material composition, free ammonia, volatile fatty acid, carbon-nitrogen ratio (Merlin Christy et al., 2014). Anaerobic digestion takes place in four steps: Hydrolysis, acidogenesis, acetogenesis, and methanogenesis.

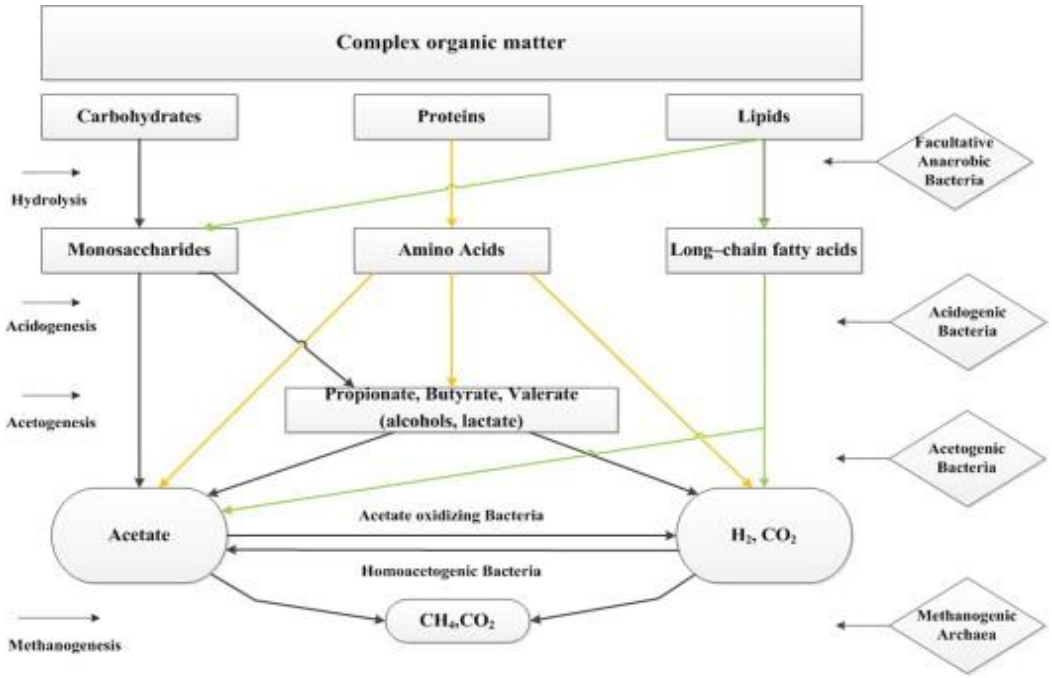

Figure 2.5 Steps of anaerobic digestion (Merlin Christy et al., 2014).

2.5.1. Hydrolysis

Anaerobic digestion starts with hydrolysis of organic polymers such as polysaccharides. Once various types of hydrolytic microorganisms (*Clostridia, Bacteroides*, *Streptococcus* etc.) secreted their extracellular enzymes including cellulase, amylase, xylanase, complex organics disintegrated to smaller subunits such as glucose, xylose or mannose (Cirne et al., 2007). It is known that the digestion of complex structures takes longer time, since the rate of degradation during hydrolysis is highly dependent on the nature of the substrate. At the same time, the degradation efficiency of the complex organic molecule is extremely critical for the fate of the next steps (Adekunle & Okolie, 2015). Nonetheless, the most vital step is hydrolysis since it is the rate-limiting (Ozturk et al., 2017).

2.5.2. Acidogenesis

Acidogenesis, the second part of anaerobic digestion, is also the fastest of the whole process. As a result of the activity of obligatory anaerobic or facultative microorganisms (*Streptococcus, Bacillus, Salmonella*) that use structures such as sugars, long chain fatty acids and amino acids that are generated during hydrolysis, alcohol, carbon dioxide and hydrogen emerge together with short-chain fatty acids including acetic, propionic, butyric (Gujer & Zehnder, 1983). Since the volatile fatty acids produced during this step are the direct precursor of methane, their concentration is vital for the methane yield of anaerobic digestion. Organic acids, carbon dioxide and hydrogen produced in proportion to the amount of substrate used cause the environment to become acidic and the pH to

drop to 4.5-6 levels. Even if this situation is favorable for acidogenic microorganisms, rapid accumulation of organic acids and a sudden increase in acidity can create an inhibitory effect for slower acetogenesis and methanogenesis steps (Merlin Christy et al., 2014).

2.5.3. Acetogenesis

In the third step, acetogenesis, the products generated during acidogenesis are further biodegraded. Thus, volatile fatty acids such as propionic and butyric acids accumulated with increasing hydrogen concentration in the previous step are converted to acetate, which can also be used by methanogens. During these reactions, carbon dioxide and hydrogen are also produced. The syntropic relationship between microorganisms operating in acetogenesis and methanogenesis depends on the hydrogen balance. While acetogenesis is dependent on low hydrogen partial pressure, methanogens constantly consume hydrogen and interspecies hydrogen transfer takes place between these microorganisms (Sekiguchi et al., 2001). Slow growing acetogenic microorganisms, which are sensitive to changing environmental conditions, are also extremely sensitive to oxygen due to their obligatory anaerobic nature (Adekunle & Okolie, 2015).

2.5.4. Methanogenesis

During methanogenesis, which is the last stage of anaerobic digestion, methane may be formed as a result of two biochemical reactions of two groups of microorganisms.

$$CO_2+4H_2 \rightarrow CH_4+2H_2O \quad (1)$$

$$CH_3COOH \rightarrow CH_4+CO_2 \quad (2)$$

Acetoclastic methanogens produce methane using acetate as seen in equation 1. On the other hand, hydrogenotrophic methanogens use carbon dioxide and hydrogen for methane production as seen in equation 2. In general, 60% of methane is generated using acetoclastic pathways, 30% using hydrogenotrophic pathways, and the remaining minor portion is produced using methyl compounds (Jain et al., 2015). Hydrogen-depleting methanogens grow much faster than acetoclastic methanogens and adapt more easily to environmental conditions. On the other hand, all methanogens are very sensitive to oxygen and lose their vitality at pH values below 6 (Ferry, 1997).

In summary, anaerobic digestion is a widely used option because it is a mature technique, as well as providing waste management, relatively high energy efficiency, and manure as a by-product as a result of the process (Lisowyj & Wright, 2020). But hydrolysis step remains as rate-limiting as

mentioned above. Considering the biochemical structure of lignocellulosic materials, which are challenging to decompose, the significance of the hydrolysis step comes to the fore even more. Consequently, pretreatment is needed to be applied to increase the AD efficiency by breaking the strong bonds between the lignin (Zheng et al., 2014).

2.6. Pretreatment Techniques for Anaerobic Digestion

Several approaches have been developed over the years to increase the efficiency of the anaerobic digestion of lignocellulosic compounds, especially lignin. Pretreatment techniques are preferred among them because they have been studied extensively for a long time (Akyol et al., 2019b; Mok & Antal, 1992; Silva et al., 2012; Uzun et al., 2021; Ziemiński et al., 2012). The choice of pretreatment affects not only the overall expenditure but also the fate of the lignocellulosic biomass. As mentioned above, the purpose of this process is to make the lignocellulosic biomass more susceptible to enzymatic attack before hydrolysis, which is the rate-determining step of AD. Therefore, during pretreatment it is expected that the crystalline cellulose will transform into an amorphous state that is more easily degraded, the lignin will be delignified, and the hemicellulose will be at least partially digested (Rodionova et al., 2022).

Although many pretreatment methods have been applied so far, only a few of them have been able to shift from pilot scale to large scale due to certain disadvantages. The ideal approach should meet criteria such as increased solubility and degradability of the substrate by reducing its size and enhancing its porosity, not producing any inhibitory compounds, having a lower energy input, being economical and suitable for scale-up (Sayara & Sánchez, 2019).

Pretreatment techniques can be categorized into four subgroups: Physical, chemical, physicochemical and biological. It is also possible to combine two or more of them.

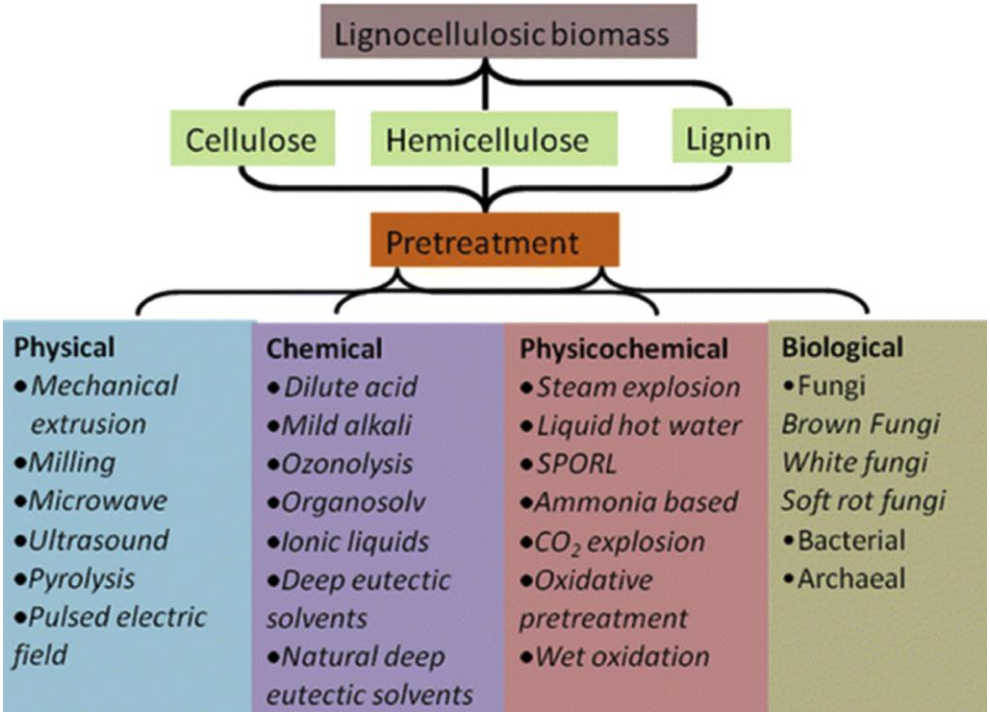

Figure 2.6 Pretreatment methods for lignocellulosic biomass (Tu & Hallett, 2019a).

2.6.1. Physical Pretreatment

Physical pretreatments can be divided into mechanical, extrusion and irradiation. Mechanical methods include grinding, milling, dry crushing and comminution. The purpose of these processes is to reduce the particle size of the substrate with the help of applied mechanical power, to increase the surface area and to deform the crystal structure of cellulose. At the end of this process, which is relatively environmentally friendly and does not produce any toxic substances at the same time, it has been determined that although the hydrolysis of the lignocellulosic structure during AD has increased slightly, the cellulose is still not fully digested (Rodionova et al., 2022). In addition, it has almost no effect on lignin degradation. Extrusion, which uses an extruder to create mechanical shear to alter composition of material in the presence of certain amount of humidity, and irradiation that includes microwave, ultrasound, gamma ray and electron beam pretreatments promise better results compared to mechanical one.

However, there are still significant drawbacks such as high energy consumption and production of inhibitor chemical even if small amount of lignin degradation is possible for these methods unlikely to mechanical one (Mankar et al., 2021). Physical pretreatments are often used in combination, with chemical techniques generally, because in addition to requiring extensive energy consumption, accumulation of volatile fatty acids causes methane production to be inhibited if the particles become

excessively small (de la Rubia et al., 2011). Physical pretreatments could be effective on lignin-free biomass.

2.6.2. Chemical Pretreatment

Chemical pretreatment involves alkali, acid, organosolv, co-solvent enhanced lignocellulosic fractionation (CELF), ionic liquids, supercritical fluids (SCFs) and deep eutectic solvents (DESs) but the first three are widely used while others are still emerging technologies and far from commercialization. Sodium hydroxide (NaOH), potassium hydroxide (KOH), calcium hydroxide ($Ca(OH)_2$) and ammonium hydroxide (NH_4OH) are used in alkali pretreatment for alteration of lignin structure, partial decrystallization of cellulose and partial degradation of hemicellulose by cleavaging of ester and glycosidic sidechain of LCC (Mansour, 2021). However, as the lignin content in the material increases, the efficiency of this technique decreases (Amin et al., 2017). On the other hand, acid pretreatment using chemicals such as sulfuric acid (H_2SO_4), hydrochloric acid (HCl), acetic acid (CH_3COOH) can be performed at high and low temperatures depending on the acid concentration, while organosolv pretreatment degrades the structure of lignocellulosic biomass by utilizing organic acids including ethanol, methanol, acetone etc. Chemical pretreatment techniques are effective in terms of degradation of cellulose and hemicellulose but formation of inhibitory compounds, requirement of additional washing step to collect substrate, corrosive nature of some of the acid used in the process and high costs are foremost drawbacks (Tu & Hallett, 2019b). It is possible to combine alkali and acid pretreatment to enhance efficiency of the technique as well as proper lignin degradation, but same disadvantages still apply.

2.6.3. Physicochemical Pretreatment

This pretreatment can essentially be defined as a combination of two different techniques. For instance, milling and alkaline processes are combined to increase the efficiency of pretreatment. However, the most commonly used physicochemical techniques are steam explosion and microwave irradiation. In the steam explosion technique, the deformation of the lignocellulosic material is ensured by following the high pressure of 160-260 degrees, followed by the gradually decreasing pressure. Acid can also be used to increase the efficiency of the process. Even when cellulose and hemicellulose undergo partial degradation, lignin remains remarkably intact in its structure. In fact, the condensation of soluble lignin components causes the production of inhibitors that make the lignocellulosic biomass less digestible (Amin et al., 2017). On the other hand, microwave irradiation method, in which magnetic and electric field forces are applied simultaneously and combined with other physical or chemical methods, has been proven to achieve better results than conventional

methods. Although it is an emerging method due to its compliance with the principles of green chemistry, many factors such as energy expenditures and the high price of the capital investment and the specific moisture required by the substrate prevent the technique from scale-up (Marx et al., 2014).

2.6.4. Biological Pretreatment

Although physical, chemical and physicochemical pretreatments are fairly rapid and can significantly increase methane yields, they are not economically and environmentally viable as these techniques require expensive chemicals, produce harmful byproducts, and are not suitable for scale-up in general. While biological pretreatment is not affected by most of these problems, it is more sustainable, albeit slowly, because it is environmentally friendly and requires low energy (Sawatdeenarunat et al., 2015). Biological pretreatment is applied to lignocellulosic biomass in order to degrade them biochemically by using bacteria, fungi, termite etc. or using enzymes of these organisms. Since the enzymatic route relies on the purification or production of the extracellular enzymes of these organisms, it eliminates the disadvantages that living organisms have, such as cellulose loss or long processing time, without microorganism growth on the biomass. However, the production of the required enzymes is often time and labor intensive and therefore quite expensive despite the ever-growing omic based approaches (Vasco-Correa et al., 2016). As a result, enzymatic pretreatment is not a widely applied method.

Fungi are the most extensively studied organisms in terms of biological pretreatment. White rot, soft rot and brown fungi are effectively delignifies cellulose, hemicellulose and especially lignin through their extracellular enzymes but white rot fungi are preferred one because it mainly attack to lignin and also degrade cellulose to some extent (Akyol, 2019; Khan et al., 2022). White rot fungi are a type of wood rot basidiomycetes that can degrade lignin as secondary metabolite as it cannot use lignin as sole carbon source. Some of the white rot fungi selectively decompose lignin over cellulose while others attack lignin and holocellulose at the same time. Since holocellulose is required for generation of biogas during AD, selective rotting fungi promises enhanced efficiency and thus, research have focused on them. *Phanerochaete chrysosporium, Pleurotus ostreatus*, *Ceriporiopsis subvermispora, Irpex lacteus,* and *Trametes versicolor* are some of the white rot fungi that are capable to degrade lignin at higher rates up to 71% (Akyol et al., 2019b; Uzun et al., 2021; Vasco-Correa et al., 2016). But biological pretreatments with fungi depends on considerable number of parameters including incubation temperature, incubation time, moisture content, type of fungi, aeration, pH, inoculum concentration and particle size (Sindhu et al., 2016). Although studies with white rot fungi,

which were found to be able to decompose lignin aerobically by using their extracellular enzymes, seem remarkably promising in terms of yield increase; the need for special environmental conditions, larger area, prolonged process period that can sometimes last for months and loss of organic matter, especially cellulose, cause this pretreatment method to be limited (Atelge et al., 2020).

Another candidate for biological pretreatment of lignocellulosic biomass would be bacteria. Even though diverse types of bacteria capable of degrading cellulose or hemicellulose such as *Cellulomonas fimi* and *Thermomonospora fusca* have already been identified, studies on bacterial lignin degradation are limited (Sharma et al., 2019). Some bacteria, including *Sphingobium sp. SYK-6, Pseudomonas putida mt-2* and *Streptomyces griseorubens* isolated from soil show capability to break down structure of lignin aerobically to a point but evaluation of their performance in terms of biological pretreatment is still lacking (Ahmad et al., 2010; Masai et al., 2007; Saritha et al., 2013). Additionally, their lignolytic system is far from being understood even if it is suggested that they may using laccases or other nonperoxidases. Using termites, worms or microbial consortium are other alternatives, but the potential use of these organisms is hard to predict since there are just a few studies related with the subject (Devi et al., 2020; Norfariha et al., 2013; Zhang et al., 2011).

On the other hand, bioaugmentation, which envisages that a special group of microorganisms including seed sludge or rumen fluid is added directly to the anaerobic digestion process and works together with the microorganisms working in the system, has recently come to the fore as newly introduced technique (Akyol et al., 2019a; Shahi et al., 2016). Although it increases the efficiency of AD, bioaugmentation also has problems such as being time-consuming method along with washout, being out of competition by other microbial groups, and regulating the number of cells required for the system (Lovato et al., 2021). In general, almost all of the techniques proposed to increase the efficiency of the rate-limiting hydrolysis step of AD come with merit and demerits. Therefore, there is still a need for an optimized method or organism that can digest lignin in an economical, environmentally friendly and effective manner to improve the efficiency of biogas production from lignocellulosic biomass.

2.7. Lignin Degrading Microorganisms

Considering the aforementioned problems of physical or chemical processes, biodegradation of lignin seems to be the best possible way. Although there is evidence that macroorganisms such as termites can digest lignin, it is clear that the potential of microorganisms is greater, and it is thought that microorganisms perform the process in termite gut. As mentioned above, it is known that white-

rot fungi or bacteria from distinct families used in pretreatment digest lignin to some extent. These microorganisms, which can carry out their degradation pathways in the presence of oxygen, use the enzymes manganese peroxidase (MnP), lignin peroxidase (LiP), versatile peroxidase (VP), laccase, and dye-decolorizing peroxidase (DyP). While these enzymes, which are usually secreted extracellularly and are small enough to penetrate the three-dimensional structure of the LCC, operate through strong redox reactions, the final product may vary depending on the electron acceptor (Khan & Ahring, 2019). However, pretreatment sometimes makes up to 40% of the entire process financially (Kucharska et al., 2018). From this point of view, considering the financial burden and energy consumption of aerobically digesting lignin requiring continuous aeration and accordingly regular mixing, it has emerged that the digestion of lignin in an oxygen-free environment may be a viable choice.

It has recently revealed that anaerobic digestion of lignin is possible, contrary to what had long been believed. As a result of several studies, the existence of anaerobic fungi and bacteria in the stomachs of ruminant animals along with termites, and also in biogas or wastewater treatment plants has been brought to the scientific literature (Dollhofer et al., 2017; Paudel et al., 2017). However, since these environments have considerably special conditions, microorganisms can lose their viability as soon as they leave their natural habitats, and for this reason, it is quite troublesome to optimize the process conditions (Sawatdeenarunat et al., 2015).

Recently, a few studies have been published on the existence of anaerobic microbial communities and bacteria thought to have a lignin digestion mechanism. These microorganisms can be seen in Table 2.2. DeAngelis et al. reported that they detected microbial communities that can digest lignin in an oxygen-free manner through soil-embedded biotrap in rainforests in Puerto Rico (DeAngelis et al., 2011). In another similar study, the presence of microbial consortia, which can digest lignin anaerobically, was revealed by enriching the sediment samples taken from paddy field and mangrove (Y. R. Wu & He, 2013). The sequencing of these studies, the first of which was cultured directly and the second from enriched samples, was performed with Phylochip, a type of microarray method. On the other hand, three species of facultative bacteria, *Enterobacter lignolyticus* SCF1, *Tolumonas lignolytica* BRL6-1^{T}, *Klebsiella* sp. strain BRL6-2 respectively, from rainforests in Puerto Rico and one type of facultative bacteria, *Mangrovibacterium lignilyticum*, from mangrove forests in Singapore were isolated recently (Billings et al., 2015; Deangelis et al., 2011; Sun et al., 2020; Woo et al., 2014). Therefore, it will be possible to discover similar microorganisms or consortium in environments similar to the habitats in which the above-mentioned microbes live.

Table 2.2 Anaerobic lignin degrading microbial community and bacteria

Bacteria/Microbial Community	Family	Substrate	Reference
Microbial consortia	--	Low-sulfonate alkali lignin	(DeAngelis et al., 2011)
Microbial consortia	--	Kraft alkali lignin	(Y. R. Wu & He, 2013)
Enterobacter lignolyticus SCF1	Enterobacteriaceae	Alkali lignin	(Deangelis et al., 2011)
Tolumonas lignolytica	Aeromonadaceae	Alkali lignin	(Billings et al., 2015)
Klebsiella sp. strain BRL6-2	Enterobacteriaceae	Alkali lignin	(Woo et al., 2014)
Mangrovibacterium lignilyticum	Prolixibacteraceae	Alkali lignin	(Sun et al., 2020)

2.8. Floodplain Forests

Floodplain forests are ecosystems that are constantly or at certain times of the year inundated by the surrounding streams. The floodplain forest subsidizes the river's food webs, with high leaf production that partially decomposes on the forest floor and a significant portion reaches the main channel (Smock et al., 2005). It is thought to have high biodiversity due to its richness in organic matter accumulation (Tockner & Stanford, 2002). Also, for this reason, it is thought that soil microorganisms carry out their biogeochemical cycles intensively. The fact that floodplain forests are one of the ecosystems where methane, one of the end products of anaerobic digestion, is produced the most is another indicator of this (Argiroff et al., 2016). In addition, floodplain forests are an ideal ecosystem to study microbial community dynamics (Doering et al., 2021). It has also been reported that floodplain forests resemble mangroves and rainforests in certain characteristics (Arekhi et al., 2019; Truong et al., 2019; Vovides et al., 2021). Considering that lignin-digesting anaerobic microorganisms or bacteria have been detected in rain and mangrove forests, it can be argued that floodplain forests may also be a hot spot in terms of hosting the organisms sought in this thesis.

İğneada floodplain forest is located within the borders of Kırklareli province in the Thrace region of northwest Turkiye. It has been determined that the İğneada floodplain forest, which has various lakes (Hamam, Erikli, Mert, Saka) and a river (Bulanık) around it, is rich in organic matter content and amount, and it is also rich in tree biodiversity, which is one of the important factors determining the soil structure (Kavgaci et al., 2011; Tecimen & Kavgacı, 2010). Our preliminary study in this region also gave the first clues about the high microbial biodiversity of the habitat (Özbayram et al., 2021).

2.9. DNA Sequencing

2.9.1. First-generation DNA Sequencing

A new threshold for molecular biology was opened in 1953 when Francis Crick and James Watson solved the 3D structure of DNA. While applications related to DNA have developed rapidly, DNA reading, or sequencing has not fully reached this speed. The sequencing of the bacteriophage ϕX174, with the technique developed by Fred Sanger and colleagues in the 1970s, was a pioneer in this regard. Based on the use of dideoxynucleotides (ddNTPs), which are radioactively labeled and modified chemical analogues of deoxyribonucleotides (dNTPs), this method generates four parallel reactions, and the results are examined on a polyacrylamide gel (Sanger et al., 1977). Although the first-generation DNA sequencing, which has developed further thanks to shotgun sequencing, has revolutionized genomics, it has left its place to developing technologies in the following years.

2.9.2. Second-generation DNA Sequencing

In parallel with the increasing interest in sequencing, especially during the Human Genome Project (HGP), state-of-the-art approaches began to emerge. Second-generation DNA sequencing, also called next-generation sequencing (NGS) or high-throughput sequencing, uses luminescent molecules instead of heavily modified ddNTP, unlike the Sanger method. This technique, which gives results by measuring the light released depending on the amount of pyrophosphate produced with the help of luciferase, employs the sequence-by-synthesis (SBS) technique, just like in the previous generation (Braslavsky et al., 2003; Nyrén & Lundin, 1985). Therefore, both require the action of DNA polymerase directly.

The fact that the company named 454 produced a machine that could handle massive amount of parallel sequencing at the same time essentially defined this generation (Shendure & Ji, 2008). In addition to the 454, many companies such as Solexa, Agencourt, Helicos and Ion Torrent enabled the rapid development of NGS through the new instruments they developed. But Solexa, which was later acquired by Illumina, developed a new system called bridge amplification, in which adapter-bracketed DNA molecules are passed over a field of complementary oligonucleotides attached to a flow cell; a subsequent solid phase PCR generates clusters of neighboring clonal populations from each of the individual original flow cell binding DNA strands (Heather & Chain, 2016; Shendure et al., 2017). As bridge amplification proved to be the most durable method, Illumina became the most used company for sequencing.

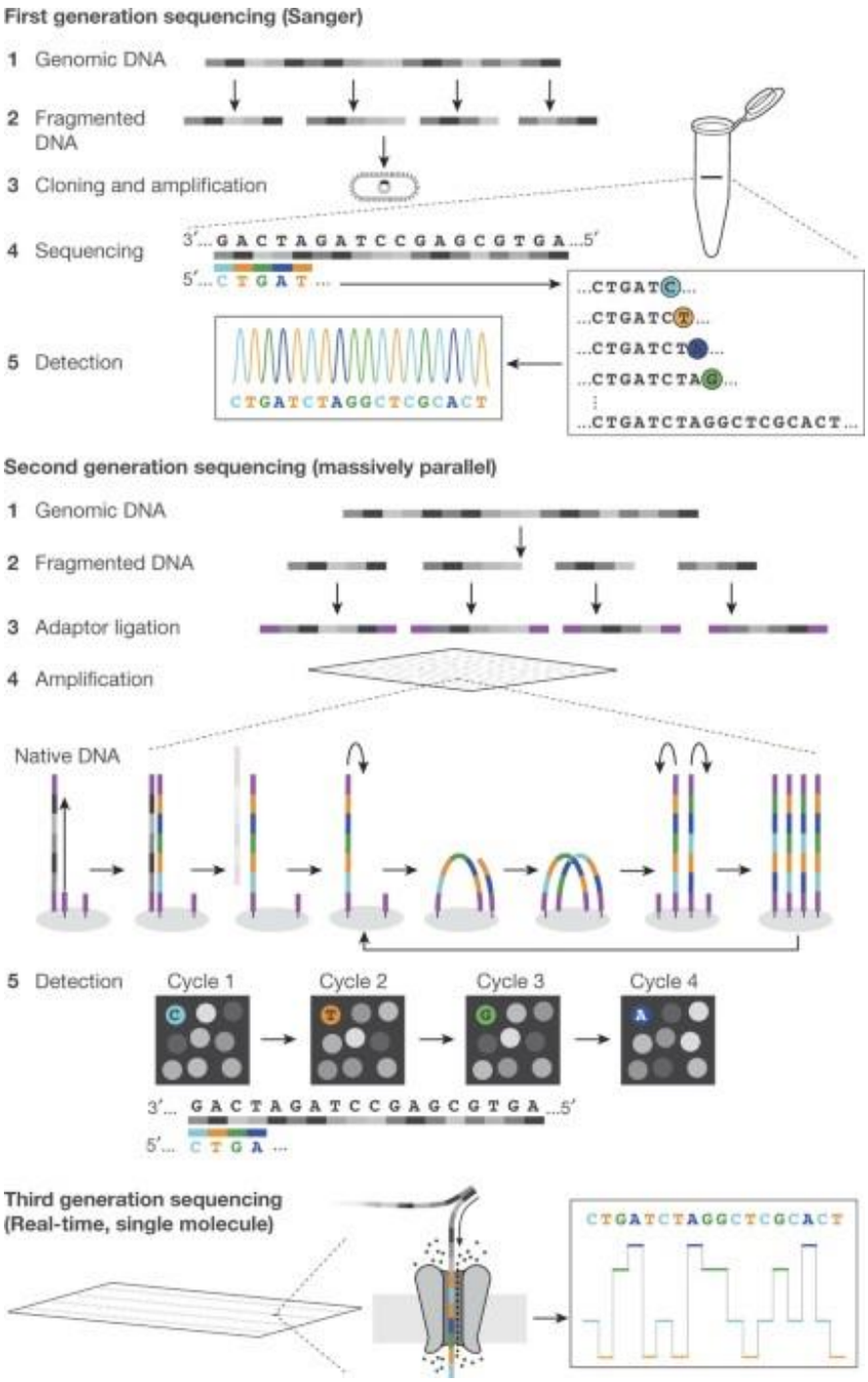

Figure 2.7 DNA sequencing technologies (Shendure et al., 2017).

2.9.3. Third-generation DNA Sequencing

Although the differences between second and third generation DNA sequencing have been discussed in the scientific literature for some time, factors such as single molecule sequencing (SMS), real-time sequencing and no need for template amplification may come to the fore in making this distinction (Karlsson et al., 2015). Similarly, the two platforms listed in the third-generation sequencing can fulfill these parameters. Thus, problems such as transcription errors caused by

amplification, bias complications, and inability to detect DNA modifications have been overcome (Shendure et al., 2017).

In technology developed by Pacific Biosciences (PacBio), DNA polymerization takes place in nanostructures called zero-mode waveguides (ZMWs). Consisting of tiny holes just small enough for a single polymerase and its template to fit, ZMW sends signals that can only be detected by adding fluorescently labeled nucleotides to the growing DNA strand, so that the sequence of a single molecule can be recorded in real time (Dijk et al., 2014; Schadt et al., 2010). It is particularly ideal for de novo genome assembly, although the risk of errors is high.

The second and perhaps the most promising third-generation sequencing approach is nanopore sequencing. Originally designed before second-generation sequencing, this technique is based on the ability of single-stranded DNA or RNA molecules to pass through the ion channel in the lipid bilayer of the cell membrane (Deamer et al., 2016). Founded in 2005, Oxford Nanopore Technologies (ONT) was the first company to commercialize this technology and manufacture nanopore sequencing devices such as MinION, GridION and PromethION. MinION, which can be carried easily due to its small size and can operate by just connecting to a computer, has been the most popular product of the company since its launch in 2014.

As mentioned above, MinION sequencing operates by recording the change in ion current as a real-time signal after translocation of a single-stranded DNA or RNA molecule through protein nanopores in a semiconductor membrane. For this, an adapter is attached to one end of each DNA or RNA that will allow translocation of the molecule across the membrane. This adapter, together with the accompanying enzyme, can unwind the double-stranded structure of DNA and binds to the docking protein, which resides in the nanopore and controls the rate at which the molecule crosses the membrane. A hairpin structure is ligated to the other end, which will allow sequencing of both sense and antisense strands (Karlsson et al., 2015). The changes in the electric current caused by DNA or RNA molecules as they pass through the nanopores are measured and the current profile is presented to the user through a software called Metrichor^{TM}. Molecules that pass through nanopores with both adapter and hairpin are considered as 2D (double stranded; template and complement) reads, molecules that pass only through adapter are considered as 1D (single stranded; template or complement) reads, while molecules that neither structure can pass are excluded from the library preparation (Kerkhof et al., 2017).

Unlike most NGS technologies, MinION does not require expensive equipment, bioinformatics training or special computing facilities, and the ability to perform extremely long reads in real time makes it a reliable option (Madoui et al., 2015). Although the risk of error is higher than Illumina, it becomes more efficient with rapidly developing technology. In fact, one study showed it could detect species with up to 93% accuracy across multiple environmental samples (Brown et al., 2017). In addition, the successful microbial profiling of scientific studies with samples taken from various environmental sources such as desert, tropical sea and fresh water is an indication that MinION can be used as an effective sequencing instrument in environmental microbiology (Curren et al., 2019; Kerkhof et al., 2017; Latorre-Pérez et al., 2021; Ma et al., 2017; Urban et al., 2021). However, this thesis will be the first scientific study to perform DNA sequencing using MinION from enriched soil samples to the best of our knowledge.

3. AIM OF THE STUDY

The aim of this study is to enrich lignin-digesting, anaerobic microbial communities living in sediments and dunes in the İğneada floodplain forest in Kırklareli province in the Thrace region of Turkiye. For the first time in this thesis, anaerobic lignin degrading microorganisms would be specified in the microbial pool in our country. In addition, microbial ecology of İğneada floodplain forests will be revealed as well with this thesis. Since Turkiye's floodplain forests present enormous microbial diversity, this will be important for not just industry, but also scientific literature.

The objectives of the thesis can be summarized as follows:

- Collection of sediment and dune samples from specific regions of İğneada floodplain forest, namely Lake Hamam and Bulanık Stream
- Enrichment of microbial consortium that can degrade lignin in anaerobic environment
- Closely monitor how microbial communities operate in real time
- Investigation of anaerobic microbial communities responsible for lignin degradation through metagenomic approach
- DNA sequencing of enriched soil samples by using MinION for the first time in scientific literature
- Revealing the anaerobic microbial diversity of İğneada floodplain forests, determination of effective organisms and their quantity within the community and bringing them to literature

4. MATERIALS AND METHODS

4.1. Sample Collection

Samples were collected from the İğneada floodplain forest in Turkiye in October 2021, with the necessary permissions from General Directorate of Nature Conservation and National Parks, the Ministry of Agriculture and Forestry of Turkiye.

The stations to be sampled were determined by evaluating the literature research, previous field work and the results of the analyzes in this study. In the field research conducted in October 2020, samples were taken from 5 different stations from various regions and as a result of the analyzes made, two stations were decided.

Samples were collected from 20 cm depth of Hamam Lake and from 20 cm depth of Bulanık Stream at intervals of several hours on the same day. Sample collection areas are shown in Figure 4.1.

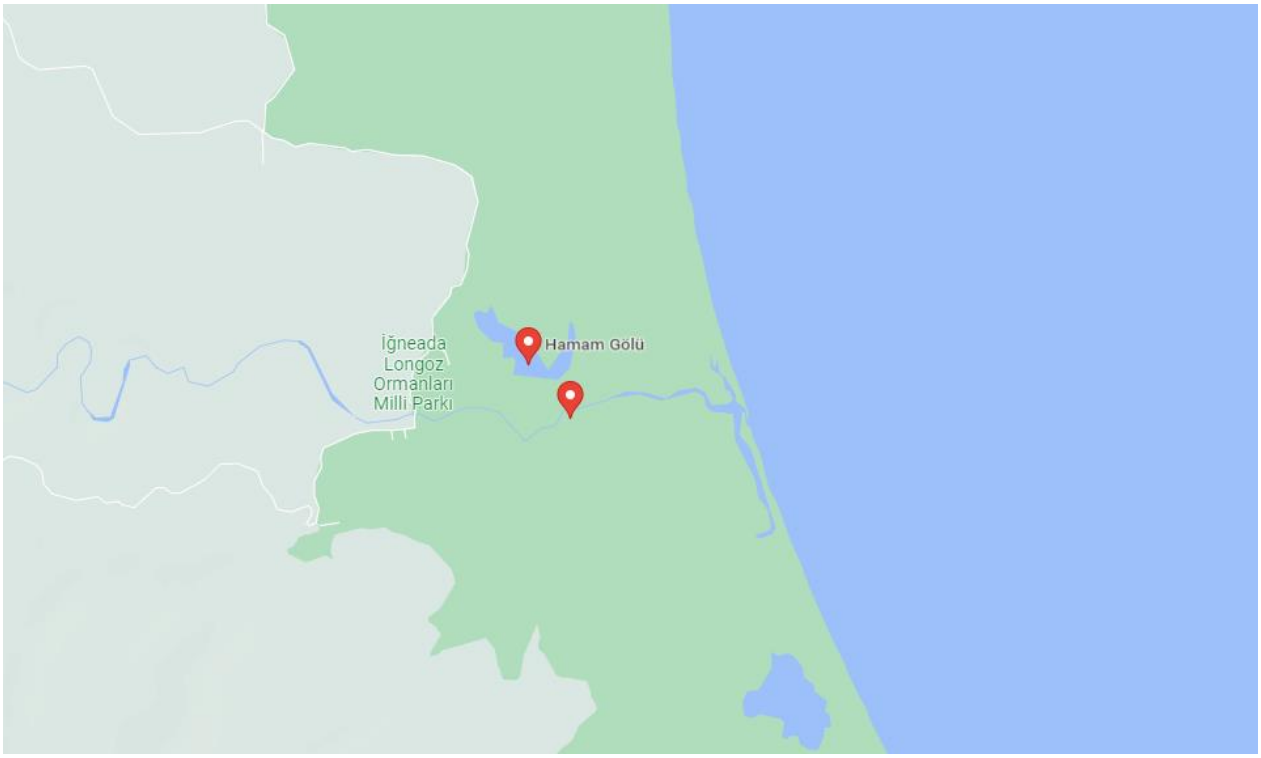

Figure 4.1 Sample collection areas (Hamam Lake at the top and Bulanık Stream at the bottom).

Water temperature and pH as well as dissolved oxygen were noted with a multi-parameter analyzer (Hach Lange, Germany) during sampling. Measurements were made in triplicate for each

sample and average results are presented. Characteristics of water bodies and sample types for both Lake Hamam and Bulanık Stream can be seen in Table 4.1.

Table 4.1 Characteristics of water bodies and sample types

Parameters	**Lake Hamam**	**Bulanık Stream**
Sample Type	Sediment	Dune
pH	7.50	8.10
Water Temperature (^{o}C)	17.2	16.1
Dissolved Oxygen (mg/L)	0.6	0.9

Both sediment and dune samples were taken into 100 ml falcons immediately. The 100 ml falcons were fully loaded with samples, and the remaining blanks were filled with enrichment medium (preparation of enrichment medium will be explained in the next section) prepared in the laboratory before the field work in order to prevent the interaction of the collected samples with oxygen as much as possible. All the falcons were sealed with parafilm to prevent oxygen ingress and placed into an 2,5 L anaerobic jar (Merk Millipore, Germany). In addition, Anaerocult® A mini (Merk Millipore, Germany) was added inside the anaerobic jar to provide anoxic environment. Finally, in order to slow down the metabolism of microorganisms as much as possible, the anaerobic jar was completely covered with ice and placed in an insulated bag, and the samples were immediately transferred to the laboratory to be ready for the enrichment process.

4.2. Enrichment Setup

As soon as the samples were transferred to the laboratory, the processes for the enrichment were started. Enrichment was done as described by Ozbayram et al. (2017) with some adjustments and modified DSMZ medium 1036 was used as in this study.

For the enrichment medium, 0.5 g NH_4Cl, 0.2 g KH_2PO_4, 0.1 g $MgCl_2$ x $6H_2O$, 0.2 g KCl, 2.0 g NaCl, 0.2 g yeast extract were weighed and added to the bottles respectively and then 850 mL high purity water is added to the bottles. 1.0 µml of trace element solution SL10 (DSMZ media 320) and 1.0 ml of resazurin (0.1 g/100 ml stock) were added to the medium and autoclaved (20 min, 121 °C) for the first time.

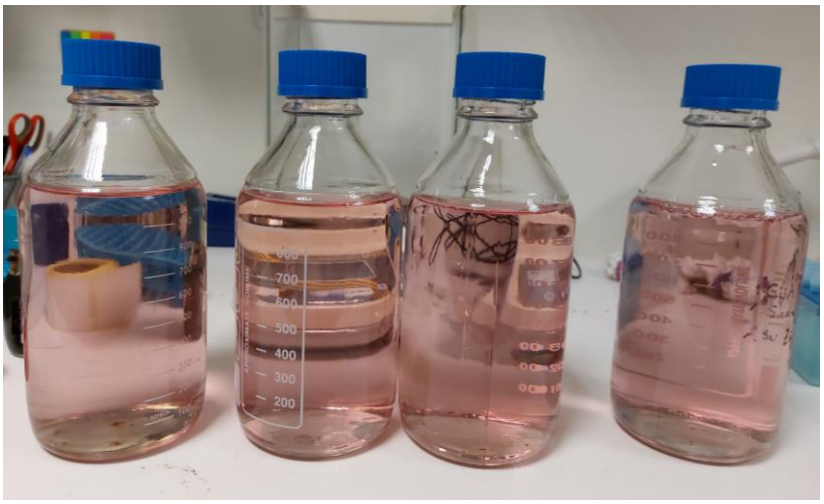

Figure 4.2 Glass bottles with enrichment medium before autoclave.

2.25 g of alkaline lignin (Merck, Germany) was weighed and added to a 250 mL glass bottle and 10-15 mL of high purity water was added to induce some dissolution. After autoclaving (30 min, 121 °C), glass bottles were filled with autoclaved enrichment medium to a total volume of 175 ml and autoclaved once again (15 min, 121 °C). Then, the 250 mL glass bottles were placed in a water bath at 100 °C for approximately 20 minutes under continuous nitrogen flushing to remove dissolved oxygen and were closed with a bromobutyl rubber stopper (Duran, Germany) as soon as they were removed from the bath and sealed with wheat kimble (Duran, Germany). 0.45 mL of reducing agent (from 60 g Na_2S/1 L stock) was added by a sterile syringe to glass bottles to maintain anoxic condition and stored overnight, making it ready for inoculum.

Figure 4.3 Alkaline lignin with 10-15 mL high purity water before autoclave.

For the first inoculum, 61 to 67 g of sediment or dune sample was added to 250 mL glass bottle including autoclaved alkaline lignin and enrichment medium under continuous nitrogen flush and the

bottles were closed with bromobutyl rubber stopper and sealed with wheat kimble again. Thereafter, all the bottles were flushed with nitrogen for 2-3 minutes to remove the oxygen penetrating inside during inoculum. Two glass bottles were prepared for each station, a total of four glass bottles. Additionally, two glass bottles of negative controls were prepared in the same way as described above but without the addition of any cultures.

Figure 4.4 250 mL glass bottles after first inoculum

All bottles were placed in an incubator at 37°C and a shaking speed of 150 rpm. Enrichment procedure was 50 days with two more inoculation. Therefore, all the samples were operated for 150 days in total.

Figure 4.5 250 mL glass bottles after second transfer.

After the first incubation of 50 days, the second enrichment period was started by transferring 50 ml of inoculum under a continuous stream of nitrogen to the fresh medium and alkaline lignin mixture prepared as described above. Thereafter, all the bottles were flushed with nitrogen for 2-3 minutes to remove the oxygen penetrating inside during inoculum. Samples remaining in glass bottles from first inoculation were filled into 50 mL falcons and stored at -20^{o}C for further analysis. The second transfer was done in the same way as the first, and the enrichment period was terminated after 150 days.

Table 4.2 Enrichment setup throughout all incubation processes

Sample Name	**Abbreviation**	**Enrichment Type**	**Station**	**Alkaline lignin amount (g)**	**Inoculum amount**	**Total volume (mL)**
Negative Control	N.C.-E1	1	-	2.25	--	225
Hamam 20cm	H20cm-E1	1	Hamam Lake	2.25	61-67 g	225
Bulanık 20cm	B20cm-E1	1	Bulanık Stream	2.25	61-67 g	225
Negative Control	N.C.-E2	2 (first transfer)	-	2.25	--	225
Hamam 20cm	H20cm-E2	2 (first transfer)	Hamam Lake	2.25	50 mL	225
Bulanık 20cm	B20cm-E2	2 (first transfer)	Bulanık Stream	2.25	50 mL	225
Negative Control	N.C.-E3	3 (second transfer)	-	2.25	--	225
Hamam 20cm	H20cm-E3	3 (second transfer)	Hamam Lake	2.25	50 mL	225
Bulanık 20cm	B20cm-E3	3 (second transfer)	Bulanık Stream	2.25	50 mL	225

4.3. Analytical Measurements

Total solids (TS) and total volatile solids (VS) were carried out according to standard methods (Rice & Baird, 2017). pH was recorded by Hach, Pocket Pro+ pH meter. Lignin content in the glass bottles were determined according to Standard Forage Analysis after every enrichment process (Goering et al., 1970).

PM-9107 7000 mbar manometer (Lutron Electronic Enterprise Co., LTD, Taiwan) is used for measurement of biogas production. The pressure in the headspace of the bottles were relieved after every measurement by using a sterile needle. After the gas discharge, the upper surface of the glass bottles was covered with vaseline to prevent oxygen from entering the tiny holes drilled in the rubber stopper. Cumulative biogas production was calculated using Equation (1) below:

$$V_{biogas} = \frac{\Delta P \times V_{headspace} \times C}{R \times T} \quad (1)$$

where V_{biogas} is the volume of produced biogas (mL), ΔP is the difference of measured pressure (kPa), $V_{headspace}$ refers to the volume of the headspace, C is the molar volume of ideal gas (22.41 L mol^{-1}), R is the universal gas constant (8.314×10^4 mbar cm^3 mol^{-1} K^{-1}), and T is the standard temperature in Kelvin (Schroeder et al., 2022a).

Approximately 0.5 mL gas was removed carefully from the headspace of the bottles using 1 mL air-tight syringe (Hamilton, USA) and loaded into HP Agilent 6850 Gas Chromatograph with a thermal conductivity detector (HP Plot Q column 30 m 0.53 mm) immediately to deter biogas composition. Helium was used at a rate of 2 mL/min as carrier gas and oven temperature was 70°C during the measurements.

For volatile fatty acid analysis, 2 mL of liquid sample was taken from glass bottles using a sterile syringe and transferred to 2 mL tubes. Tubes were centrifuged at 14000 rpm for 2-3 minutes. 10 N phosphoric acid was added to supernatant with 1:10 dilution rate in order to fix all biological activity. Then, the supernatants were filtered through 0.22 μm pore size membrane filter and VFA concentration was determined by a gas chromatograph (GC-2025, Shimadzu Co., Japan) equipped with an auto injector (AOC-20i, Shimadzu Co., Japan). N_2 was the carrier gas connected to the instrument and 1 μL gas sample was injected by 0.5 mL syringe. For the calibration, 10 mM standard mix including acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproic, isocaproic and heptanoic acids (Sigma-Aldrich, St. Louis, MO) was used. Total acetic acid concentration was calculated according to COD (Chemical oxygen demand) equivalent of each VFA which are 1.067 g COD/g acid for acetic acid, 1.514 g COD/g acid for propionic acid, 1.818 g COD/g acid for *iso*-butyric and butyric acids, 2.039 g COD/g acid for *iso*-valeric and valeric acids, 2.207 g COD/g acid for *iso*-caproic and caproic acids (Atasoy et al., 2020).

Biogas production, pH measurements, gas composition analysis along with VFA analysis were performed every 7 days during the whole enrichment process. pH measurement and sample collection for VFA analysis was also made immediately after the enrichments were set up.

4.4. Metagenomic Analysis

4.4.1. DNA Isolation

Total genomic DNA of raw samples as well as samples taken after each transfer is analyzed by using Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA) and a ribolyser (Fast PrepTM FP120 Bio 101 Thermo Electron Corporation, Belgium) according to manufacturer's protocol with minor adjustments.

Approximately 250 mg of soil sample was added to a ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm). Then, 400 µl BashingBead™ Buffer was added to the tube. Tubes were secured in a bead beater fitted with a 2 mL tube holder assembly and vortex at 2000 rpm for 40 minutes. Samples were placed in a heat block at 90°C for 10 minutes. ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm) was centrifuged at 13.000 x g for a minute. 400-500 µl supernatant was transferred to a Zymo-Spin™ III-F Filter in a Collection Tube and centrifuged at 8,000 x g for 1 minute. 1.2-1.5 mL Genomic Lysis Buffer was added to the filtrate in the Collection Tube and mixed vigorously. 800 µl of the mixture was transferred into a Zymo-Spin™ IICR Column4 in a Collection Tube and centrifuged at 10,000 x g for 1 minute. Flow through was discarded from the Collection Tube and 1.2-1.5 mL Genomic Lysis Buffer was added until the mixture is completely filtered. 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin™ IICR Column in a new Collection Tube 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 at 10,000 x g for 1 minute. Zymo-Spin™ IICR Column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl (pre-heated to 50°C) DNA Elution Buffer was added directly to the column matrix. Tubes were centrifuged at 10,000 x g for 30 seconds to elute the DNA. Zymo-Spin™ III-HRC Filter is placed in a clean Collection Tube and 600 µl Prep Solution was added immediately. Tubes were centrifuged at 8,000 x g for 3 minutes. The eluted DNA was transferred to a prepared Zymo-Spin™ III-HRC Filter in a clean 1.5 ml microcentrifuge tube and centrifuged at exactly 16,000 x g for 3 minutes.

4.4.2. PCR Amplification

The method previously followed by Cuscó et al. (2019) was applied for metagenomic analysis. The primer pair targeting a region of approximately 1450 bp spanning the V1-V9 region of the 16S rRNA gene will be used to construct amplicon libraries (Klindworth et al., 2013; Zeng et al., 2013). Oxford Nanopore Technologies Nanopore barcode DNA sequences of the constructed library were added to the 5' end of the target-specific primer pairs. Forward primer was TTTCTGTTGGTGCTGATATTGC - AGRGTTTGATYHTGGCTCAG -3' and reverse primer was 5'-ACTTGCCTGTCGCTCTCTCTCTTCTC - TACCTTGTTAYGACTT -3' for the target-specific primer binding sequences specific to 16S rRNA. Evidence Read DNA Polymerase 2x Reaction Mix and 200 nm from each primer were used for initial PCR.

The thermal cycling program for following PCR was: 3 minutes at 95°C; 25 cycles for 30 seconds at 95°C, 30 seconds at 55°C, and 90 seconds at 72°C; 5 minutes at 72°C. After that, amplicon was run on an agarose gel to confirm its size (~1450 bp) and purified using the PCR Product Purification Kit.

Table 4.3 Primer pairs used for PCR

Oligo Name	ONT Universal Tag	Sequence (5' -> 3')	Amplicon
16S-27F	TTTCTGTTGGTGCTGATATTGC	AGRGTTTGATYHTGGCTCAG	16S
16S-1492R	ACTTGCCTGTCGCTCTATCTTC	TACCTTGTTAYGACTT	16S

4.4.3. Library Preparation

During the study of the sample, Ligation sequencing kit 1D (SQK-LSK108; Oxford Nanopore Technologies) was used with DNAs and the DNA library was loaded into the MinIONTM (Oxford Nanopore Technologies). The device was loaded with 45 µl of barcoded DNA mix with 1–1.5 µg DNA content and 5 µl of phage DNA on a slide as a positive control. The NEBNext End Repair/dA-tailing Module (New England Biolabs) kit was used for DNA end repair and dA splicing. Agencourt AMPure XP beads (Beckman Coulter) kit was used for purification.

For the adapter ligation step, a total of 0.2 pmol tips were prepared, DNA was added to 50 µl of Blunt/TA ligase master mix (New England Biolabs) and 20 µl of adapter mixture was added and incubated for 10 minutes at room temperature. Final purification to obtain the cDNA library was completed using Adapter Bead Binding buffer (provided in the SQK-LSK108 kit) and 0.5X Agencourt AMPure XP beads (Beckman Coulter) kits.

The sequencing mix (14 µl of DNA library) is mixed with Loading beads (25.5 µl) and Running Buffer mix (35.5 µl). The R9.4 flow cell to be used was primed to make it ready for loading and the prepared sequencing mixture was transferred to the sample loading section of the flow cell. A 48-hour (R9.4) sequencing protocol was performed using MinION™ control software, MinKNOW™ version 0.46.1.9 (R9.4). Read data was obtained based on 1.2.2 rev 1.5 workflow and software Metrichor™ agent (version 0.16.37960).

4.4.4. Bioinformatic Analysis

After sequencing, the results obtained in fast5 format were converted to fastq format using latest version of guppy software (base-calling and de-multiplexing). Barcode and adapter sequences were cleared using guppy software, and universal primers and tags were also deleted by deleting 15 bases from both ends of the sequences. After clearing the sequences, reads 1000-2000 bp long were filtered with Trimmomatic, and the remaining reads were excluded from the analysis.

The cleaned reads were analyzed with a customized workflow using the python programming language. With this workflow, each sequence was matched with the BLAST algorithm during the filtering process. A .biom file was created by taking taxonomic data of sequences with more than 40% coverage and 60% similarity in sequence matching in the results. In order to perform phylogenetic analyzes with the created .biom file, krona plot, abundance analysis and alpha diversity analyzes, were performed together with the tools provided by the qiime2 platform. The graphics and tables in the analyzes were made with the libraries of the python programming language.

4.4.5. Statistical Analysis

Experimental data are expressed as the mean ± standard deviation of the triplicate measurements. One-way-analysis of variance (ANOVA) was conducted using SPSS 21 software, and statistical significance was assumed at a level of ($p < 0.05$). The differences in the microbial community dynamics were evaluated by principal coordinates analysis (PCoA) in Fast UniFrac (http://bmf.colorado. Edu/fastunifrac/).

5. RESULTS AND DISCUSSION

The aim of the thesis was detection of lignin-degrading anaerobic microbial communities from sediment and dune samples collected from İğneada floodplain forest in Kırklareli province in the Thrace region of northwest Turkiye. For that purpose, sediment and dune samples were processed with DSMZ medium 1036 as well as alkaline lignin in oxygen-free environment at 37^{o}C in order to enrichment of microbial communities of interest. Inoculums were transferred twice and physiological characteristics including biogas production, pH, gas composition, VS and VFA analysis were monitored through the whole enrichment cycle. DNA isolation of samples was performed according to manual of standardized isolation kit and genetic material was sequenced by using MinION. The physicochemical and metagenomic profiles of the microbial community intended to be found were obtained by this way. Thus, it was aimed not only to provide isolation and characterization of microbial organisms that can digest alkaline lignin in an anaerobic environment, but also to propose an inexpensive and effective guideline for the isolation and identification of such organisms from environmental samples.

5.1. Physiological Characterization of Enrichment Cultures

5.1.1. TS, VS and VS Removal

After the collection of the samples from İğneada floodplain forest, TS, VS and VS/TS were measured according to standard methods as mentioned above. Total solid, volatile solid and VS/TS ratio of raw samples can be seen in Table 5.1.

Table 5.1 Total solid, volatile solid and VS/TS ratio of raw samples

Raw samples	TS (% w/w)	VS (% w/w)	VS/TS (% w/w)
Hamam 20cm	63,98	2,66	4
Bulanık 20cm	71,03	0,74	0,11
Alkaline lignin	93,86	33,45	36

Amount of total solid and volatile solid are the highest for alkaline lignin among raw samples. On the other hand, TS value of Bulanık 20cm (B20cm) is bigger than that of Hamam 20cm (H20cm), although the opposite is true for VS, so the VS/TS ratio of H20cm is higher than B20cm. These

measurements were done before enrichment step to adjust ideal inoculum to substrate (I:S) ratio which is suggested between 1:1 to 3:1 for anaerobic digesters (Sri Bala Kameswari et al., 2012). Amount of alkaline lignin as well as sediment and dune samples put into glass bottles were calculated accordingly. I:S ratio within anaerobic digesters is critical since excess inoculum loading may cause problems including VFA accumulation and substrate inhibition (Akyol, 2020).

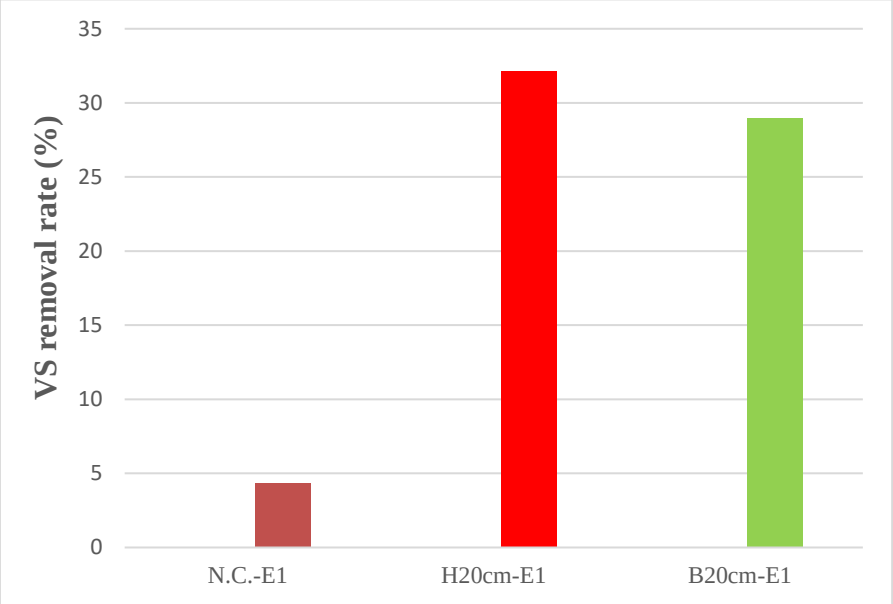

Figure 5.1 VS removal rate (%) for the first enrichment.

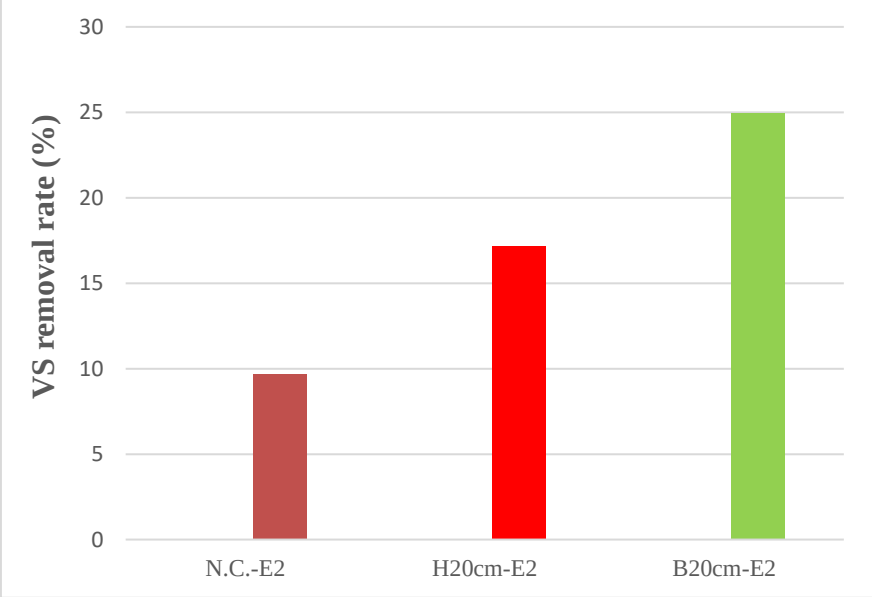

Figure 5.2 VS removal rate (%) after the end of the first transfer.

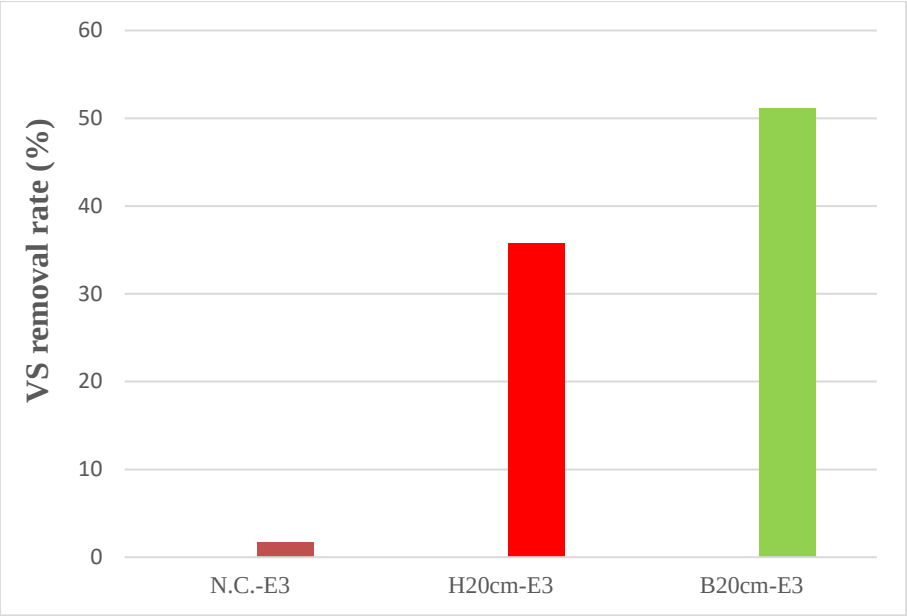

Figure 5.3 VS removal rate (%) after the end of the second transfer.

Amount of volatile solid was measured after each enrichment and then the removal rate was calculated. Figure 5.1. illustrates VS removal rate for the first enrichment while Figure 5.2. and Figure 5.3. show VS removal rate after the first transfer and after the second transfer respectively. VS removal rate for negative controls (N.C.) for all enrichment is under 10% which is acceptable considering two-step autoclave process and the relatively longer treatment time of 50 days. Removal rate is higher for H20cm (32,13%) than B20cm (28,99%) for the first enrichment but the vice versa is applicable for the rest of the enrichment period. The highest VS removal rate is achieved after the end of the second transfer for B20cm (51,11%) while the lowest was recorded for the H20cm with 17,18% after the end of the first transfer.

VS removal rate can be regarded as one of the parameters which provide clues about hydrolysis efficiency of substrate during anaerobic digestion (Hallaji et al., 2019). Hence, it can be said that the best degradation efficiency belongs to B20 after the end of the second transfer and the worst degradation efficiency belongs to H20 after the end of the first transfer in terms of VS removal.

5.1.2. Lignin Content

Alkaline lignin content of the samples was determined according to Standard Forage Analysis as mentioned earlier. All of the samples, of which lignin content is given as TS% value below, were analyzed using the liquid phase collected at the end of the incubation period.

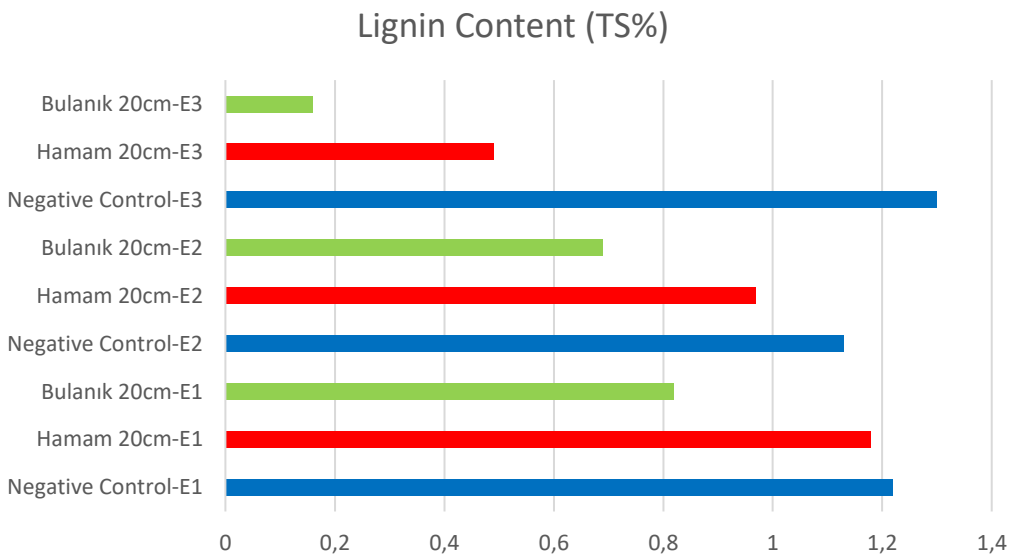

Figure 5.4 Alkaline lignin content of all samples after each enrichment period.

When the negative controls were examined, it was found that the amount of lignin in each enrichment period was higher than in the samples. Only in the first enrichment, H20cm was found to be very close to the negative control in terms of lignin value. This may be due to extra lignin sources from the sediment. Likewise, after the first and second transfers of H20cm, it was determined that it contained much less lignin than the negative controls. Among all samples, the second transfer of B20cm has the lowest lignin content, in other words the one with the highest lignin removal.

When the enrichment performances of the samples were compared among themselves, it was determined that the lignin removal after the second transfer of H20cm was 58% better than the first enrichment, 49% better than the first transfer. Lignin removal for B20cm after the second transfer compared to other incubation periods was 80% and 77%. Therefore, the third enrichment turned out to be more efficient than the second in terms of lignin removal.

5.1.3. Biogas Production

The gas volume in each glass bottle, including negative controls, was measured weekly with a manometer, and then the cumulative biogas production was calculated by adding the values at the end of each enrichment. The total biogas production was converted to ml gas with the aforementioned formula. Cumulative gas production of H20cm and B20cm after the first, second and third enrichment can be seen in Figure 5.5., Figure 5.6. and Figure 5.7. respectively.

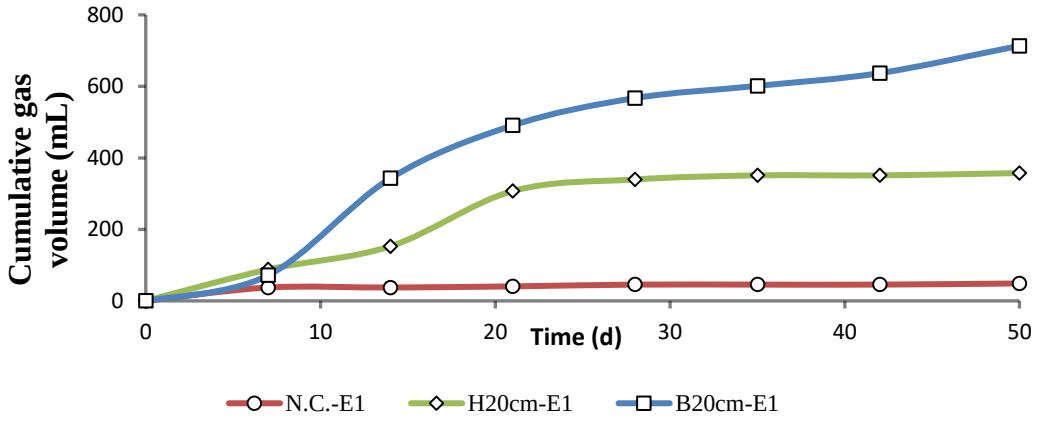

Figure 5.5 Cumulative biogas production of H20cm and B20cm in the first enrichment period in 50 days normalized to standard pressure (101.325 kPa) and temperature (273.15 K).

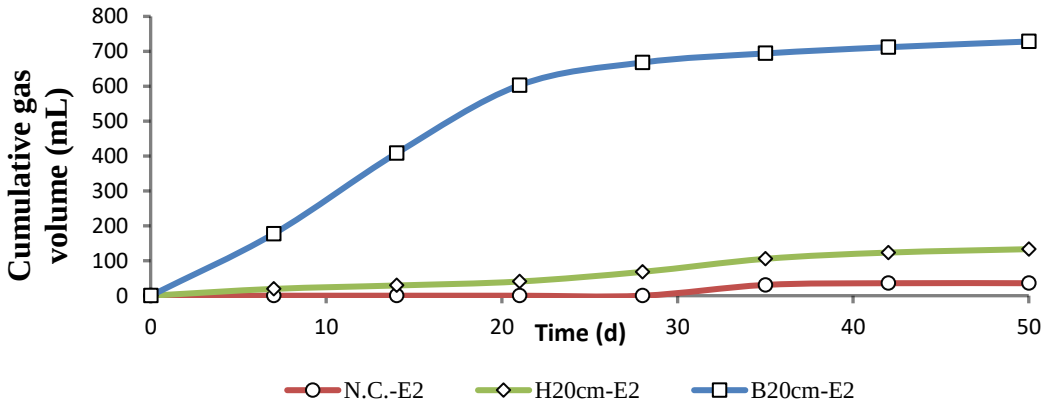

Figure 5.6 Cumulative biogas production of H20cm and B20cm after the first transfer in 50 days normalized to standard pressure (101.325 kPa) and temperature (273.15 K).

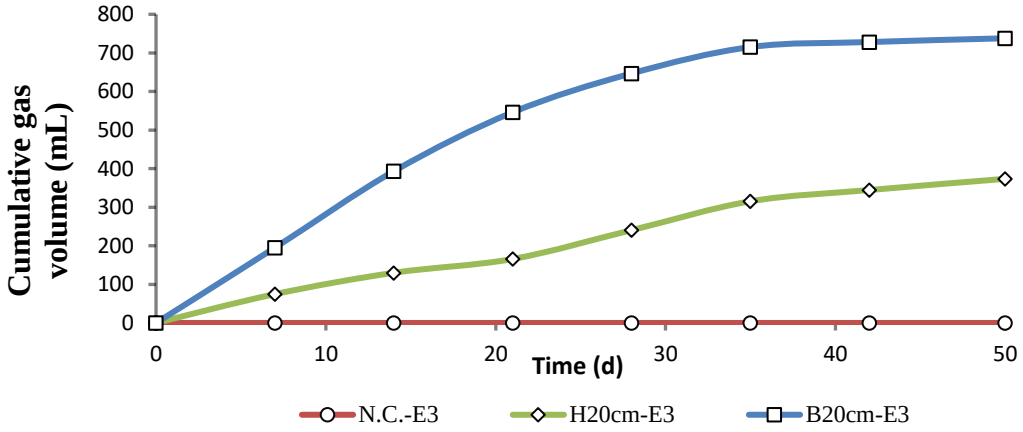

Figure 5.7 Cumulative biogas production of H20cm and B20cm after the second transfer in 50 days normalized to standard pressure (101.325 kPa) and temperature (273.15 K).

Biogas production was observed to occur during the incubation period of almost 50 days. It has been determined that while the production is relatively fast in the first 30 days, it continues even if it slows down significantly in the remaining 20 days, and in some samples, gas output continues even after the 50th day. While the cumulative biogas production was the most at the end of the second transfer of B20cm, the minimum generation was realized at the end of the first transfer of H20cm. In the negative controls, no or negligible amount of biogas production was observed. Therefore, it can be said that the two-step autoclave method applied in the methodology is successful in preventing microbial activity in the control bottles (Ozbayram et al., 2017b; Porsch et al., 2015).

Maximum biogas production in all three enrichments for B20cm occurred in the second week and then gradually decreased. The only exception was during the first enrichment and after the 5th week, the biogas production started to rise again and reached the level of the 4th week on the 50th day. The reason for this can be shown as microorganisms that consume the extra carbon source from the sediment begin to adapt slowly to lignin digestion. On the other hand, there is no similar trend for H20cm. Interestingly, biogas production decreased in the second week in all three enrichment processes, increased again in the first two enrichments, and decreased in the following week in the final enrichment. Afterwards, a reduction in biogas production was observed in the first enrichment and a rise in the other two enrichments, reaching a peak in 4 or 5 weeks, and then entering a period of decline again.

5.1.4. Gas Composition

The gas composition in the headspace was initially the same for all enrichment processes at the beginning because each glass bottles was flushed with nitrogen to render it anaerobic. As time progressed, the content changed with the output of CO_2 and CH_4, and these gases generally reached their maximum levels at the end of the incubation periods.

Below are graphs (Figure 5.8.-5.10.) of the resulting gas composition at the end of each enrichment process.

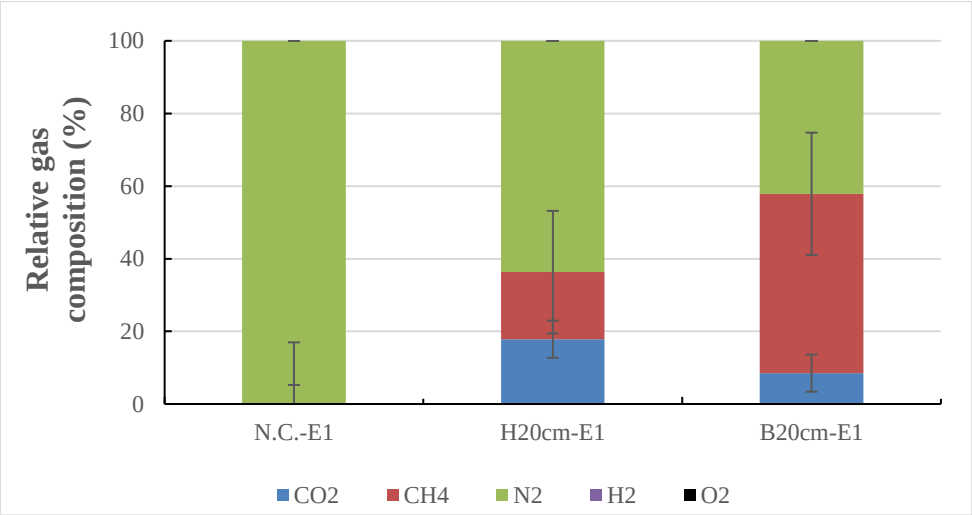

Figure 5.8 Relative gas composition in the headspace after first enrichment.

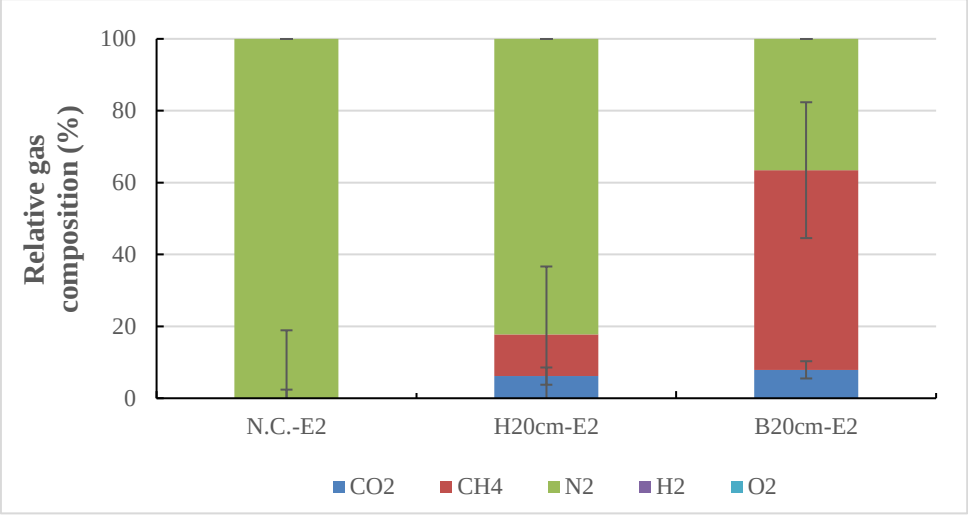

Figure 5.9 Relative gas composition in the headspace after the end of the first transfer.

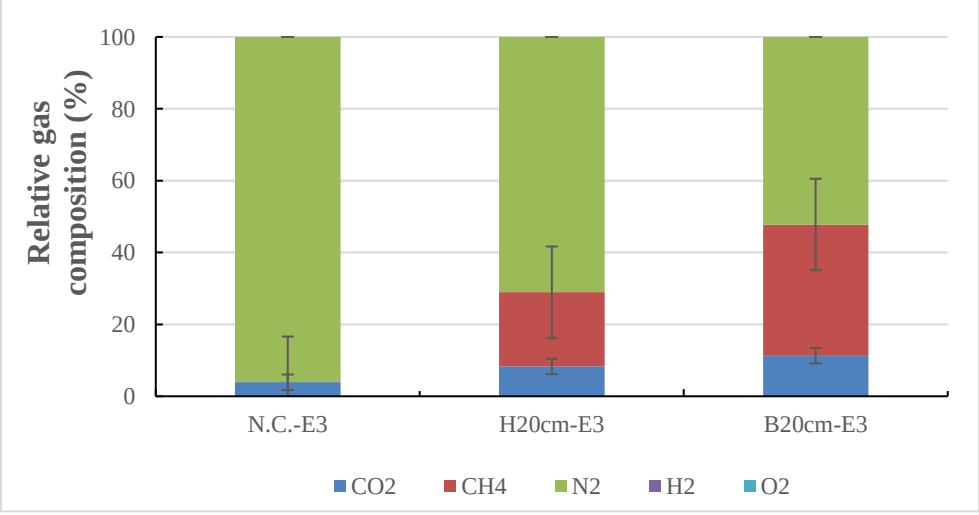

Figure 5.10 Relative gas composition in the headspace after the end of the second transfer.

No methane output was observed in any of the negative controls, while traces of carbon dioxide output were detected in some. The amount of carbon dioxide in the negative controls never exceeded 4%, so it was negligible. Likewise, the presence of similar amounts of carbon dioxide has been reported in various studies (Ozbayram et al., 2018; Schroeder et al., 2022b). This may also be further proof that the two-step autoclave method significantly reduces the risk of contamination in glass bottles.

Except for the first H20cm enrichment, it was determined that the methane ratio was higher than the carbon dioxide ratio in all glass bottles during the incubation period. Therefore, it is possible to say that methanogenic microorganisms are collected and enriched in addition to the anaerobic microbial community that digests lignin. Considering that these two species are in a syntropic relationship in nature, it is clear that this situation is quite normal. The highest methane composition was obtained at the end of the first transfer of B20cm with 55%, while the lowest methane composition was obtained at the end of the first transfer of H20cm with 9%. In the initial enrichment of H20cm, the carbon dioxide content peaked at 17%, while no more than 10% carbon dioxide was detected in any glass bottle for the remainder of the entire process. On the other hand, while the presence of oxygen could not be detected in any of the samples, trace amounts of hydrogen were observed in some glass bottles.

5.1.5. pH

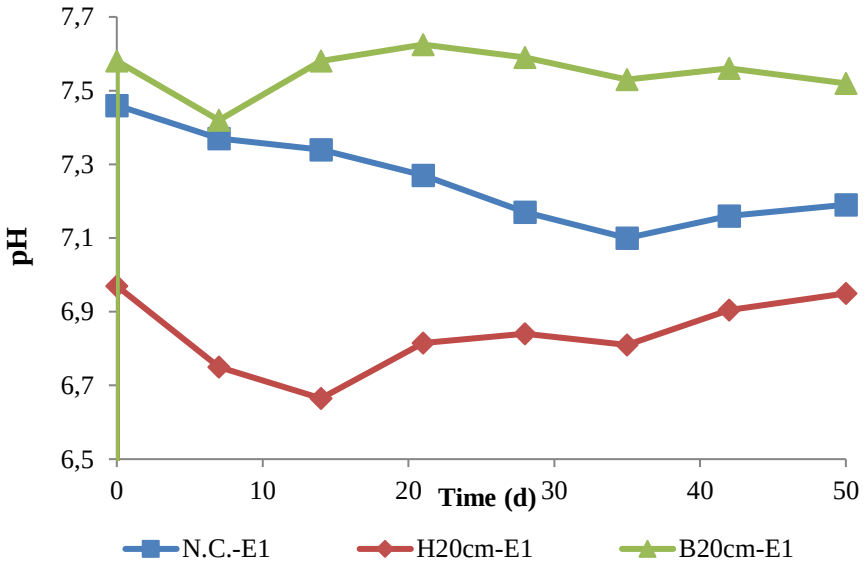

Figure 5.11 Time course of pH values in the first enrichment.

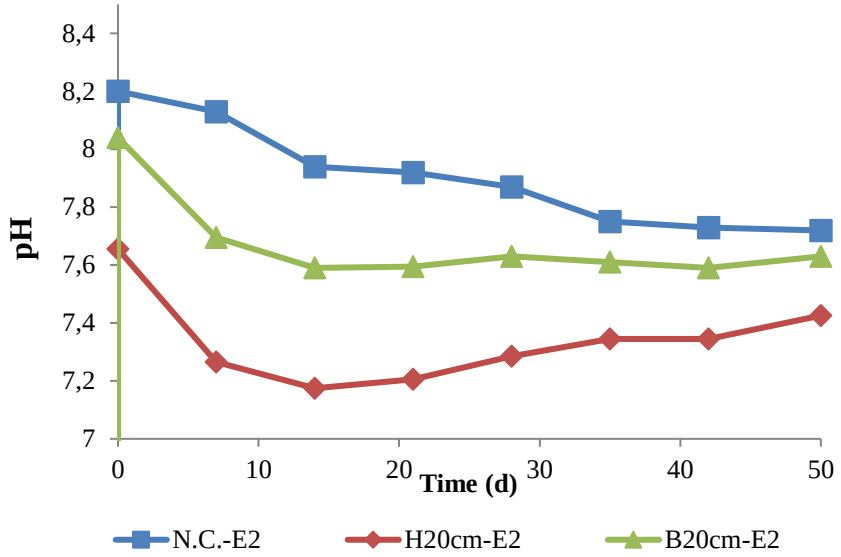

Figure 5.12 Time course of pH values after the first transfer.

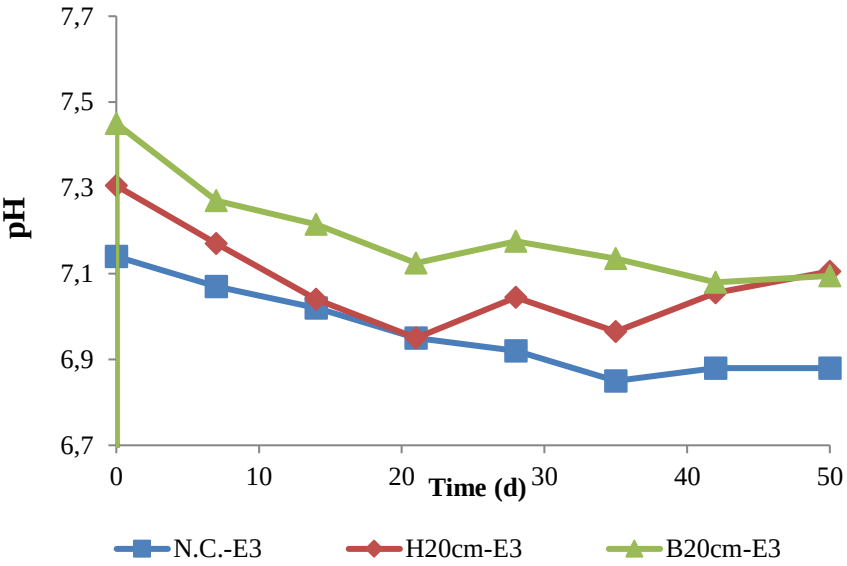

Figure 5.13 Time course of pH values after the second transfer.

The pH values of the entire enrichment process can be seen in the graphs (Figure 5.11.-5.13.) above. In all glass bottles with inoculum, pH values started to decline by the 2nd week, and this trend continued at the 3rd week, with only one exception (B20cm in the first enrichment). The reason for this situation is the accumulation of volatile fatty acids formed during acidogenesis (Kothari et al., 2014). It was observed that the pH values rise again with the increase in the efficiency of methanogens using VFAs as substrates. Likewise, the maximum biogas production and methane production efficiency in most glass bottles increased rapidly, especially in the 2nd and 3rd weeks. However, a correlation between biogas production and pH change was also detected. For example, as mentioned above, there was an unexpected increase in biogas production in the last week of the initial enrichment

of B20cm. Similarly, although the pH values of this sample had increased in the previous weeks, they started to decrease again in the last week.

On the other hand, it was observed that the samples taken from the Hamam Lake showed activity in the pH range of 6.6-7.6, while the samples taken from the Bulanık Stream worked in the range of 7-8 and therefore it can be said that the microorganisms in the samples taken from the Bulanık Stream are better adapted to the alkaline conditions. Correspondingly, the reporting of a similar result in pH measurements made in areas where sediment and sand samples were taken can be shown as an explanation for this situation.

Last but the least, although the pH values for all samples were within the above-mentioned range, it was determined that there were significant fluctuations within this range. The reason for this is that the pH value is not kept at the desired point with the help of any buffer. The fact that the alkaline lignin used as the substrate kept the pH of the medium slightly above 7 may have had a positive effect on the enrichment process, since lignin was reported to be relatively better soluble in alkaline environment (X. Li & Brune, 2005).

5.1.6. VFA Production and Removal

Propionic and butyric acid were the main VFAs in the glass bottles, with acetic acid predominant throughout the entire process. Acetic acid was up to 400 mg/L in some samples, while the highest value that propionic and butyric acids could reach was only around 60 mg/L. Although there are occasional fluctuations in some fatty acids such as isobutyric, isovaleric and valeric, a regular fluctuation trend has not been detected.

During the whole process, the amount of acetic acid enhanced in the first three weeks and reached its maximum point and then entered a downtrend. Although some samples showed small rises in the amount of acetic acid after the third week or remained constant, the values never returned to their maximum values during this time.

The graphs of the acetic acid profiles of all samples (Figure 5.14.-5.16.) can be seen below.

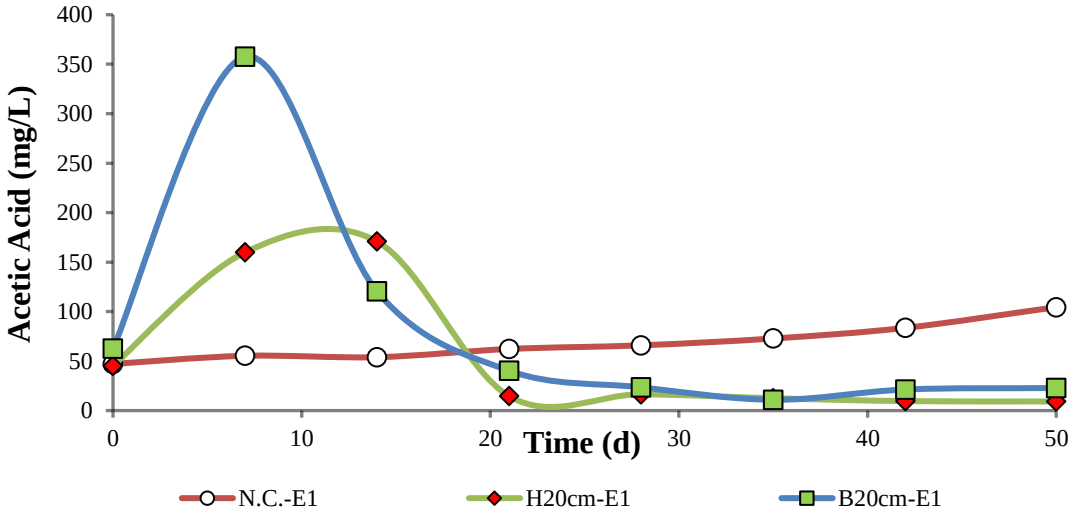

Figure 5.14 Acetic acid concentration in the liquid phase in the first enrichment.

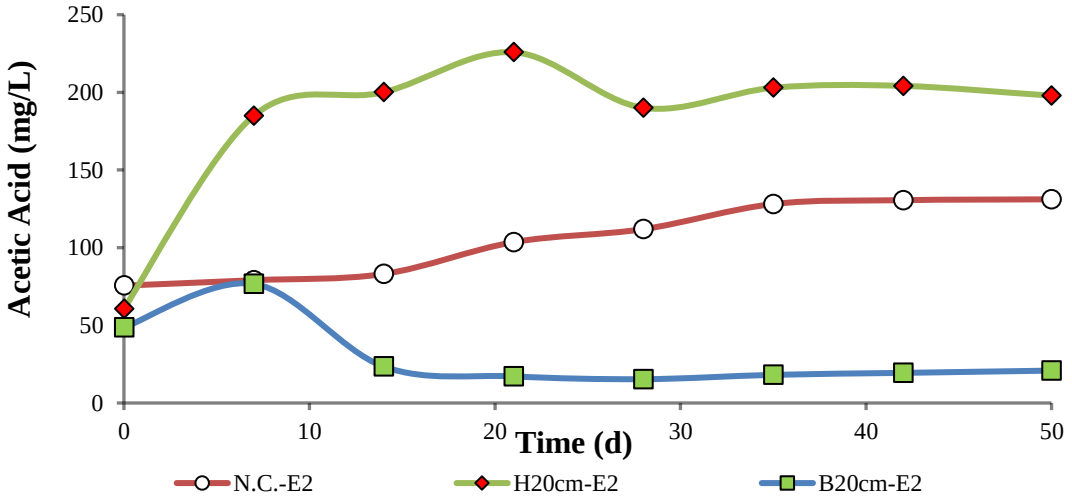

Figure 5.15 Acetic acid concentration in the liquid phase after the first transfer.

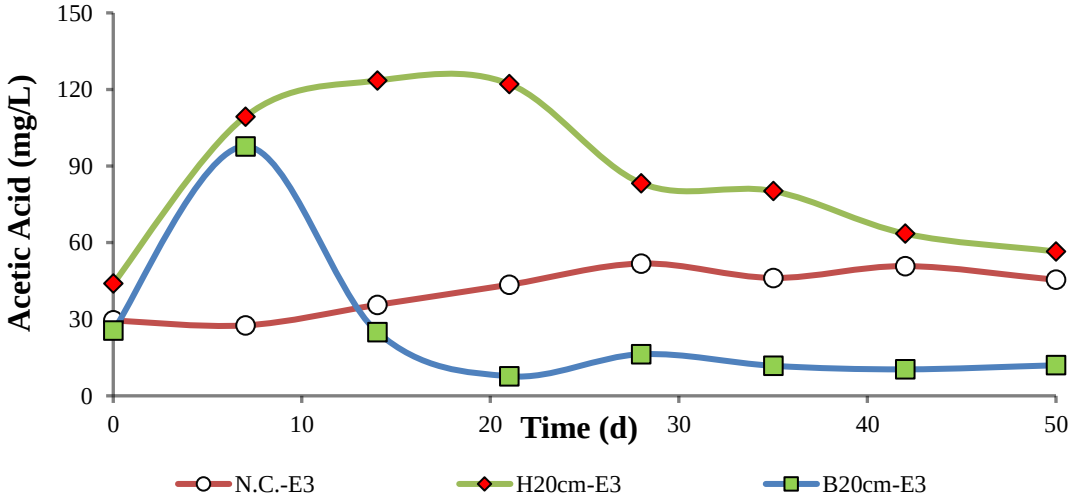

Figure 5.16 Acetic acid concentration in the liquid phase after the second transfer.

Minor increases were seen in the acetic acid content of the negative controls as the weeks progressed, but unlike the inoculum bottles, no significant decrease was noted. Therefore, it cannot be said that there is a digestion as a result of any microbial activity in negative controls. The reason for this situation may be that the lignin dissolves in a small amount due to the effect of temperature and shaker or the alkalinity of the medium during the relatively long enrichment period of 50 days. Likewise, the fact that the increase in the third enrichment of negative control, where the pH changes between 7.1 and 6.9, is less than the other two may be an indicator of this.

The highest amount of acetic acid was approximately 360 mg/L in the first enrichment of B20cm, and the lowest with approximately 80 mg/L after the first transfer of B20cm. The amount of acetic acid for B20cm increased logarithmically in the first 10 days, then decreased rapidly and became almost completely stable from the 40th day. On the other hand, for H20cm, the amount of acetic acid rise between the 10th and 20th days in the first enrichment and then fell, and for the other two enrichments, the upward trend continued until the 20th day and then decreased. Even though it became relatively stable after the 30th day, a complete equilibrium state could not be observed unlike B20cm. It was unexpected that H20cm undergoes remarkably limited acetic acid reduction after the first transfer. This may be due to the partial inhibition of methanogens during the second enrichment due to the relatively high pH.

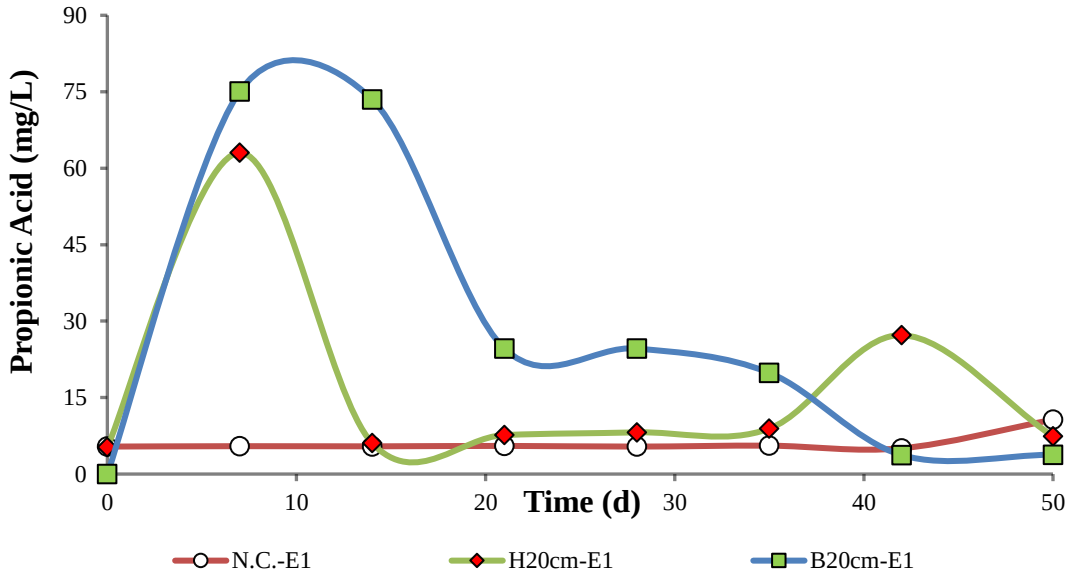

Figure 5.17 Propionic acid concentration in the liquid phase in the first enrichment.

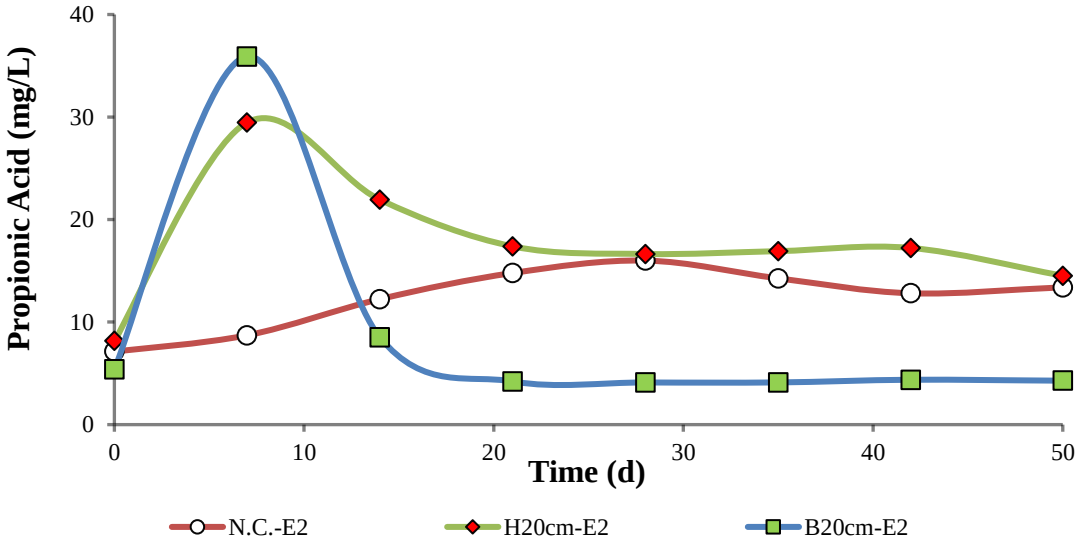

Figure 5.18 Propionic acid concentration in the liquid phase after the first transfer.

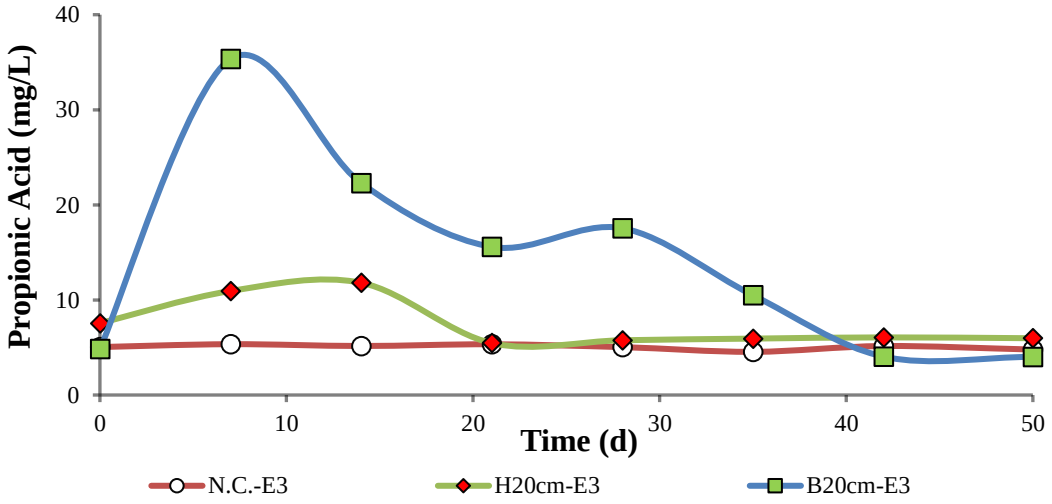

Figure 5.19 Propionic acid concentration in the liquid phase after the second transfer.

When the propionic acid profiles of the samples are examined, it seems that 75 mg/L of B20cm reached in the second week of the first enrichment is the maximum achieved. With one exception (H20cm after the second transfer), the propionic acid concentration began to decline after peaking in the second week and remained on a downward trend with occasional slight increases until the end of the enrichment. Only the significant increase in H20cm at week six during the initial enrichment appears to be an abnormality. However, when the pH and biogas production profiles of this sample in the same period are examined, it is possible to say that this increase can be explained.

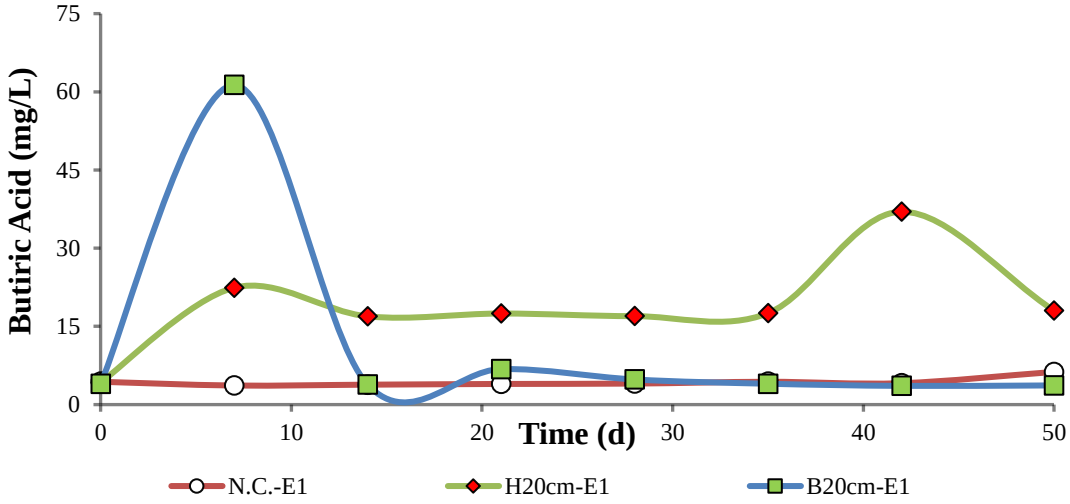

Figure 5.20 Butiric acid concentration in the liquid phase in the first enrichment.

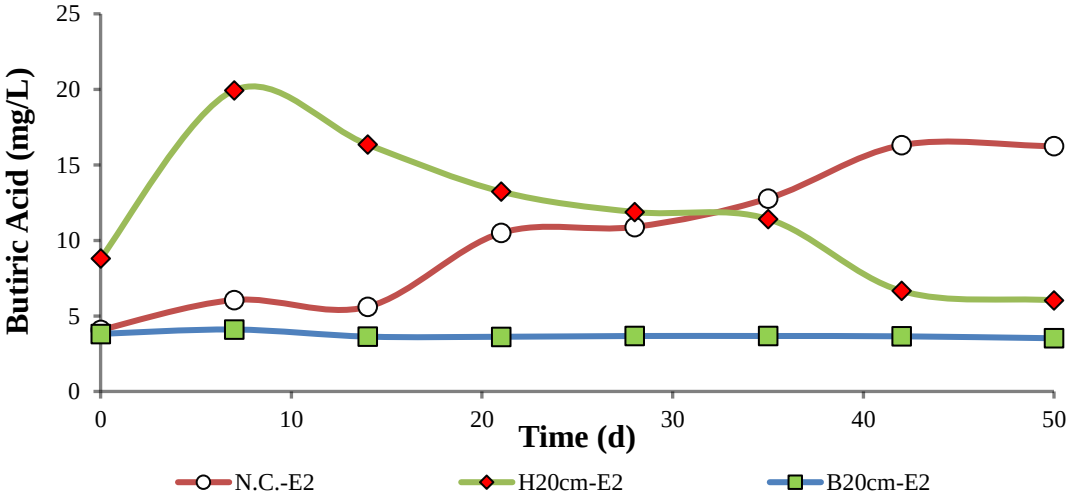

Figure 5.21 Butiric acid concentration in the liquid phase after the first transfer.

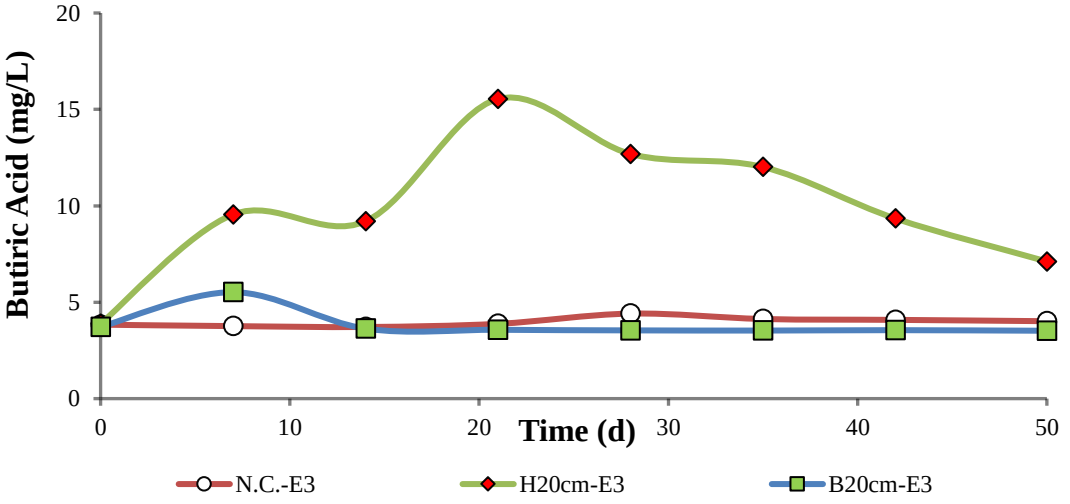

Figure 5.22 Butiric acid concentration in the liquid phase after the second transfer.

Butyric acid, the third most common VFA in this study, remained in less concentration and activity than the other two fatty acids. For instance, the butyric acid values of B20cm remained almost the same after the first and second transfer. However, in the first enrichment of B20cm, it reached the highest concentration (61.31 mg/L) throughout the entire process. This may be because the extra carbon source from the dune has been digested.

On the other hand, H20cm shows a more consistent butyric acid profile. Surprisingly, H20cm, which showed very similar butyric acid values in all three stages, reached its maximum value at week 4, not at week 2, unlike the other two enrichment treatments after the second transfer. Likewise, the amount of butyric acid reached its peak in the second week in all samples except H20cm after the second transfer.

5.2. Microbial Community Composition and Dynamics

After DNA isolation of all incubated sample as well as raw samples, the isolates were sequenced with the MinION system. Then, comparative metagenomic analyzes including Krona analysis, genus based analyzes, similarity analyzes, alpha diversity analyzes, principal component analysis (PCA) based analyzes and beta diversity analyzes were performed.

5.2.1. Krona Analysis

The metagenome is represented as concentric rings forming a circle in the Krona analysis. While each of the rings corresponds to a single taxonomic level, they are represented as part of the ring in proportion to the abundance of that taxonomic level found in the sample (Ondov et al., 2011).

The graphs below show the Krona analysis of sediment and dune samples collected from the İğneada floodplain forests in October 2021.

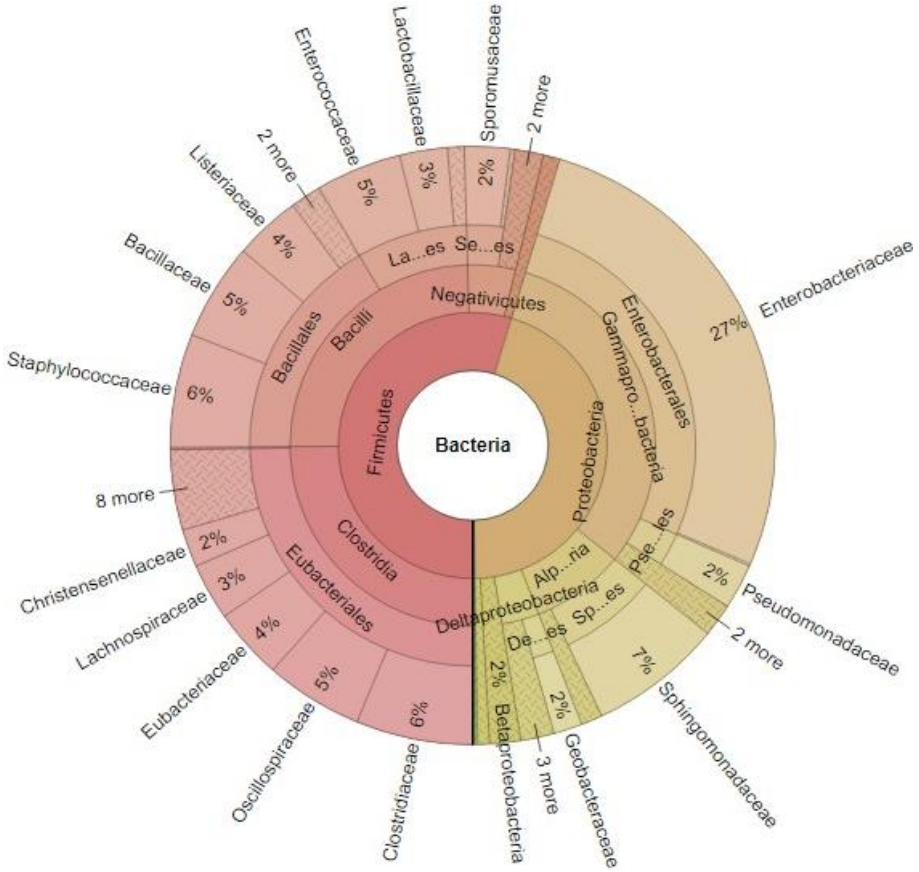

Figure 5.23 The bacterial communities in phylum, class, order, and family levels of the H20cm.

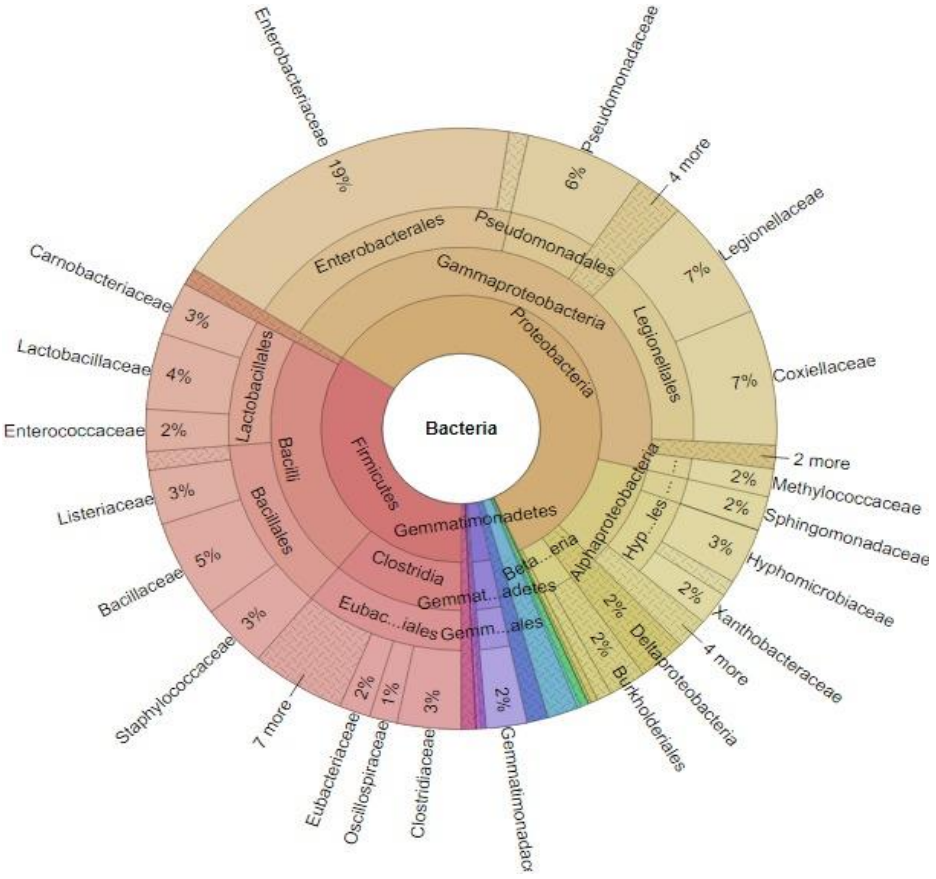

Figure 5.24 The bacterial communities in phylum, class, order, and family levels of the B20cm.

Firmicutes (55%) was the most dominant phylum in sediment samples taken from Hamam Lake, while *Proteobacteria* (59%) were prominent in dune samples taken from Bulanık Stream. The second most common phylum is again *Proteobacteria* and *Firmicutes*, respectively. *Proteobacteria* are one of the most common phyla in freshwater ecosystems (Newton et al., 2011). *Firmicutes*, although not a common phylum in such environments, have been shown to be present in sediment samples taken from the deep (X. Wu et al., 2007).

Surprisingly, the most common class for both stations is *Gammaproteobacteria*, which is rarely seen in these habitats as opposed to *Betaproteobacteria*. There have been reports of this class, including *Enterobacteriales*, which includes many enteric microorganisms, temporarily entering the ecosystem through anthropogenic or zoonotic sources (Zwart et al., 2002). Likewise, the prevalence of opportunistic pathogens such as *Salmonella enterica*, *Escherichia coli* and *Pseudomonas aeruginosa* at both stations is an indication of this (Harris & Brooks, 2013; W. Wu et al., 2015). In addition, the predominance of *Proteobacteria* and *Actinobacteria* in Hamam Lake in our previous field studies can be shown as proof of this. (Özbayram et al., 2021).

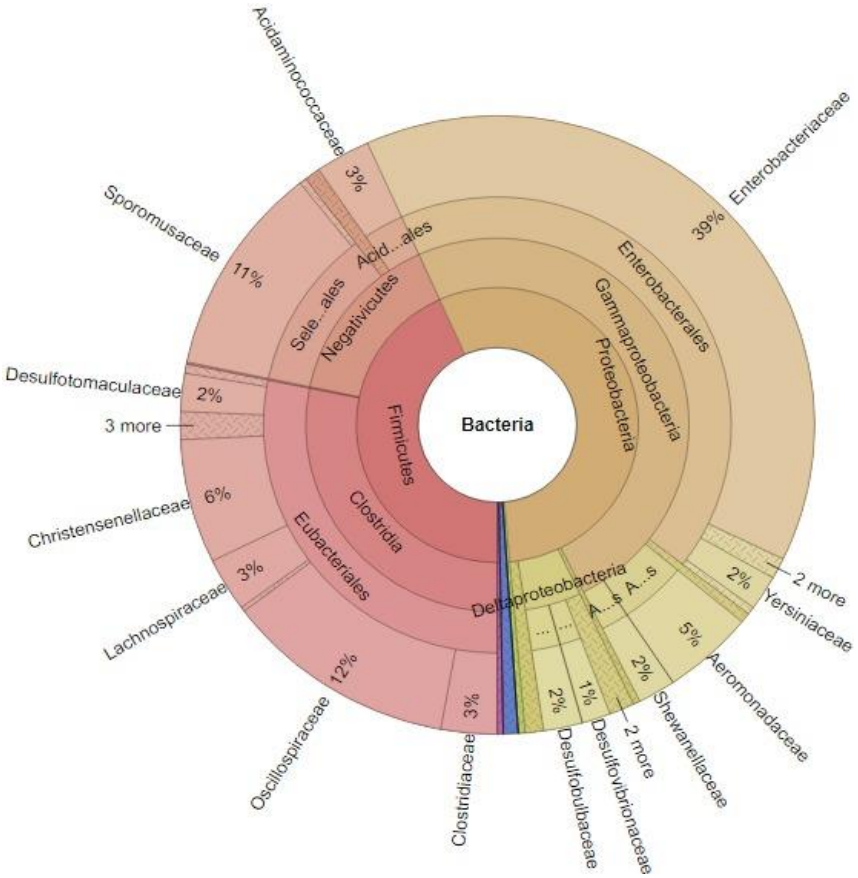

Figure 5.25 The bacterial communities in phylum, class, order, and family levels of the H20cm after the first enrichment.

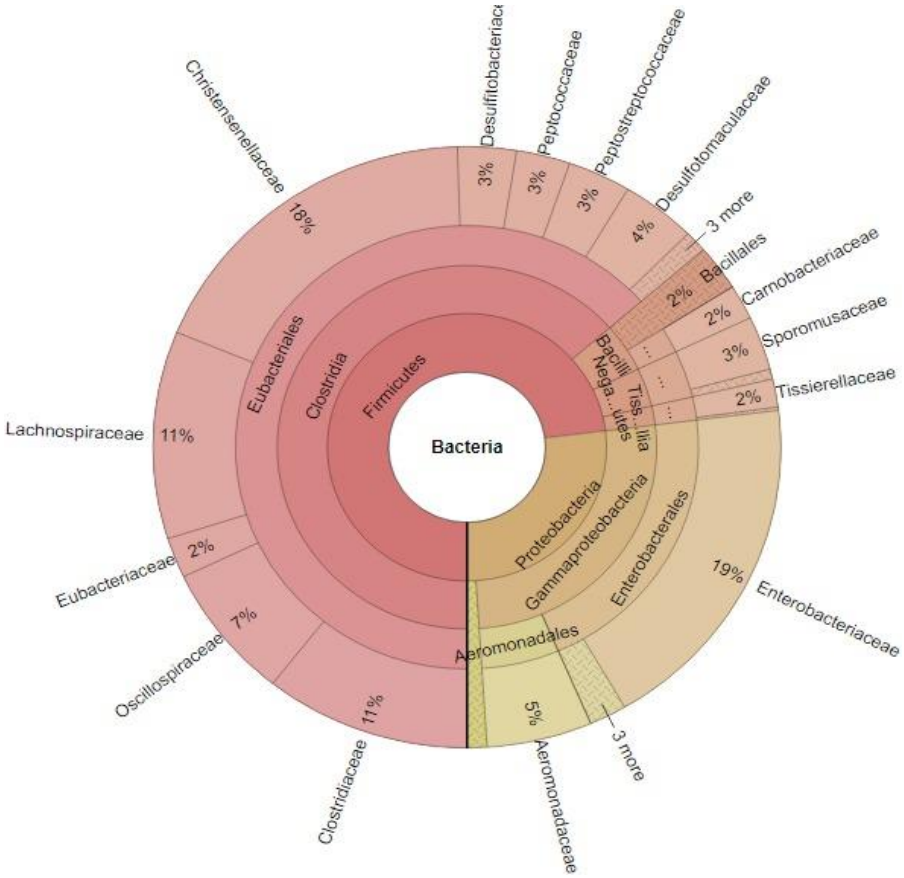

Figure 5.26 The bacterial communities in phylum, class, order, and family levels of the B20cm after the first enrichment.

After the initial enrichment period, the common phyla for both stations were *Proteobacteria* for H20cm and *Firmicutes* for B20cm, in contrast to the crude samples. Even though the prevalence of *Gammaproteobacteria* increased even more for H20cm, when we looked at the species basis, it was determined that pathogens such as *Salmonella enterica*, *Escherichia coli* and *Pseudomonas aeruginosa* started to disappear gradually. They have been replaced by *Enterobacter asburiae* and *Enterobacter roggenkampii*, again from the *Enterobacteriales* class. *Enterobacter asburiae*, a facultative anaerobe, is known in nature to digest polyethylene plastic, even if it has harmful effects on the human body (Sato et al., 2016). *Enterobacter roggenkampii*, on the other hand, takes part in the nitrogen cycle in the soil and also has the cellulase enzyme (D. J. Guo et al., 2020).

For B20cm, 64% of the total readings were assigned to *Clostridia*. The most prominent family of the class, which consists entirely of the order *Eubacteriales*, is *Christensenella*. This family generally includes oxygen-tolerant anaerobic bacteria (Ndongo et al., 2016a). In addition, *Anaerocolumn*a taxa containing anaerobic species, which are generally isolated from methanogenic reactors and can degrade cellulose and predicted to digest hemicellulose, were found to be significantly enriched (Kim et al., 2021; Ueki et al., 2016).

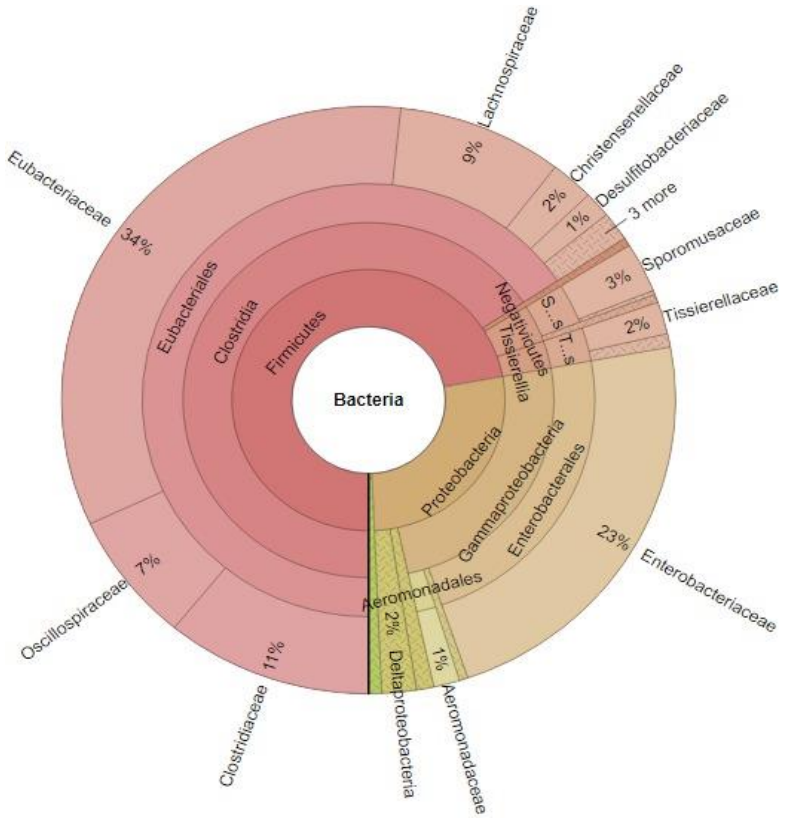

Figure 5.27 The bacterial communities in phylum, class, order, and family levels of the H20cm after the first transfer.

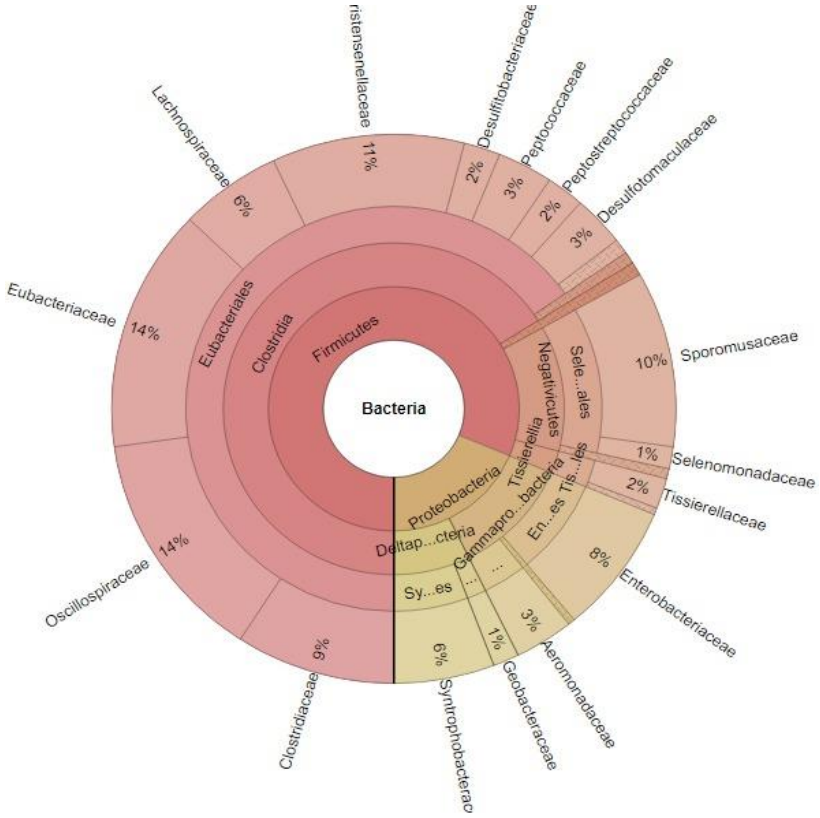

Figure 5.28 The bacterial communities in phylum, class, order, and family levels of the B20cm after the first transfer.

After the first transfer, the dominant phylum of H20cm differentiated once more, as in the raw sample, *Firmicutes*, while for B20cm, as in the previous incubation, *Firmicutes* stood out and even increased its dominance by assigning 81% of all readings. As a result, the share of *Gammaproteobacteria* decreased further and the pathogens seen in the sediment or dune almost completely disappeared. The most dominant class and order in both stations were *Clostridia* and *Eubacteriales*, respectively.

One of the most striking points after the second enrichment is that *Alkalibacter sp. ES005* has been revealed as the dominant species. This species, which assigns 31% of all readings for H20cm and 13% for B20cm, came to the fore as the dominant species after the first transfer, even though it was found in small amounts in the raw samples and after the first enrichment.

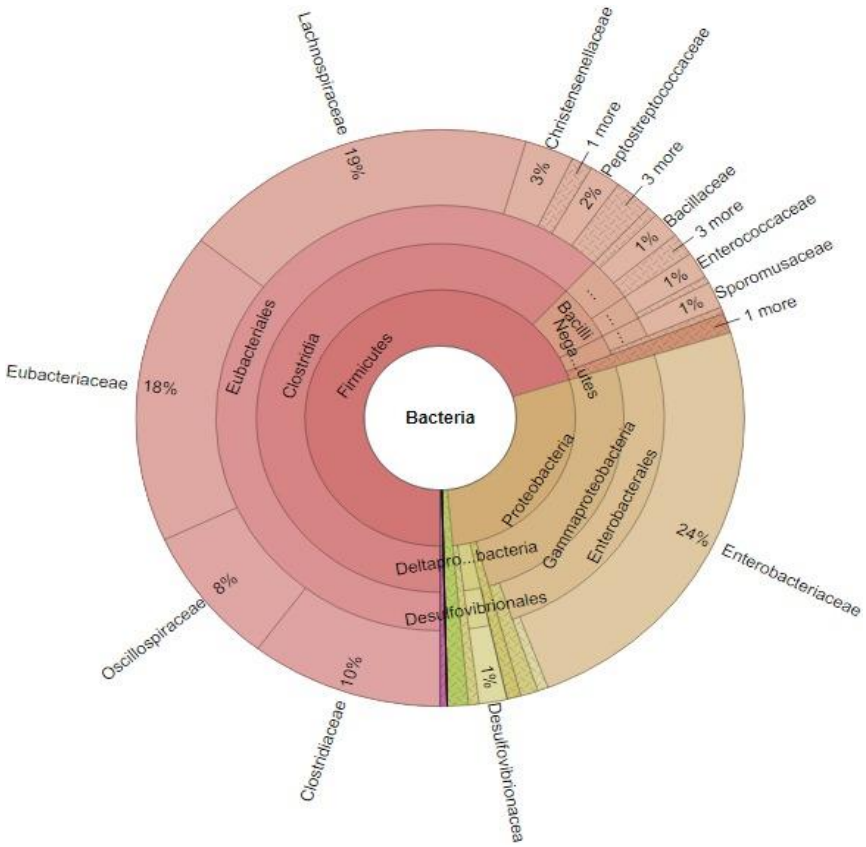

Figure 5.29 The bacterial communities in phylum, class, order, and family levels of the H20cm after the second transfer.

On the other hand, it has been determined that the ratio of obligate or oxygen tolerant anaerobic microorganisms such as *Methylomusa anaerophila*, *Anaerotignum propionicum*, *Geosporobacter ferrireducens* and *Clostridium argentinense* in various phyla, as well as anaerobic cellulose-digesting species such as *Acetivibrio thermocellus*, is increasing among all bacteria (Amano et al., 2018; Halpin

et al., 2017; Hong et al., 2015; Ueki et al., 2017a; Wang et al., 2022). This may be an indication that anaerobic conditions are successfully maintained during the incubation period and the microbial community may slowly adapting to lignin digestion.

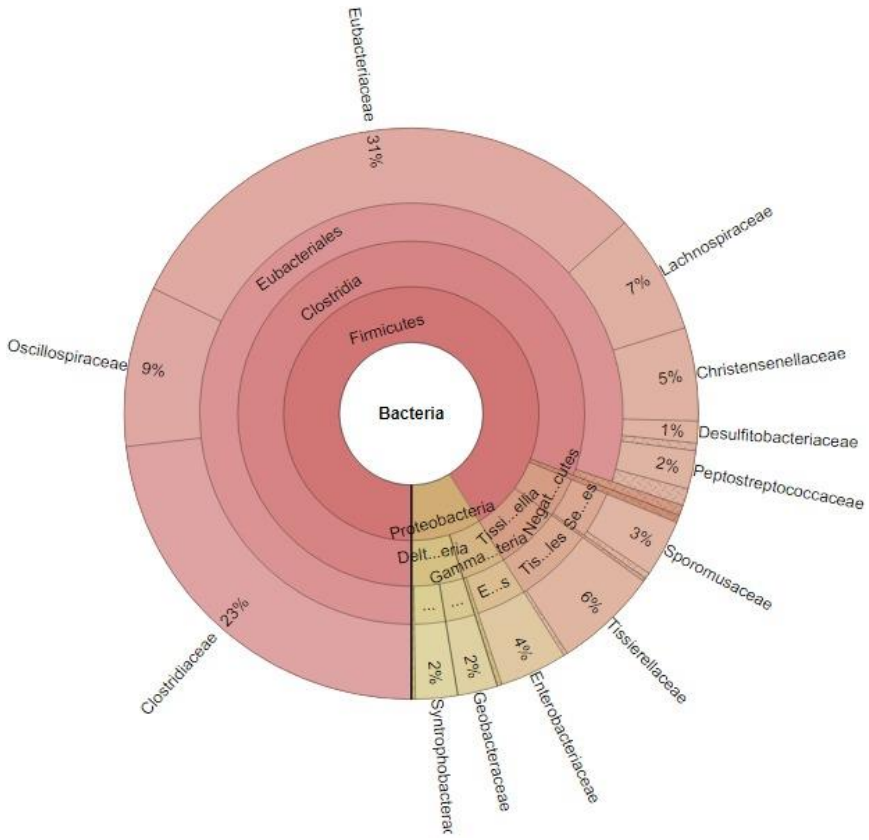

Figure 5.30 The bacterial communities in phylum, class, order, and family levels of the B20cm after the second transfer.

After the second transfer, *Firmicutes* remained the dominant phylum for both stations, while it remained almost the same for H20cm and enhanced from 81% to 91% in all bacteria for B20cm. As in the previous transfer, *Clostridia* and *Eubacteriales* stood out, while the share remained almost the same for H20cm, but increased even more for B20cm.

When metagenomic data are analyzed on the basis of species, it is revealed that *Alkalibacter sp. ES005* is in the first place for both of the samples. Assigning 30% of all readings for B20cm and 15% for H20cm, this strain is in the genus *Alkalibacter* with only a few known species. *Anaerotignium propionicum* was the second dominant species, representing 13% of the bacterial community, while *Clostridium perfringens*, representing 19% of the bacterial community, was the second most abundant species.

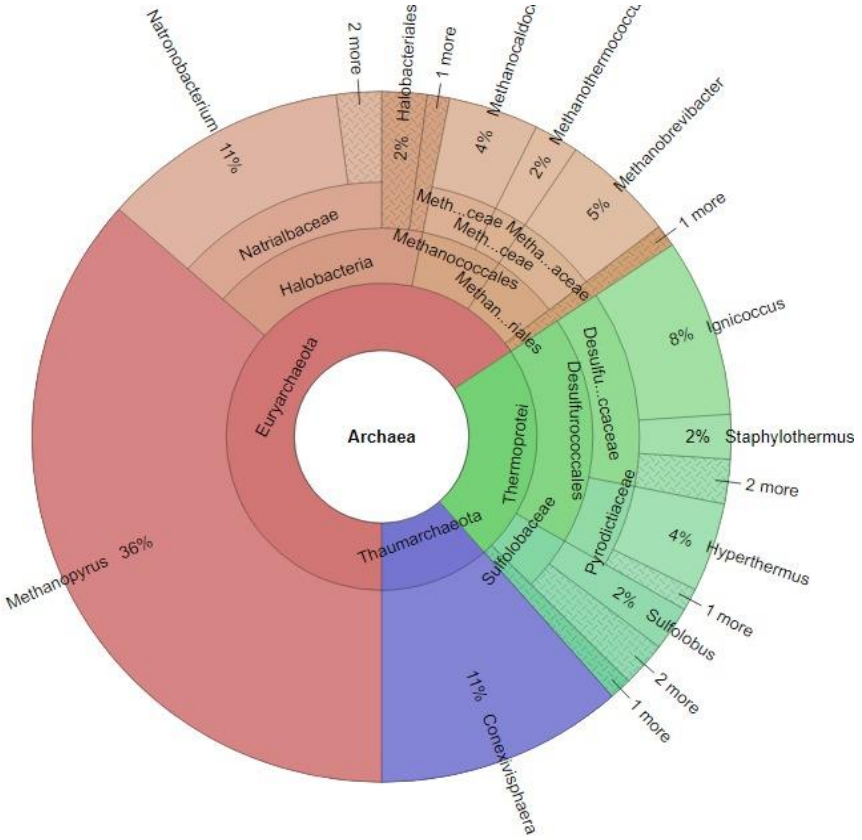

Figure 5.31 The archaeal communities in phylum, class, order, and family levels of the H20cm

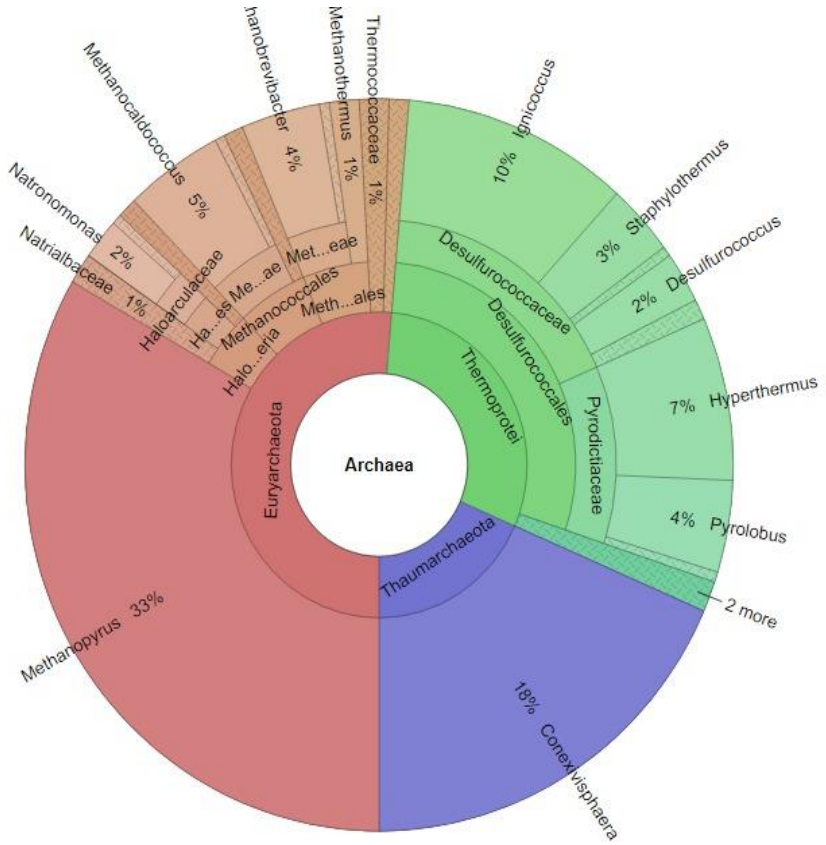

Figure 5.32 The archaeal communities in phylum, class, order, and family levels of the B20cm

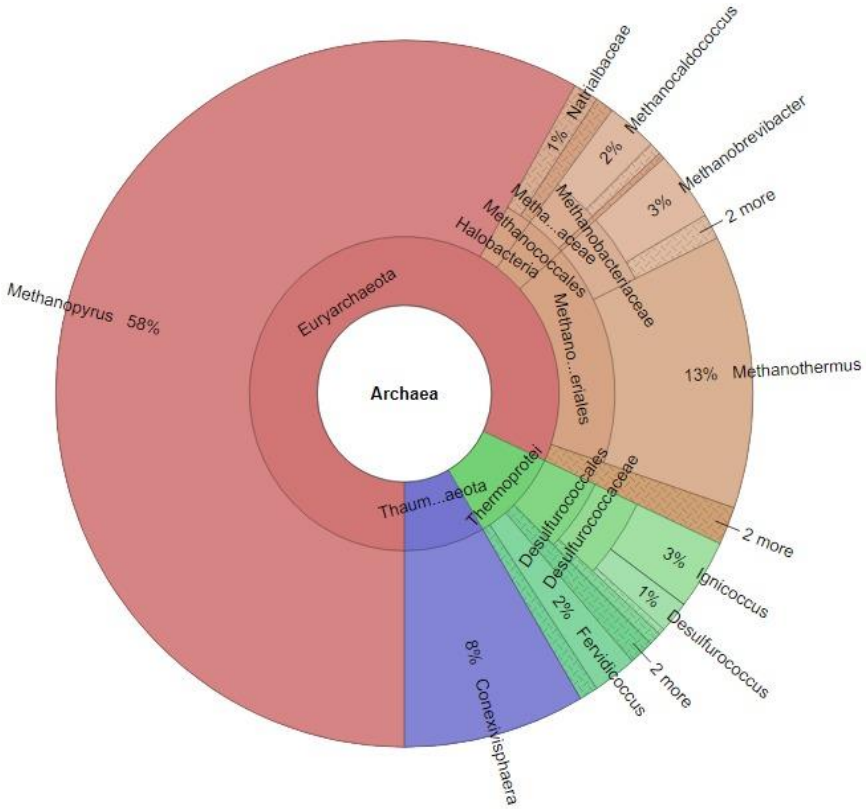

Figure 5.33 The archaeal communities in phylum, class, order, and family levels of the H20cm after the first enrichment

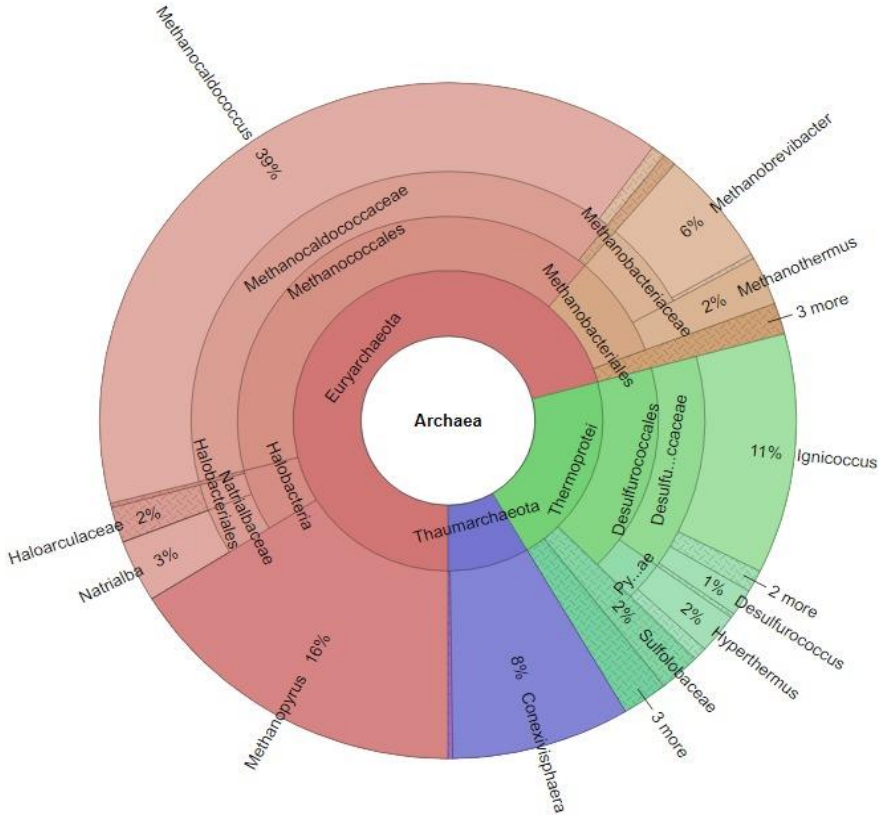

Figure 5.34 The archaeal communities in phylum, class, order, and family levels of the B20cm after the first enrichment

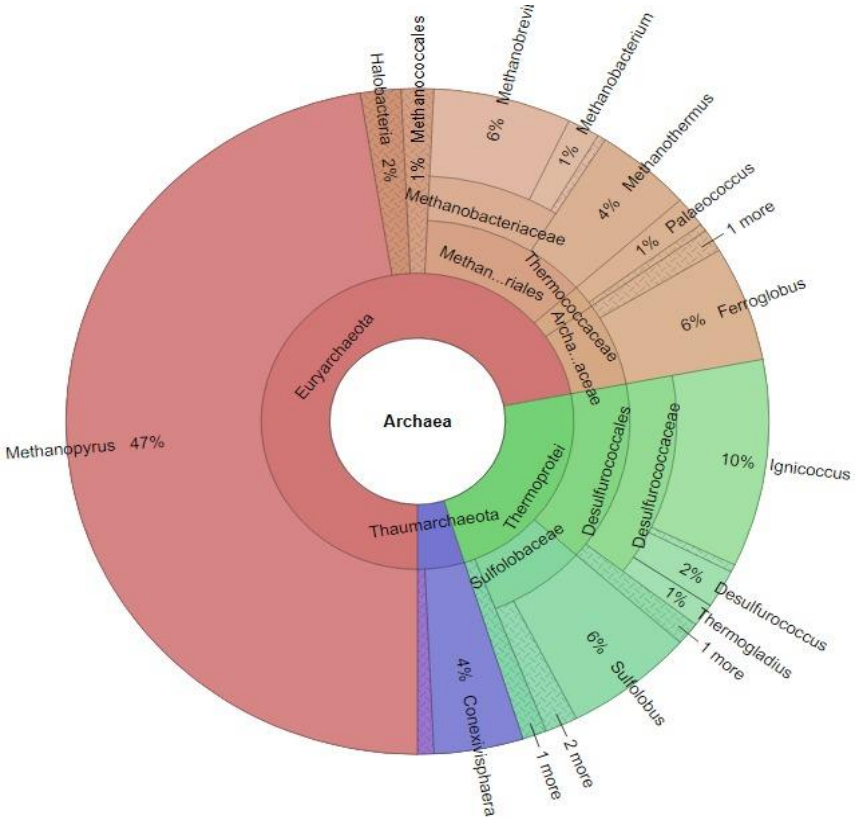

Figure 5.35 The archaeal communities in phylum, class, order, and family levels of the H20cm after the first transfer

Figure 5.36 The archaeal communities in phylum, class, order, and family levels of the B20cm after the first transfer

Figure 5.37 The archaeal communities in phylum, class, order, and family levels of the H20cm after the second transfer

Figure 5.38 The archaeal communities in phylum, class, order, and family levels of the B20cm after the second transfer

Figure 5.31. and Figure 5.38. show archaeal communities for H20cm and B20cm respectively for all enrichment process.

At the end of the incubation period, the most dominant family for both stations was emerged as *Methanopyraceae*. Even though most of the reads were assigned to same family in the raw samples, it was also determined that the families including *Methanococcales* and *Methanobacteriales* were enriched in the last culture. It is known that these three families have the ability to produce methane in an oxygen-free environment (Angelidaki et al., 2011; DasSarma et al., 2009).

5.2.2. Similarity Analysis

The dendrogram given in Figure 5.31 was created by analyzing the proximity of the samples according to their diversity and amount. The sample denoted as mean was created by averaging all OTUs, expressed as a percentage, and added to represent average variation. The samples on the horizontal and vertical axis of the graph are colored so that the similarity ratio with the other samples is between 0 and 1. The higher the similarity ratio, the darker the color, thus approaching 1, on the contrary, the samples diverge from each other.

According to given dendogram, two main groups and four subgroups were formed. Accordingly, the two groups that differed the most were raw sample of B20cm and B20cm after the second transfer. On the other hand, with the first transfer of H20cm and B20cm, the raw sample of H20cm and after the first enrichment were found to be in different groups in the same branch. B20cm started to differ from the dune samples with the first enrichment, unlike H20cm, and continued in this way. Therefore, considering the similarity analysis, it can be said that the enrichment steps were more efficient for B20cm than for H20cm.

PCA based analysis in the Figure 5.40. and dendogram results may have differed due to better enrichment of alkaline bacteria due to relatively higher pH in the initial transfer process. Since more parameters are considered in the PCA graph, the results were formed in a way that did not have a major effect on the cultures.

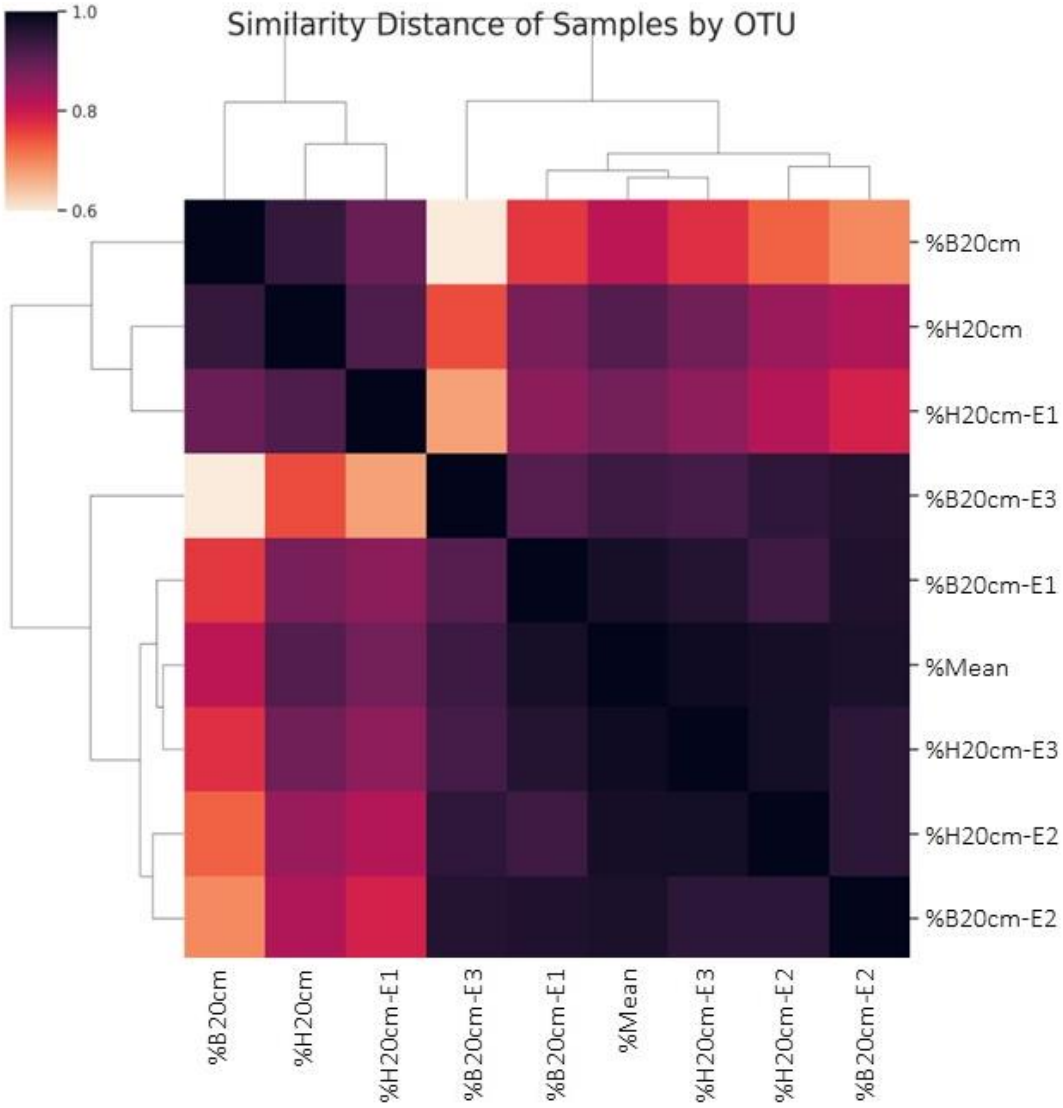

Figure 5.39 Dendogram displaying similarity distance of samples by OTU

5.2.3. Alpha Diversity Analysis

Alpha diversity refers to the diversity of species found in a particular specimen or ecosystem. Various indices are used to calculate alpha diversity. With the Shannon index, alpha diversity is measured in terms of species richness, which depends on the number of various species, and in terms of species diversity, which determines the distribution of microorganisms in a sample. The Chao1 index, on the other hand, predicts the diversity in the available data and the species that may be overlooked due to insufficient data. The Simpson index calculates the richness of the sample with the relative abundance of the different species that make up the sample (Willis, 2019). Table 5.2. indicates number of reads, OTUs, Chao1 index, Shannon index as well as Simpson index.

Table 5.2 Bacterial alpha diversity indices of the samples

Samples	Total number of reads	Chao1	Shannon	Simpson
H20cm	783	250	6,43	0,976
B20cm	1194	205	6,67	0,981
H20cm-E1	1712	112	5,82	0,969
B20cm-E1	1658	136	6,16	0,972
H20cm-E2	1753	104	4,98	0,889
B20cm-E2	1005	109	5,64	0,96
H20cm-E3	537	119	5,57	0,948
B20cm-E3	2578	90	4,34	0,866

The highest total number of reads belongs to B20cm-E3, and the lowest one belongs to H20cm-E3. According to Shannon and Simpson index, dune and sediment samples collected from the floodplain forest were found to have the highest diversity, respectively. However, with one exception (the diversity of H20cm at the end of the second transfer was higher than at the first transfer), it was found that the diversity started to decrease as transfers progressed. This is an expected situation which is known as “microcosm effect”, and it has been reported in several studies in the literature that the diversity decreases as the process progresses (Jacquiod et al., 2013a; Vorob’ev & Dedysh, 2008).

According to the Chao1 index, it was determined that the raw samples came to the fore in species evenness, and that, unlike only the diversity indices, the samples from Hamam Lake were ahead of Bulanık Stream. Species evenness, like diversity, decreases as enrichments progress, but this time the evenness of H20cm after the second transfer is an exception.

5.2.4. PCA Based Analysis

PCA analysis relies on the size reduction method by finding the maximum variance to detect the relationship between variables in multidimensional data. The purpose of this analysis is to reduce the data to 2 or 3 dimensional planes and thus to observe the patterns found among the data. The main feature of PCA analysis is to reveal as much variance as possible with a minimum of variables.

Figure 5.40 Two-dimensional plot of a 3D PCA analysis of bacterial communities in raw samples and enrichment cultures.

According to PCA analysis, it is possible to say that microbial communities show a certain trend. It can be easily seen that the raw sample of H20cm in the upper part of the graph and the raw sample of B20cm in the lower right of the graph are separated from each other and the remaining cultures. All cultures subjected to enrichment, on the other hand, entered a trend in the same direction and moved away from the raw samples, and were located very close to each other. Therefore, enrichment cultures differed considerably from the raw samples and became similar to each other.

In addition to the clustering of the samples according to the given enrichment conditions, the fact that the inoculums taken from the floodplain forest were away from the enrichment cultures is a crucial factor in terms of the reproducibility of the experiment and shows that the microbial community varies depending on the substrate (Wong et al., 2016).

5.2.5. Beta Diversity Analysis

The main purpose of beta diversity analysis is to observe the structural status between the groups or samples by comparing the samples both within themselves and the groups they are in. Unlike PCA analyzes, PCoA also take into account the distances between the bacteria in the samples.

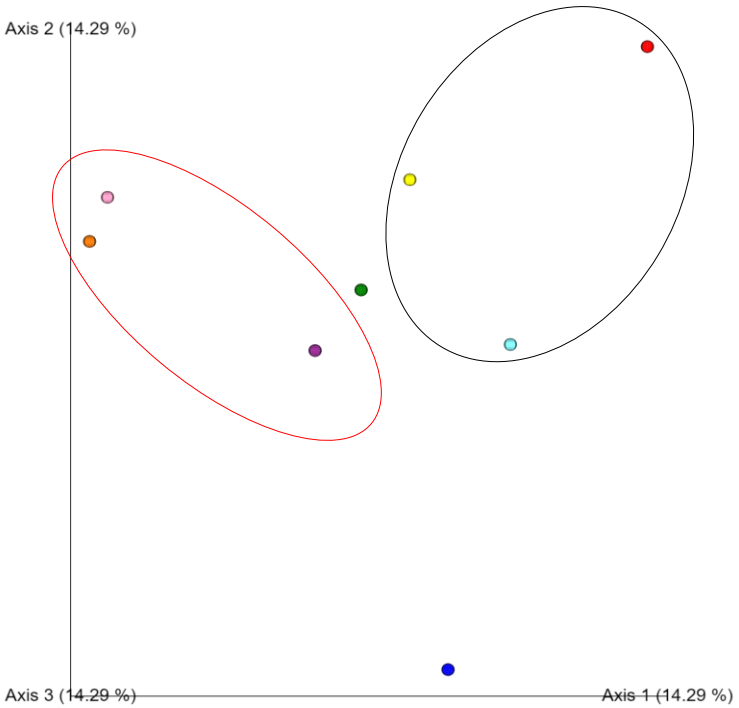

Figure 5.41 Beta diversity analysis according to the Bray-Curtis index (Green: H20cm, turquoise: B20cm, pink: H20cm-E1, red: B20cm-E1, blue: H20cm-E2, orange: B20cm-E3, purple: H20cm-E3, yellow: H20cm-E3)

The Bray-Curtis PCoA analysis is generated using a statistic based on the finding of shared bacteria, taking into account the proportions of bacteria in the samples. On the other hand, Weighted-Unifrac calculates the distance by creating a phylogenetic tree with the sequences of the genomes of the bacteria in the samples and analyzes them together with the bacteria's presence rates.

The Bray-Curtis PCoA analysis shows that, with the exception of two, samples can be split into two clusters. The cluster drawn with black circle contains the raw sample of B20cm together with the first enrichment and after the second transfer, while the circle with red circle contains the points representing the sediment sample of H20cm as well as the first enrichment and after the second transfer of H20cm. According to beta diversity analysis, after the first transfer of the samples, B20cm was located closer to the other samples, while H20cm remained quite far away from the cluster.

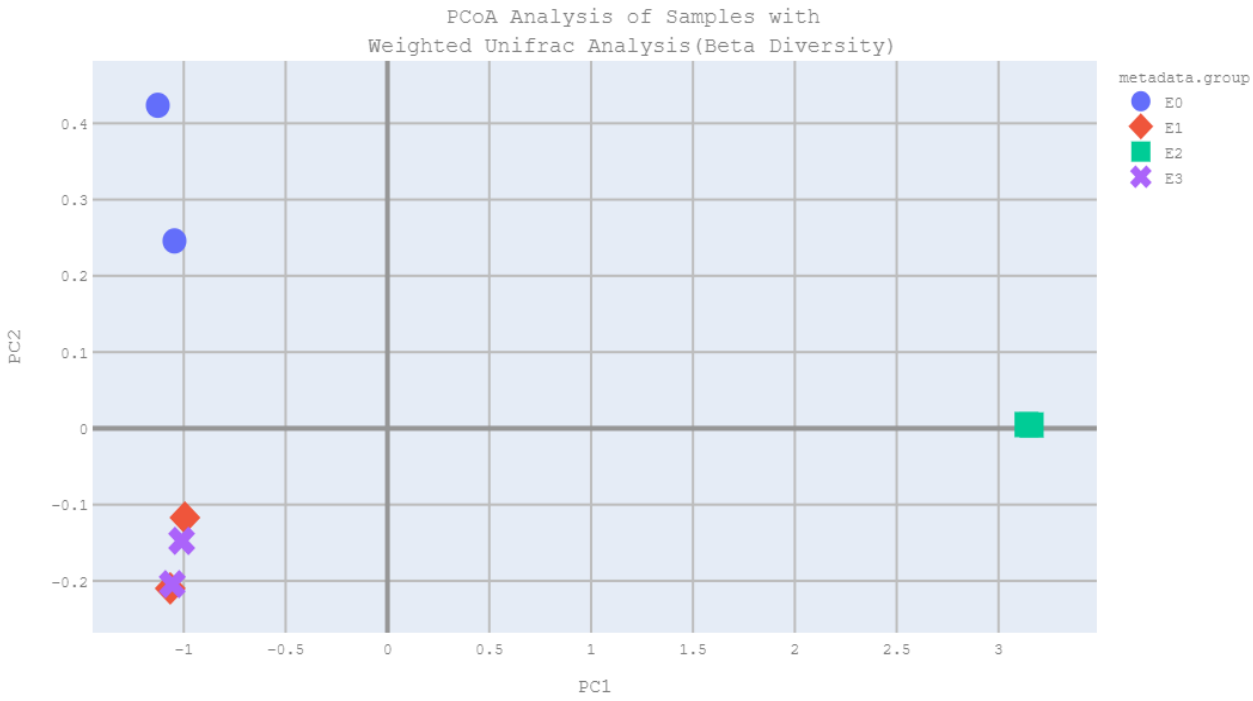

Figure 5.42 Beta diversity analysis according to the Weighted Unifrac distance index (Green: H20cm, turquoise: B20cm, pink: H20cm-E1, red: B20cm-E1, blue: H20cm-E2, orange: B20cm-E3, purple: H20cm-E3, yellow: H20cm-E3)

Weighted Unifrac PCoA analysis is comparable to The Bray-Curtis PCoA analysis. Accordingly, the first and third enrichments of the two stations are located close to each other. The raw samples of the stations and the second enrichments formed a separate group. The crude samples are relatively close to the first group, while the second enrichment group is quite far from both. Therefore, beta diversity analysis indicates that the microbial communities formed as a result of the second enrichment differed from the rest

5.3. Comparison Between Samples and Enrichment Cycles

In this section, the overall performance of the sediment and dune inoculation collected during the enrichment process, as well as a comparison of the two stations will be made. The graph below (Figure 5.35.) shows the biogas production efficiency of all samples per milliliter medium volume.

Overall, each microcosm enriched with the dune sample collected from Bulanık Stream showed up to six times better biogas production performance than those enriched with the sediment sample from Hamam Lake.

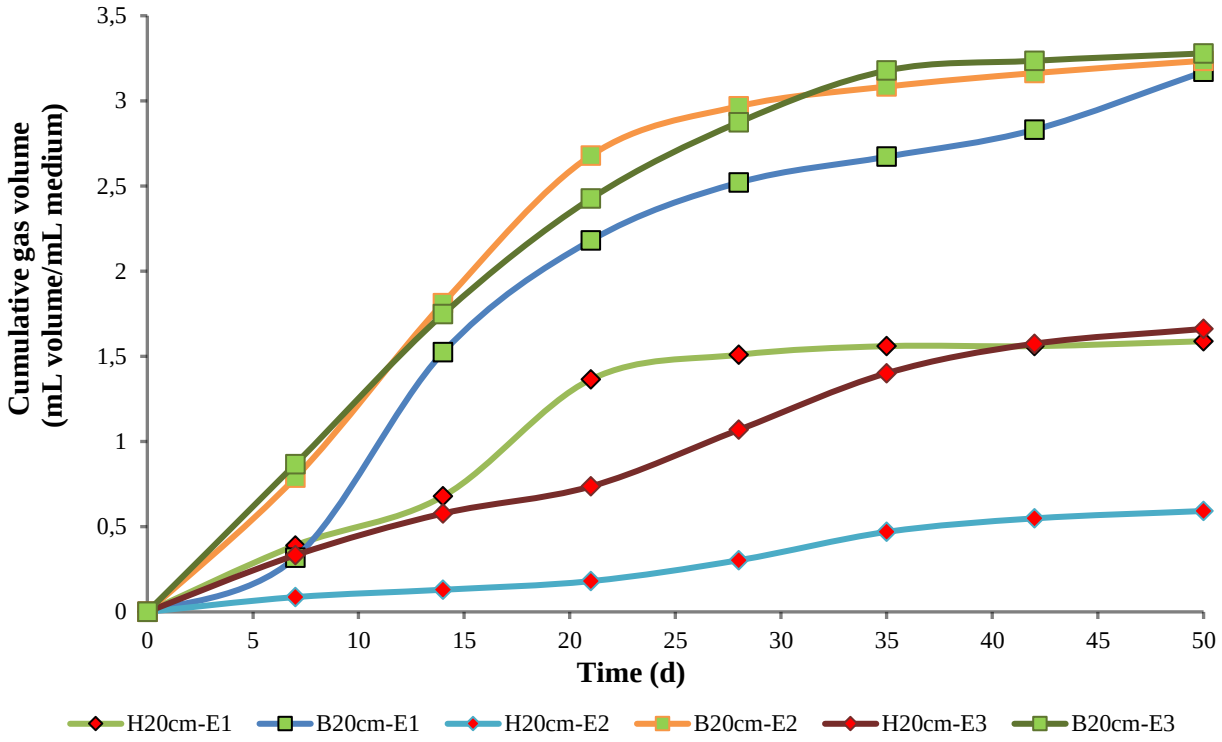

Figure 5.43 Cumulative biogas volume per ml medium for all samples (except negative controls)

When we compared cultures from Bulanık Stream among themselves, it was determined that they were ranked as second transfer, first transfer and first enrichment respectively. Also, biogas production took less time to switch to the logarithmic phase after initial enrichment, which may be due to the microbial consortium adapting to lignin digestion. However, as can be seen in the graph, there are quite low difference between the biogas production efficiency of these three samples. It is an expected phenomenon that the amount of biogas production between transfers will increase, but this difference can be expected to be larger. The high amount of biogas output after the first inoculum may have been achieved as a result of the digestion of extra carbon sources from the dune sample.

On the other hand, the reason for the difference between the first transfer and the second transfer may be the pH value. As in Figure 5.12., it will be seen that this incubation takes place at a higher pH than the others, although it is still below 8. The reason why biogas production was quite high after the first transfer may be that the methanogens in this sample work better at pH close to 8. As seen in the gas composition graphs, methanogens from the soil microbial community were also enriched, although this was not the main purpose of this thesis. With some exceptions, methanogens are known to work at pH values between 6.6 and 7.8 (Jiunn-Jyi et al., 1997). However, the pH measurement made during the sampling in Bulanık Stream shows the alkalinity of the dune. Therefore, methanogens can be expected to perform better at alkaline pH values. Figure 5.36 shows that B20cm achieved maximum methane yield at the end of the first transfer, outpacing the other two enrichments.

Finally, although the CH_4 amount of B20cm after the second transfer remains lower than the other two treatments for the reasons mentioned above, the CO_2 amount, on the contrary, is at its maximum value. Therefore, it may be an indication that the microorganisms in the inoculum have begun to adapt to lignin digestion.

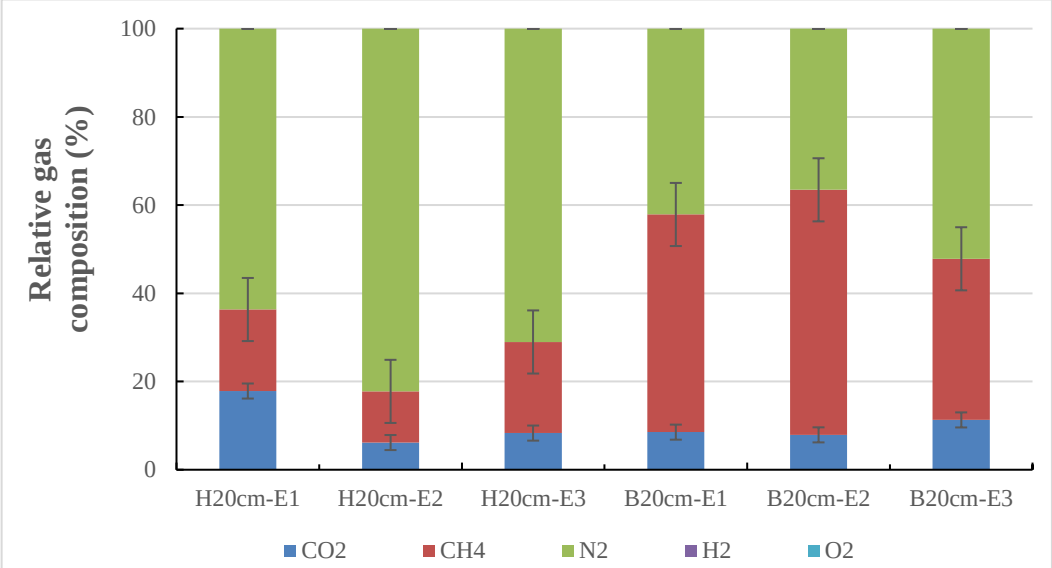

Figure 5.44 Relative gas composition in the headspace for all samples.

When the cultures of Hamam Lake were examined, it was reported that there was no linear trend in terms of biogas production efficiency. It was determined that the first enrichment was the highest, followed by the first enrichment, and the first transfer was half as efficient as these two. It is stated in various articles that there are problems in terms of biogas production from time to time in the intermediate incubations (Ozbayram et al., 2017b; Porsch et al., 2015).

Although it is not easy to determine the reason for this, pH may be the reason for the decrease in this study. It can be seen at Figure 5.12. that the pH is higher after the first transfer compared to other incubations. It can be estimated that the yield of methanogens may decrease in alkaline conditions, since the samples taken from the Hamam Lake, unlike the dune samples of Bulanık Stream, are closer to neutral pH. It was seen that lignolytic microorganisms continue their activity. The pH close to 8 did not prevent the lignolytic microorganisms from continuing their activities, but it caused a decrease in the performance of methanogens. Therefore, although lignin digestion continued, CH_4 production decreased. While methane production decreased, the fact that the removal of VFA remained at low levels after reaching the peak, as seen in Figure 5.37, can be a proof of this theory. However, even after the relatively unsuccessful enrichment step, the cultures did not change significantly as the

cultures were recovered in the next incubation, but the microbial community differed slightly on a genus basis, as can be seen in Figure 5.38.

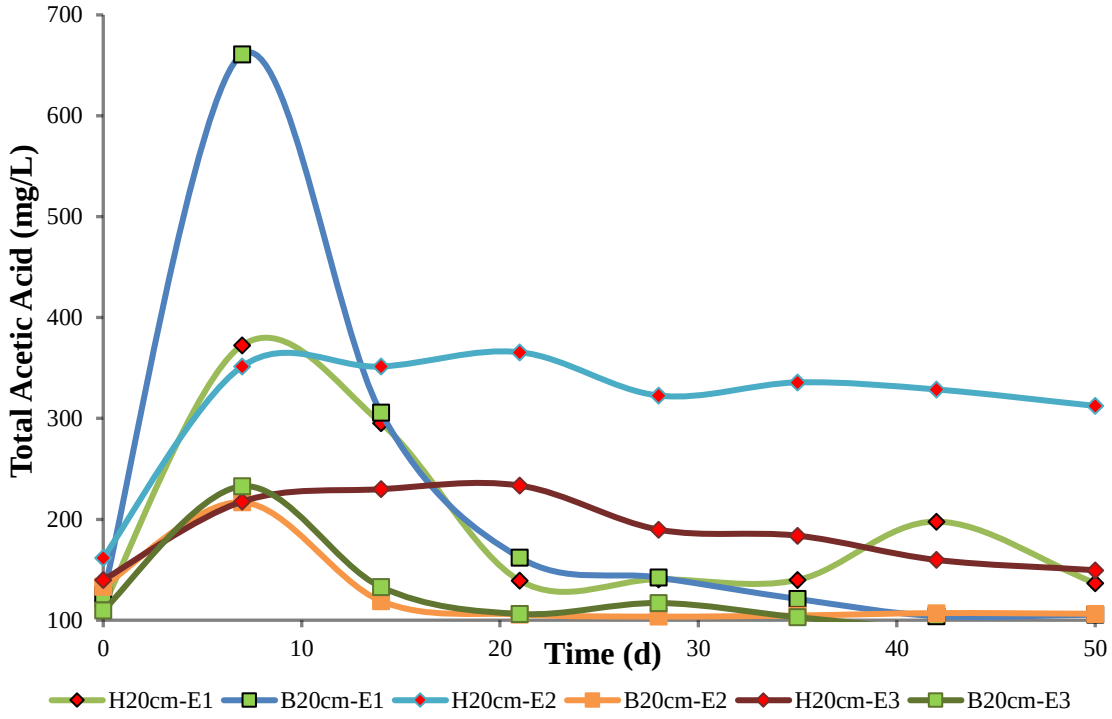

Figure 5.45 Total acetic acid concentration in the liquid phase for all samples.

The main VFA throughout the entire process has been acetic acid. Although there were fluctuations in propionic and butyric acid, their concentrations remained much lower than acetate. Therefore, it can be said that acidogenic fermentation predominates (Nagarajan et al., 2022). In addition, the decrease of acetate concentration after reaching its maximum value and the observation of methane formation in the same time interval indicate that methanogens are acetoclastic (Kurade et al., 2019).

Since the main VFA is acetate, all other volatile fatty acids were converted to acetic acid by the aforementioned method and compared. The total amount of acetic acid for both stations peaked at the first incubation and entered a downward trend as the transfers progressed. A similar situation has been reported during different studies (Ozbayram et al., 2018; Porsch et al., 2015; Schroeder et al., 2022b). Especially for the B20cm samples, the total acetic acid value decreased to almost one third after the first incubation and remained that way. There was no such a dramatic decrease for H20cm. In this particular study, this may be due to the breakdown of lignin as well as extra carbon sources from the sampled regions during first two enrichments. After the second transfer, the capacity of the

microorganisms to produce VFA may have become more limited, since there is no other carbon source other than lignin.

On the other hand, the time to reach the maximum total acetic acid value increased from 2 weeks to 4 weeks for H20cm, while it was always 2 weeks for B20cm. This may be because the bacterial community in the B20cm adapts more quickly to lignin digestion, as well as the fact that methanogens digest the produced VFA fairly quickly without accumulating. Likewise, the methane production efficiency of B20cm is quite high compared to H20cm.

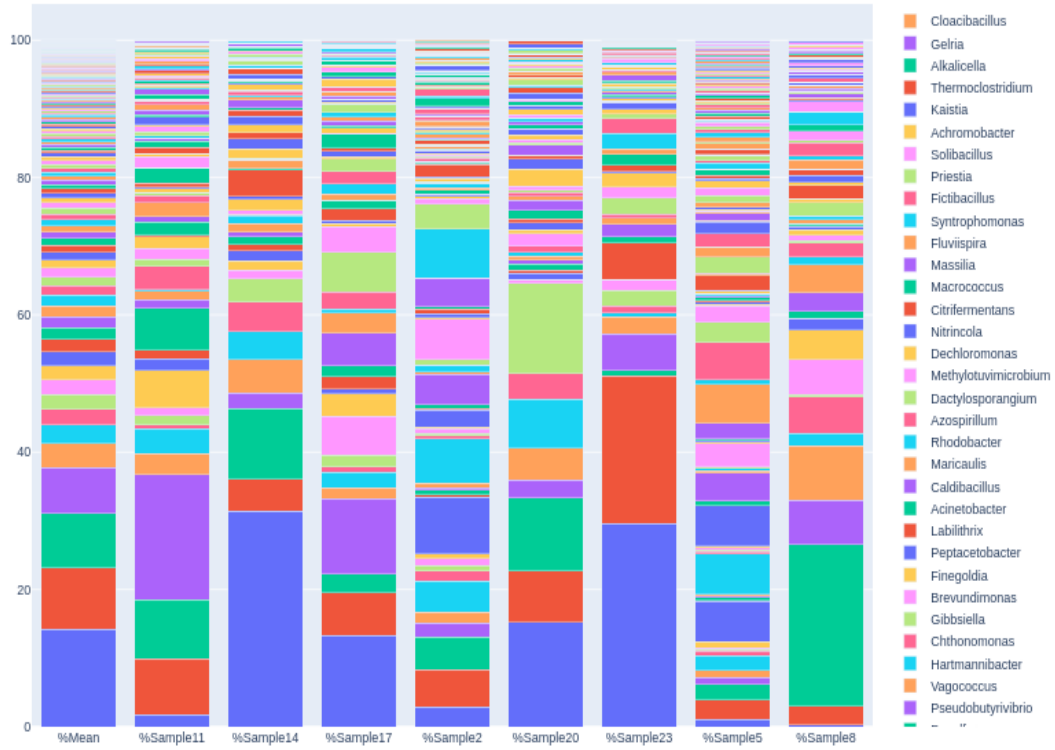

Figure 5.46 Percentage display of the microbial community diversity and amount graphs at the genus level of the samples (Sample2: H20cm, Sample5: B20cm, Sample8: H20cm-E1, Sample11: B20cm-E1, Sample 14: H20cm-E2, Sample17: B20cm-E2, Sample20: H20cm-E3, Sample23: B20cm-E3).

Although the dominant phyla at the sampled stations differed, *Firmicutes*' dominance gradually increased as the transfers progressed. It is known that the share of *Firmicutes* among other microorganisms in the biogas plant is generally high and its abundance tends to enhance with treatment in anaerobic digesters (Zhu et al., 2016). Gao et al. also reported a similar situation when adapting the microbial community collected from mangrove soil to cellulose digestion (Gao et al., 2014). At the same time, a major difference was detected between the bacterial community dynamics of the raw samples and the bacterial community of the final culture obtained. Likewise, this is a

situation that has been mentioned in various articles (Jacquiod et al., 2013b; Wong et al., 2016). According to the beta diversity analysis, the bacterial composition formed for both stations after the first transfer altered from the remaining enrichment cultures. The reason for this situation can be shown as the taking advantage of microorganisms that show optimum efficiency in alkaline environments from the pH of the medium close to 8. The fact that the first and third enrichment cultures appear close in terms of bacterial community dynamics can be considered as an indicator of this.

More than half of the reads assigned for *Firmicutes* belong to the class *Clostridia* for both stations, especially starting from the end of the first transfer. *Clostridia* are anaerobic, Gram-positive, rod-shaped, endospore-forming bacteria and several members of this class are known to degrade lignocellulosic biomass (Tracy et al., 2012). *Clostridium thermocellum* is one of the most widely used cellulose decomposers because of its relatively higher efficiency compared to other microorganisms (H. Akinosho et al., 2014; Ecem Öner et al., 2018; Ichikawa et al., 2017). It is also reported that *Clostridium thermocellum* can degrade hemicellulose and modify lignin without hydrolyzing it (H. O. Akinosho et al., 2017; Mazzoli & Olson, 2020). It would therefore come as no surprise that a species from this class can digest lignin anaerobically.

In addition, taxa containing species that can digest cellulose and hemicellulose without oxygen, such as *Anaerocolumna*, and species that digest anaerobic cellulose, such as *Acetivibrio thermocellus*, have been found to become more dominant in the microbial community as transfers progress. This may be a sign that the microbial community is slowly adapting to lignin digestion while confirming that anaerobic conditions have been successfully maintained.

Figure 5.38. shows that share of *Alkalibacter* genus in the *Clostridia* starts to rise after the first transfer for both stations. The only species within the genus was *Alkalibacter sp. ES005* (NCBI:txid2815577) and %15 and %30 of all reads was assigned to it for H20cm and B20cm respectively after the second transfer. This species, which does not yet have characterization, was recently discovered (Schoch et al., 2020). In fact, it is only the third individual after *Alkalibacter mobilis* and *Alkalibacter saccharofermentans*, in the *Alkalibacter* genus. Isolated from a lake habitat, *Alkalibacter mobilis* is a chemoorganotrophic organism which can degrade lignin-derived monomers such as vanillic acid and methylated lignin derivatives such as 3,4-dimethoxybenzoic acid (Khomyakova et al., 2021). *Alkalibacter saccharofermentans* has also been isolated from a lake in Russia, but there are no studies on the ability of this organism to digest lignocellulosic compounds (Garnova et al., 2004). Main VFA for both microorganism is acetic acid.

In the light of all these findings, it can be predicted that *Alkalibacter sp. ES005* may be one of the microorganisms sought in this study. However, since anaerobic microorganisms cannot benefit from the high redox reactions provided by oxygen, lignin digestion in the anoxic environment is believed to result from the synergistic roles of the microbial community, similar to anaerobic cellulose degradation (Gao et al., 2014; Zhu et al., 2016).

Clostridium perfringens was assigned to 19% of the total reads for B20cm after the second transfer while it was not enriched for H20cm even though raw sample of H20cm includes small amount. *Clostridium perfringens* has been isolated from a variety of habitats including soil along with sediments and is thought to be directly or indirectly involved in cellulose degradation (Adams et al., 2008; Y. Guo et al., 2017).

Anaerotignum propionicum, which is the second most dominant species after the second transfer for H20cm, is known to produce acetic and propionic acids by digesting amino acids in an oxygen-free environment (Ueki et al., 2017b). There is no study in the literature on whether this organism can digest lignocellulosic material.

Oscillibacter valericigenes, which increases its presence in the bacterial community as the transfers progress, belongs to clostridial cluster IV. Isolated from environments where lignin is partially digested, such as wood-fed termites, anaerobic sewage sludge, and anaerobic digesters, this species plays a role in the digestion of durable structures such as xylan and pectin, as well as cellulose (Iino et al., 2007; Ziemer, 2013).

Christensenella is also one of the genera that is relatively abundant in both specimens. With only a few members, this group is usually isolated from human gut (Morotomi et al., 2011; Ndongo et al., 2016b). It was noted that it was obtained from seed sludge in only one study. Therefore, to our knowledge, this is the first study in which both *Christensenella massiliensis* and *Christensenella minuta* were collected from soil. While the functions of this species are not still being discovered, it is estimated that it plays a role in the digestion of cellulose and hemicellulose (W. Li et al., 2018).

It was revealed that the *Tissierellaceae* family constituted 6% of the B20cm enrichment culture and less than 1% of the H20cm enrichment culture as *Gudongella oleilytica* most dominant species. This family, which has been isolated from methanogenic reactor, anaerobic digesters, freshwater hot springs, human and animal microbiota, is thought to play a critical role in anaerobic digestion (K. Wu

et al., 2020). However, it is not yet clear whether this role is in the degradation or during the formation of methane (Dalantai et al., 2022; Granada et al., 2018).

While the first three steps of anaerobic digestion, namely hydrolysis, acidogenesis and acetogenesis, are orchestrated by bacteria, the realization of methanogenesis depends on archaea (Weiland, 2010). Therefore, it is normal for these two organisms to coexist in nature due to the synergistic relationship between them. Likewise, the enrichment of methanogens along with hydrolyzing bacteria in this thesis can be explained in this respect. Considering that methanogens comprise almost half of the entire archaeal population with archaeal families including *Methanopyraceae*, *Methanococcales* and *Methanobacteriales*, it is possible to explain the high amounts of methane formed at the end of the incubation processes.

However, it is known that archaea can be found in lesser amounts in the natural ecosystem compared to bacteria (Aller & Kemp, 2008). In this study, the number of archaea detected in metagenomic data is much less than bacteria. In addition, while universal primers reproduce bacteria better, they may work with a lower performance for archaea. The results will therefore be confirmed with the NGS.

6. CONCLUSIONS AND RECOMMENDATIONS

The main focus of this thesis was isolation and characterization of anaerobic lignin-degrading microorganisms or microbial communities. For that purpose, analytical evaluations such as biogas production, VFA production and removal, gas composition as well as alterations in the microbial dynamics were evaluated. The major contributions of this thesis are summarized below.

B20cm station was showed much better performance than H20cm in terms of cumulative biogas production. Overall, cumulative gas production rate was enhanced for both stations after the second transfer compared to first enrichment. Methane formation in high amounts, especially for B20cm, according to gas composition measurements indicates that methanogens were also enriched even if this was not the primary objective of this thesis.

VFA for this study was acetic acid, propionic acid and butyric acid but the most abundant was acetic acid. Concentration of acetic acid was in a range of 90 to 350 mg/L. It was detected that amount of VFA was increased in the beginning and then, decreased until the end of each incubation. This shows that lignin first breaks down into volatile fatty acids and then turns into methane. Correspondingly, VS and lignin removal measurements were also demonstrated that the amount of carbon source become lowered as transfers progressed suggesting lignin degradation had taken place in the glass bottles.

On the other hand, microbial community dynamics changed after each transfer and as a result of enrichment, certain phyla such as *Firmicutes* that was present in small amounts in raw samples became dominant. *Clostridia* class, whose many members are known to perform anaerobic digestion of lignocellulosic biomass, has become quite dominant for both stations. *Alkalibacter sp*. ES005 became the most dominant species for both stations after the second transfer. Since it is known that other members of the same genus digest lignin derivatives in oxygen-free fashion, it can be stated that *Alkalibacter sp*. ES005 can degrade lignin in an anaerobic environment.

It was estimated that *Clostridium perfringens*, *Anaerotignum propionicum*, *Oscillibacter valericigenes*, *Christensenella massiliensis*, *Christensenella minuta* and *Gudongella oleilytica* might be also involved in the anaerobic degradation of lignin since these species have been reported to be associated with the hydrolysis of lignocellulosic biomass. Additionally, the archaeal community appeared to be dominated by methanogenic families including *Methanopyraceae* and

Methanococcales which indicates that methane output occurs after lignin degradation.

In the light of all findings, it can be concluded that the samples collected from the İğneada floodplain forests are enriched on the basis of the bacterial community in such a way as to ensure the degradation of lignin anaerobically, and that the products resulting from hydrolysis are converted to methane by methanogenic archaea.

In future studies, first of all, it is necessary to culture the samples with alkaline lignin as the sole carbon source to determine which species are involved in the degradation of lignin. The genomic sequence of that species must then be definitively determined by DNA isolation and sequencing. The next step will be to find out which gene or genes are responsible for the digestion of lignin with omics approach and uncover the pathway. Finally, the enzyme that digests lignin will also be found and can be made suitable for biotechnological applications.

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