IMPROVEMENT OF BIOGAS PRODUCTION BY FUNGAL TREATMENT DURING THE CONVERSION OF AGRICULTURAL BIOMASS INTO ENERGY AND DIGESTATE

by

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dedicated to my dear mom,
Aydan Atabay

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ABSTRACT

IMPROVEMENT OF BIOGAS PRODUCTION BY FUNGAL TREATMENT DURING THE CONVERSION OF AGRICULTURAL BIOMASS INTO ENERGY AND DIGESTATE

In this PhD study, the advantage of highly-cellulolytic white-rot fungus *Trametes versicolor* and anaerobic rumen fungus *Orpinomyces* sp. was taken by aerobic pretreatment and bioaugmentation approaches in lignocellulose-based anaerobic digestion (AD) trials, respectively. Selected cereal crop materials (i.e. wheat, rye, barley, triticale) were harvested at different stages and subjected to AD with cow manure as the co-substrate. In all AD tests, early-harvested barley was found to be the highest methane-yielding crop material. Changes in the quantity of selected key functional enzyme groups responsible for lignocellulose degradation and biomethanation were further determined in AD using quantitative real time PCR (qPCR). 16S rRNA gene amplicon sequencing revealed a more diverse microbial community in fungal-treated anaerobic digesters. Comparatively more unique microbiome of biogas reactors upon fungal treatment synergistically affected VFA production, cellulose degradation and eventually methane yield in an affirmative way. Following the AD tests, anaerobic digestates were collected on crop-basis and composted. All compost piles exhibited same abiotic profiles. Based on 16S and 18S rRNA gene amplicon sequencing, similar predominant bacterial and fungal genera detected that were mostly composed of lignocellulose degraders. In the last step, each final compost product was amended to agricultural fields where the crops were planted, and changes in soil microbiome was monitored using 16S rRNA gene amplicon sequencing. *Proteobacteria* was the most abundant bacterial phylum; whereas a shift in the predominance from *Actinobacteria* to *Acidobacteria* was observed following the compost amendment. In addition, there was a remarkable increase in the relative abundance of *Bacteroidetes* upon compost amendment.

ÖZET

FUNGAL ARITIM UYGULANAN LİGNOSELÜLOZİK BİYOKÜTLEDEN BİYOGAZ ÜRETİMİNİN ARTIRILMASI VE SON ÜRÜNLERİN DEĞERLENDİRİLMESİ

Bu doktora tezi kapsamında, beyaz çürükçül mantar *Trametes versicolor* ve anaerobik rumen mantar *Orpinomyces*'in yüksek selolüzik aktivitesinden yararlanılarak lignoselülozik biyokütle ile işletilen anaerobik çürütücülerde sırasıyla aerobik ön arıtım ve biyoaugmentasyon uygulamalarına gidilmiştir. Seçilen soğuk iklim tarla bitkileri (arpa, tritikale, buğday, arpa) farklı hasat dönemlerinde toplanmış ve büyükbaş hayvan dışkısı ile beraber anaerobik çürütmeye bırakılmıştır. İşletilen tüm anaerobik çürütücülerde en yüksek metan verimi, erken hasat edilen arpadan elde edilmiştir. Anaerobik çürütme sırasında lignoselülozik yapıların parçalanmasından ve metan oluşumundan sorumlu anahtar enzim gruplarının kantitatif olarak değişimi, gerçek zamanlı polimeraz zincir reaksiyonu ile belirlenmiştir. 16S rRNA gen amplikon sekans analizi sonuçlarına göre, fungal ön arıtım yapılan anaerobic çürütücülerde daha yüksek çeşitliliğe sahip mikrobiyal topluluk gözlemlenmiştir. Görece daha çeşitli mikrobiyal topluluğa sahip olan fungal arıtım izlenen çürütücülerde, daha verimli UYA üretimi, daha yüksek selüloz giderimi ve beraberinde yüksek metan verimi elde edilmiştir. Anaerobik çürütücü çalışmalarını takiben, bitki türü baz alınarak çürütücü çamurları ayrı ayrı toplanmış ve kompostlaştırılmıştır. Bütün kompost prosesleri aynı abiyotik özellikleri göstermiştir. 16S ve 18S rRNA gen amplikon sekansına göre, çoğu lignoselüloz parçalayıcılardan oluşan benzer bakteri ve mantar grupları tespit edilmiştir. Son adım olarak, elde edilen her bir kompost ürünü, çalışılan bitki türlerinin yetiştirildiği tarla arazisine uygulanmıştır ve toprak mikrobiyotasında meydana gelen değişimler 16S rRNA gen amplikon sekans analizi ile izlenmiştir. *Proteobacteria* en yaygın olarak tespit edilen şube olurken, kompost uygulaması öncesinden sonraki zamana kadar baskın olan şubelerde *Actinobacteria*'dan *Acidobacteria*'ya bir geçiş gözlemlenmiştir. Buna ek olarak, kompost uygulamasını takiben bütün toprak örneklerinde *Bacteroidetes*'in göreceli bolluğunda artış olmuştur.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Unit
ABTS	2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic acid	
$CaCl_2$	Calcium Chloride	
CH_4	Methane	
CO_2	Carbon Dioxide	
H_2	Dihydrogen	
HCl	Hydrochloric Acid	
KH_2PO_4	Potassium Dihydrogen Phosphate	
$MgSO_4$	Magnesium Sulphate	
$NaHCO_3$	Sodium Bicarbonate	
N_2	Dinitrogen	
NaCl	Sodium Chloride	
NaOH	Sodium Hyroxide	
$(NH_4)_2SO_4$	Ammonium Sulphate	
O_2	Dioxygen	
μg	Microgram	
μL	Microliter	

Abbreviation	Explanation	
AD	Anaerobic Digestion	
BC	Barley Compost	
BMP	Biomethane Potential Test	
BS	Barley Soil	
cDNA	Complementary DNA	
COD	Chemical Oxygen Demand	mg/L
DNA	Deoxyribonucleic Acid	
DNase	Deoxyribonuclease	
EB	Early Barley	
EC	Electrical Conductivity	
ET	Early Triticale	

GC	Gas Chromatograph	
GH	Glycoside Hydrolase	
LB	Late Barley	
LT	Late Triticale	
MC	Moisture Content	%
NGS	Next Generation Sequencing	
PCR	Polymerase Chain Reaction	
QPCR	Quantitative PCR	
RB	Residual Barley	
RC	Rye Compost	
RNA	Ribonucleic Acid	
RR	Residual Rye	
RS	Rye Soil	
RT	Residual Triticale	
RW	Residual Wheat	
SAO	Syntrophic Acetate Oxidation	
SAOB	Syntrophic Acetate Oxidizing Bacteria	
TC	Triticale Compost	
TKN	Total Kjeldahl Nitrogen	mg/L
TS	Total Solids	%
UV	Ultraviolet	
VFA	Volatile Fatty Acids	mg/L
VS	Volatile Solids	%
WC	Wheat Compost	
WS	Wheat Soil	

1. INTRODUCTION

Global energy demand has increased rapidly in recent years due to rising populations and developing technologies. Worldwide energy consumption was calculated as 524 QBtu in 2010 and is estimated to reach 800 QBtu by 2040, which shows an average increase of 1.5% per year (Sawatdeenarunat et al., 2015). A significant amount of this energy demand is supported by non-renewable fossil fuels. These resources are not only limited but also have negative impacts on the environment mainly due to the emission of greenhouse gases (GHGs), which further contributes to global warming.

Alternative energies are necessary to cease the dependence of fossil fuels and overcome the limitations they bring out. Transformation of lignocellulose-rich materials into biofuel is an attractive strategy to accomplish growing energy demands and mitigate GHGs emissions. Bioenergy, specifically biogas production through anaerobic digestion (AD) is considered to be highly-promising and reliable technology with its less-energy consuming process. AD has been adopted to society over the last century in terms of waste management and stabilization. Thousands of full-scale biogas plants are successfully in operation worldwide and converting many biodegradable wastes/feedstock into energy such as agro-wastes, sewage sludge, organic fraction of municipal solid waste etc. The potential of energy recovery in AD is different among each substrate (Sawatdeenarunat et al., 2017; Paul and Dutta, 2018).

Lignocellulosic biomass have been gaining more attention in recent years for producing bioenergy. Lignocellulosic biomass includes agricultural residues (crop residues), animal manure, energy crops and forest residues. Lignocellulosic biomass sources can be easily distinguished from other AD substrates by their abundance, low price, more consistent composition, and relatively high yield. However, the composition of lignocellulosic biomass, consisting cellulose, hemicellulose and lignin, creates a highly recalcitrant structure. Consequently, the hydrolysis becomes the rate-limiting step in AD processes fed with lignocellulosic feedstock. To design an efficient and stable anaerobic digester treating lignocellulosic biomass, and to overcome the slow hydrolysis rate, it is important to identify key microbial players as well as the metabolic pathways involved during hydrolysis (Shrestha et al., 2017).

Innovative methods are still being developed to overcome the refractory structure of lignocellulosic biomass, which stand as one of the main bottlenecks for their frequent utilization in

AD. Research on AD of lignocellulosic biomass has elevated greatly during the last decade and a number of strategies have been proposed recently, such as physical and chemical pretreatment of feedstock. Since the metabolic efficiency of microorganisms is in the highlight for a successful AD, process microbiology should be placed in the centre. Process engineering and microbiological approach should be encapsulated and integrated to improve the AD of lignocellulosic biomass (Nzila, 2017; Shrestha et al., 2017).

Biological strategies are often preferred in lignocellulose-based AD systems to improve the rate and extent of hydrolysis since they are comparatively cost-effective and environment-friendly. Biological pretreatment, co-digestion with other substrates and/or bioaugmentation are among these approaches to maintain a highly active microbial community capable of performing hydrolysis efficiently (Shrestha et al., 2017). Accordingly, biological treatment utilizing the lignocellulose-degrading capacity of fungi can be a good alternative. Aerobic fungi, such as white-rot fungi, has been considerably studied for the breakdown of lignocellulosic materials and further achieve improvements in biogas production. Meanwhile, anaerobic fungi, is a novel approach to be considered as a biological agent for successful anaerobic biodegradation (Dollhofer et al., 2018). Both applications contribute to significant affirmations in energy recovery, while having their own advantages and disadvantages that are discussed further. However, lack of information exists in the literature on the effects of these treatment methods on indigenous AD microbiome. Afore-mentioned reasons created a big motivation for this dissertation.

Within the framework of a sustainable agricultural bioenergy system, anaerobic digestate obtained from AD process can be used as a soil conditioner in the fields of related crops. However, in order to obtain a more suitable and humus-like so-called fertilizer, composting can be good practise to further treat anaerobic digestates. At the end of composting, these final compost products can be amended to soils to improve soil function and affirm soil microbiome.

The aim of this study was to provide a comprehensive “closed-loop” sustainable bioenergy approach, starting from the cultivation of selected crops to accelerated biomethane production in AD using fungi, ending with bio-fertilizer production via composting of anaerobic digestates and their amendment to soils belonged to each crop. A further and deeper insight was gained and discussed with metagenomic analysis of each step. The results may provide useful information regarding the management of agricultural residues and enhance methane recovery in lignocellulose-based biogas plants within a sustainable agriculture concept.

2. THEORETICAL BACKGROUND

2.1. A Brief Overview of Anaerobic Digestion Process

In AD process, organic substrates are metabolized in the absence of oxygen mainly into two molecules: methane and carbon dioxide, together with other trace gases (e.g. ammonia, hydrogen, hydrogen sulphide). AD is a commonly-applied and reliable process that is driven by bacteria and archaea; however, due to the complex interactions between the microbiome, a huge number of unexplored species still exist (Treu et al., 2016b). This biological process can be simply categorized in four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. These specific stages are depicted in Figure 2.1.

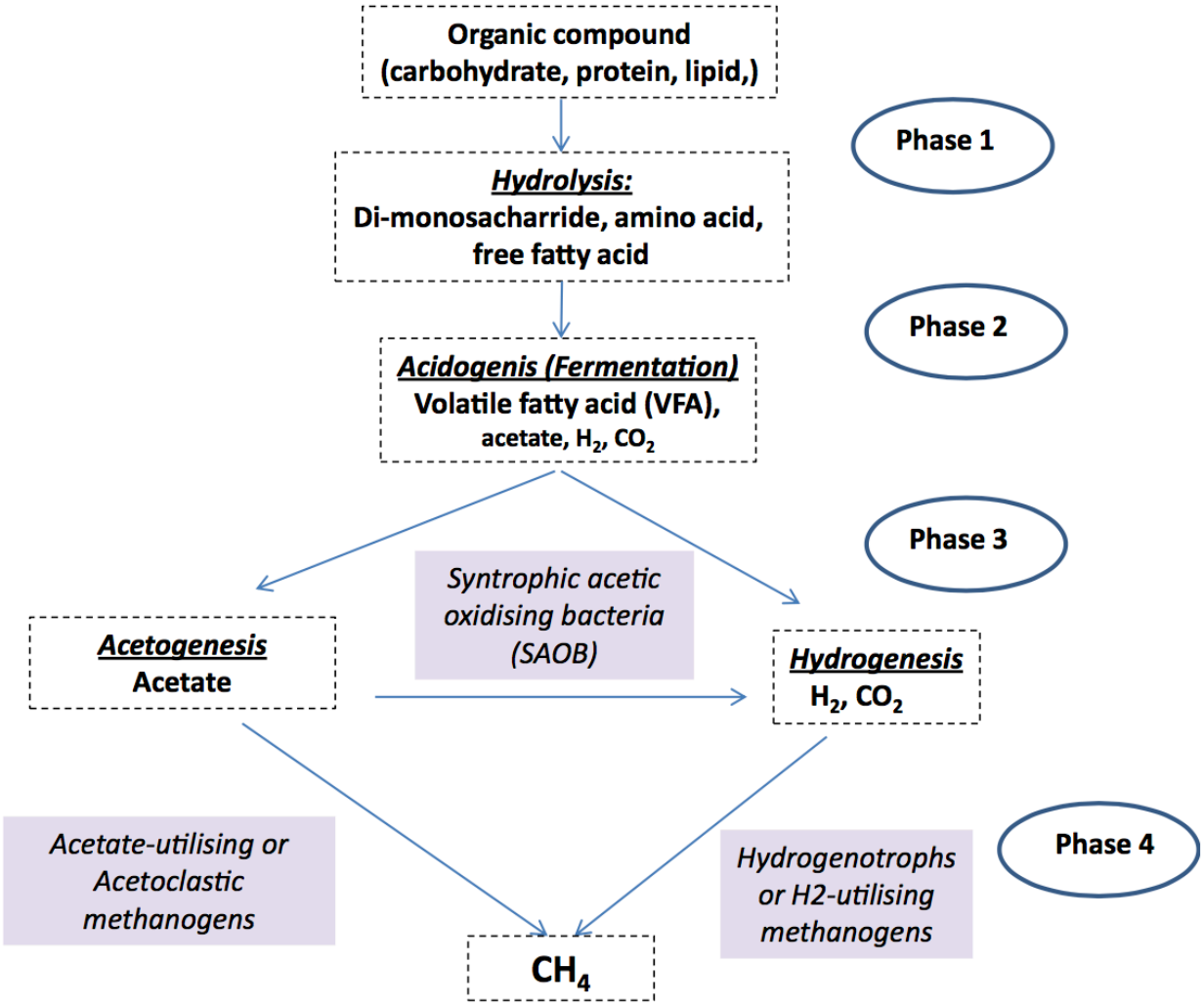


Figure 2.1. Summary of biomethane formation in an anaerobic process (Nzila 2017).

2.1.1. Hydrolysis

The first step of AD process involves an enzyme-mediated transformation of insoluble organic materials and macromolecules such as lipids, polyssacharides, proteins, fats, nucleic acid etc. into soluble organic materials (compounds suitable for the use as source of energy and cell carbon) such as monosaccharides, amino acids and other simple organic compounds. This step is carried out by hydrolytic and fermentative bacteria (Nzila, 2017) . This first stage is important since large organic molecules cannot be directly used by microorganisms as a substrate/food source. To accomplish biodegradation, certain microorganisms secrete extracellular enzymes. For instance, hydrolytic enzymes (e.g. cellulase, β- glucosidase, xylanase) or complex enzyme systems (e.g. cellulosome) are effective on polysaccharides, while protease and lipase degrade proteins and lipids, respectively (Azman et al., 2015). The degradation efficiency is based on the presence and activity of hydrolytic and fermentative microbes to excrete these extracellular enzymes.

2.1.2. Acidogenesis

The hydrolyzed products of the macromolecules in the first phase are then subjected to the fermentation step by various metabolic pathways and produce volatile fatty acids (VFA), hydrogen, carbon dioxide and alcohols. In general, during the acidogenesis phase, simple sugars, fatty acids and amino acids are converted into organic acids and alcohols. Hence, sugars and amino acids are the major substrates. The results of glycerol fermentation are propionate production and biomass generation (Angelidaki et al., 1999) are given below:

$$C_{57}H_{104}O_6 + 3H_2O \rightarrow C_3H_8O_3 + 3C_{18}H_{34}O_2 \quad (2.1)$$

A coupled oxidation-reduction reaction occurs in pairs for the acidogenic fermentation of amino acids and release NH_3 (Angelidaki et al., 2011). In this so-called Stickland reaction, different amino acids act either as an electron donor or electron acceptor. The type of the final products depends on the concentration of hydrogen formed as an intermediate product in the step. For example, if the hydrogen partial pressure is too high, the amount of reduced compounds can decrease (Botheju, 2011).

Hydrolysed sugars are transformed through the Emben–Meyerhof–Parnas (EMP) or Entner Doudoroff (ED) pathway (Angelidaki et al., 2011). Lactic acid and propionic acid are produced through EMP pathway, meanwhile acetic acid, butyric acid and caproic acid are fermented through

acetyl coenzyme A (acetyl-CoA). Contrary to amino acids, glucose can act both as electron acceptor for oxidation (e.g. acetate) and donor for reduction (e.g. propionic acid, ethanol etc.). Fermentative strains of glucose can metabolize the available monosaccharide through different pathways ending up to varying amounts of energy and products. Examples of glucose fermentation products (i.e. acetic acid, propionic acid, butyric acid, lactic acid, ethanol) are presented below (Schink, 1997).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2.2)$$

$$3C_6H_{12}O_6 \rightarrow 4CH_3CHCOOH + 2CH_3COOH + 2CO_2 + 2H_2O \quad (2.3)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2.4)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH \quad (2.5)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (2.6)$$

Based on the responsible microbial community for the acidogenesis, a wide range of phyla among fermentative microbes exists. However, *Firmicutes* are stated to be dominant bacterial phylum in biogas microbiome; that can degrade oligosaccharides into the aforementioned products (Treu et al., 2016b). Environmental and operating conditions, such as pH, temperature, feedstock composition and hydrogen pressure significantly influence the diversity of biogas microbiome (Rodríguez et al., 2006). Thus, specific microbial community differs among the various AD systems.

2.1.3. Acetogenesis

During acetogenesis, products which cannot be directly converted to methane by methanogenic bacteria (VFAs with carbon chains longer than one unit and alcohols) are converted into methanogenic substrates like acetate, hydrogen and carbon dioxide (Jain et al., 2015). So mainly, acetate is formed by different microbial groups, either by the hydrogen-producing acetogens or the hydrogen-utilizing acetogens. This bioconversion process is endergonic that a syntrophic relationship with methanogens is mandatory to maintain low H_2 partial pressure for acetogenic reactions to be energetically favorable (Treu et al., 2016a). It is crucial that the microorganisms carrying out the anaerobic oxidation reactions collaborate with the next group, namely methanogens; which depends on the H_2 partial pressure of the hydrogen present in the system. For instance, acetogens and methanogenic archaea should co-operate for the degradation of propionic acid and butyric acid which are oxidized through the methyl-malonyl-CoA pathway to produce acetate, H_2 and CO_2 and through β-oxidation to acetate, respectively (Wang et al., 2009). Additionally, sulphate reducers consume hydrogen and improve hydrogen concentrations for the acetogenesis process. On the other hand, hydrogen-utilizing acetogens use the acetyl-CoA pathway to form acetate by the reduction of CO_2.

These microbial members compete with the hydrogenotrophic methanogens for the utilization of hydrogen, methanol and formic acid (Batstone et al., 2006). Moreover, acetic acid and hydrogen is also produced from lipids degradation, as the VFAs go through β-oxidation (Kim et al., 2004; Treu et al., 2016a):

$$CH_3(CH_2)_nCOOH + 2H_2O \rightarrow CH_3(CH_2)_{n-2}COOH + CH_3COOH + 2H_2 \quad (2.7)$$

Acetogenesis step is conducted by various bacteria; in which *Clostridium* and *Bacteroides* are dominantly involved (Snell-Castro et al., 2005).

2.1.4. Methanogenesis

In the methanogenesis phase, the production of methane and carbon dioxide from intermediate products (mainly acetate and H_2/CO_2) is carried out by methanogenic archaea under strict anaerobic conditions; however, to less extent, substrates as formic acid and alcohols are also used (Schink, 1997). The larger portion of methane is derived from the conversion of acetate (acetoclastic pathway) and the rest is primarily produced from H_2/CO_2 (Angelidaki et al., 2011). Extended methane production can be conducted via the hydrogenotrophic pathway based on the conditions (i.e. temperature, feedstock characteristics etc.) (Campanaro et al., 2016).

The acetoclastic and syntrophic acetate oxidation (SAO) are the two potential pathways for methanogenesis that consume acetic acid. In the first pathway, the acetoclastic methanogens consume acetate to produce methane and carbon dioxide (Angelidaki et al., 2011):

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \quad \Delta G^{0'} = -31.0 \text{ kJ/mol} \quad (2.8)$$

In the SAO pathway, the syntrophic acetate oxidation bacteria (SAOB) convert acetate into hydrogen and carbon dioxide, where these products are further consumed by the hydrogenotrophic methanogens and converted into methane (Kougias et al., 2016):

$$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+ \quad \Delta G^{0'} = +104.6 \text{ kJ/mol} \quad (2.9)$$

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \quad \Delta G^{0'} = -135.6 \text{kJ/mol} \quad (2.10)$$

Overall, more than 60% of methane is produced through acetate and about 30% by H_2/CO_2. Only a small proportion of methane is generated from other methyl compounds (Jain et al., 2015). Acetoclastic methanogenesis is most active and important in freshwater sediments and anaerobic digesters. Methylotrohic methanogenesis is important in some marine sediments and other anoxic systems where methylated substrates occur. In sulfate-depleted, anaerobic habitats – especially in freshwater sediments, sewage digesters, in the rumen and at some depth in marine sediments – methanogens play a central role as H_2-scavengers and in the terminal mineralization of acetate. Thus, they contribute significantly to the carbon cycle (Fenchel et al., 2012).

Methanogens are more sensitive to environmental changes such as variations in pH or temperature, an increase in salt, metal ions or organic matter concentration, or differentiation in the loading rate (Demirel and Scherer, 2008). pH between 6.5 and 7.5 are found to be optimal in anaerobic process for methanogens, and pH change of 0.5 unit or less can be detrimental to the process (De Vrieze et al., 2012). For instance, if the rate of acetate generation is higher than their utilisation by methanogens, this leads to VFA accumulation and eventually a decrease in pH, which latter inhibits methanogenic growth. Thus, the coupling of acid/acetate generation and their utilisation is critical for an efficient AD process (Nzila, 2017).

2.2. Biogas Production from Lignocellulosic Biomass

Lignocellulosic biomass, namely agricultural residues and energy crops, is considered one of the most applicable renewable resource due to its abundance and availability as a fuel source (Table 2.1). The global biomass supply was estimated at 59.2 EJ in 2014, accounting for 10.3% of the world's energy supply. Of the total biomass supply, contribution of agricultural products and by-products was approximately 9% in 2013 (Paudel et al., 2017). As a renewable and abundant resource, cereal residues include both on-site residues and processing residues. On-site residues refer to materials left on cropland after harvesting and include straw, stems, leaves, and seedpods. Process residues such as bagasse, husk, roots, and seeds are materials that remain after processing the cereal crop. Harvesting of cereals produces a huge amount of agricultural residues. Major agricultural residues include rice straw, wheat straw, corn stover, and sugarcane bagasse (Kim and Dale, 2004). Meanwhile, animals manure holds a great potential as feedstock for biofuel production. Bioenergy production from animal manure is more than 5995 Mt of animal manure utilized as a feedstock for biofuel in 27 EU countries (Holm-Nielsen et al., 2009). Another important point with animal manure is that, if not managed properly, causes substantial environmental pollution. Of many disposal and recycling strategies, bioenergy from animal manure can be most attractive with many benefits such as preventing

environmental contamination, reducing greenhouse gases (GHGs) emission and gaining valuable energy as by-products (Paudel et al., 2017).

Table 2.1. Annual production and estimated energy potential of agricultural residues (adopted from Paudel et al., 2017).

Source		Crop/animal production (Mt)	Main residue type	Residue production (Mt)	Residue energy potential (Ej)
Cereal	Barley	141	Barley straw	51	0.4-1.5
	Corn	962	Corn stover	377	5.0-60.0
	Oat	23	Oat straw	10	-
	Rice	3363	Rice straw	658	2.0-22.3
	Sorghum	62	Sorghum straw	12	0.3-2.6
	Wheat	706	Wheat straw	472	2.5-9.5
	Sugarcane	1741	Sugarcane bagasse	1045	0.7-14.5
Animal	Cattle	1818	Cattle manure	4006	56.3
	Pig	1466	Swine manure	1382	21.0
	Poultry	28,213	Poultry manure	607	5.5

AD of sole energy crops result significantly low CH_4 yield since these crop materials are often rich in carbohydrates but low in nitrogen. Furthermore, mono-digestion of crops lacks the essential trace elements such as iron, cobalt, nickel, which are considered vital for methanogens. In this regard, the supplementation of nutrients and trace elements improves CH_4 yield and digestion stability. Meanwhile, mono-digestion of animal manure is also not recommended since it can cause instable digestion operation due to ammonia toxicity. Thus, anaerobic- co-digestion of carbohydrate-rich lignocellulosic biomass with nitrogen/trace element-rich animal manure leads significant affirmative implications to maintain optimal C:N ratio and achieves high CH_4 yields (Sawatdeenarunat et al., 2015). Some examples of anaerobic co-digestion processes are given in Table 2.2. A scheme of anaerobic co-digestion plant in which manure and lignocellulosic residues are co-digested to obtain energy and fuel is illustrated in Figure 2.2.

Table 2.2. Anaerobic co-digestion of selected lignocellulosic biomass and animal manure (adopted from Sawatdeenarunat et al., 2015).

Co-substrate	Reactor operation mode	Co-substrate mixing ratio	C:N ratio	CH_4 yield (L/Kg VS $_{added}$)
Swine manure and rica straw	Batch	2:1 VS basis	21.7	350
Chicken manure and corn stover	Batch	1/3 VS basis	27.3	298
Chicken manure and corn stover	Semi-continuous CSTR	1:1.4 VS basis	20.0	223
Chicken manure and agricultural waste	Batch	7.0/0.5/1.3/0.3 wet weight basis (Chicken manure/ coconut/ coffee grounds/ wheat straw)	17.1	506
Chiken manure, dairy manure and wheat straw	Batch	2.7/2.7/1.0 VS basis	25.0	235
Cattle slurry and raw cheese whey	Semi-continuous CSTR	1:1 wet weight basis	-	343
Cow manure and crop silage	Semi-continuous CSTR	4:1 wet weight basis	-	249

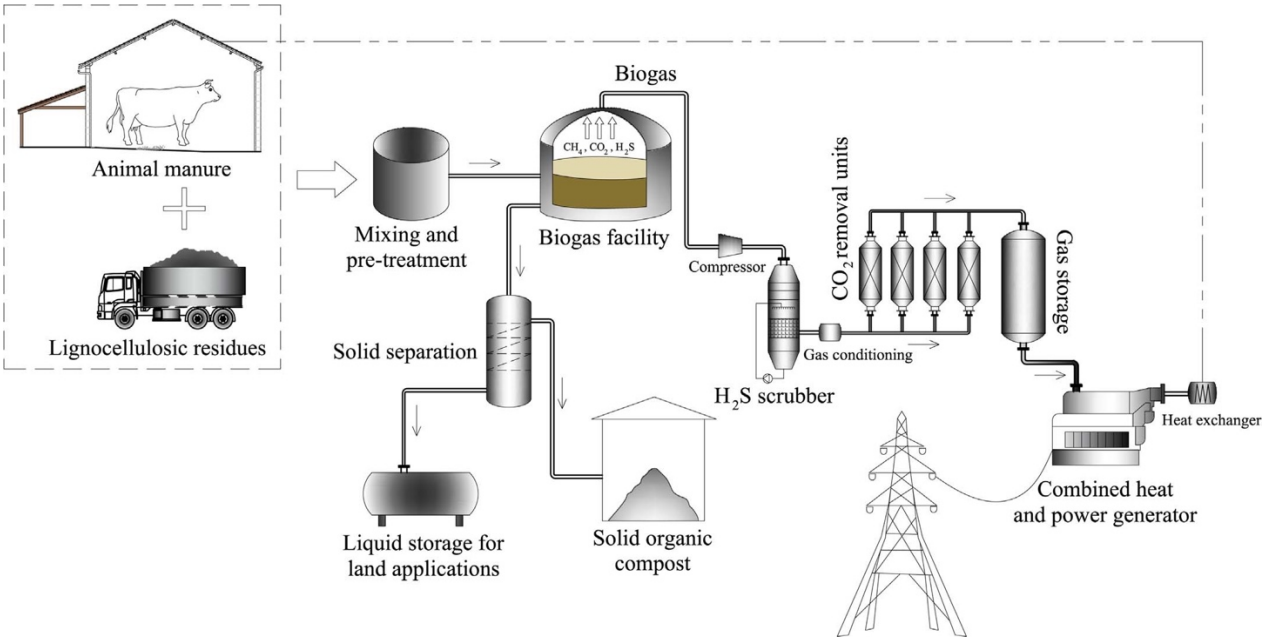


Figure 2.2. A scheme of anaerobic co-digestion process few with animal manure and lignocellulosic residues for biogas production and its potential applications (Neshat et al., 2017).

Table 2.3. Lignocellulose composition of agricultural residues adopted from (Paudel et al., 2017; Paul and Dutta, 2018; Sawatdeenarunat et al., 2015).

Source		Cellulose (%)	Hemicellulose (%)	Lignin (%)	C:N ratio
Cereal	Barley straw	38-48	21-25	11-26	71
	Corn stover	40	25-31	14-17	50-63
	Oat straw	33	23	21	95
	Rice straw	35-44	27-34	12-13	47-67
	Rye straw	38	36.9	17.6	20
	Wheat straw	38-42	20-27	20-22	50-60
	Sugarcane bagasse	40-45	20-24	25-30	118-150
Animal	Cattle manure	14.2-27.4	12.2-21.4	6.1-13.0	24
	Swine manure	13.2-13.9	20.4-21.9	5.1-6.4	17
	Poultry manure	7.7-12.0	16.4-21.5	4.1-7.2	10

A biorefinery process using lignocellulose needs various pretreatment steps due to the high level of crystallinity of cellulose, as well as the cross-linking of carbohydrates and lignin (Paul and Dutta, 2018). Methane yield obtained from AD process highly depend on the type and composition of the substrate, so that the characteristics of the lignocellulosic biomass affect the process efficiency as well as the selection of the pretreatment method. In lignocellulose-based AD, hydrolysis is the key rate-limiting step since lignocellulose is insoluble in water and has a complex and rough structure that resists mechanical stress and enzymatic attack (Paudel et al., 2017). Compared to cellulose and hemicellulose, lignin is more a recalcitrant component itself while forming tight bonds that reduces the surface area needed for enzymatic reactions to hinder the degradation of carbohydrates (Agbor et al., 2011) (Agbor, et al., 2011). Hence, pretreatment is a crucial process prior to AD to improve the biodegradability of carbohydrates and lignocellulose (Jönsson and Martín, 2016). Lignocellulose composition as well as C:N raio of some of agricultural residues are given in Table 2.3. A schematic diagram of various strategies to enhance hydrolysis of lignocellulosic biomass is shown in Figure 2.3.

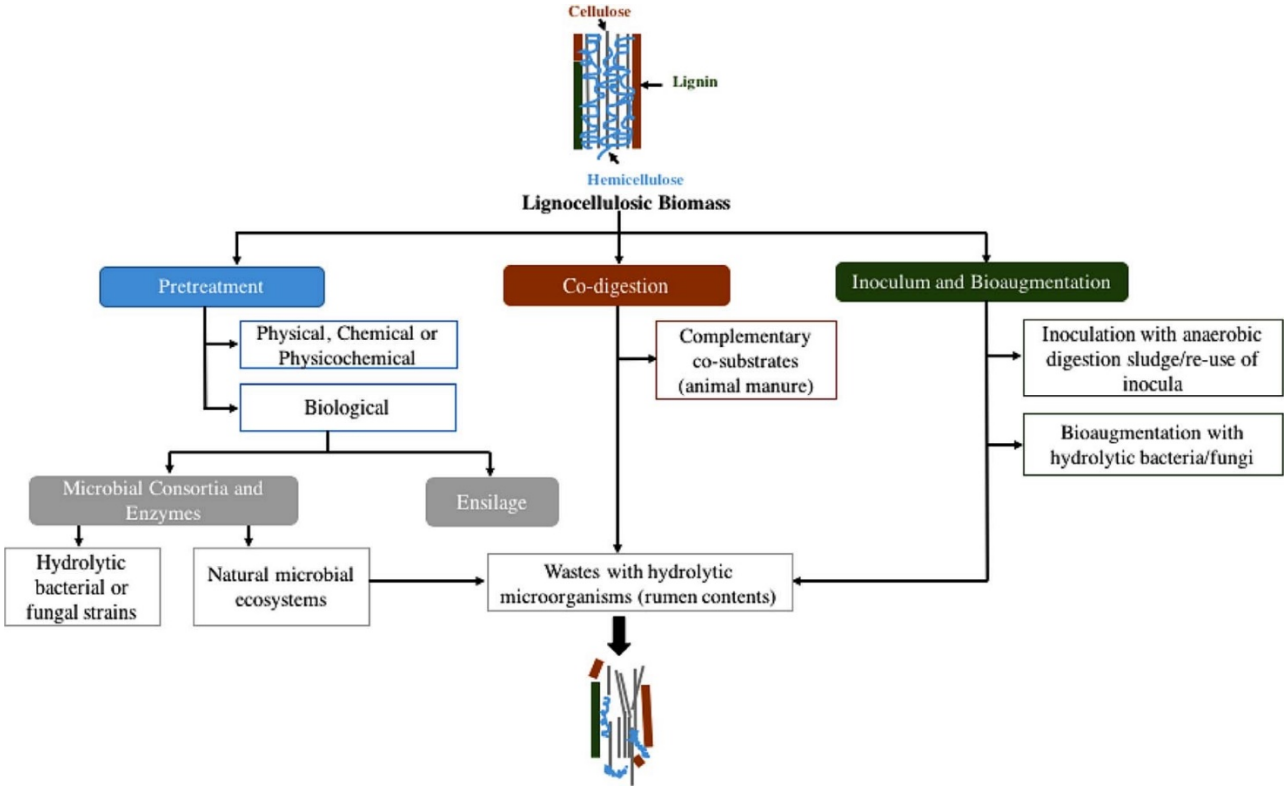


Figure 2.3. Shematic illustration of pretreatment, co-digestion, and inoculum and bioaugmentation strategies to enhance hydrolysis of lignocellulosic biomass (Shrestha et al., 2017).

2.2.1. Composition of Lignocellulosic Biomass

The major component of plant matter, cellulose, is an important source of biomass that stores large amount of energy through photosynthesis. It is widely found in the wooden structure of plant tissue as a fibrous, rigid and water-insoluble substance. It is a linear polymer in which 3,000-14,000 glucose monomers are linked via β-1,4 glycosidic bonds. Elementary fibrils are formed by 60–70 of those cellulose polymers interconnected with hydrogen bonds. These elementary fibrils build up and form microfibrils and then end up in supramolecular fibers network, which is a high tensile strength and a partially crystalline structure. The crystalline structure is an important feature of cellulose and the degree crystallinity varies depending the type of plant tissue. This structure restricts the degradation of cellulose and even permits the access of enzymatic attack, including cellulose (Wagner et al., 2018).

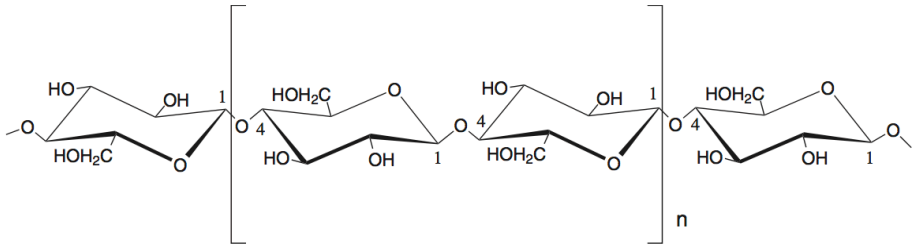


Figure 2.4. The structure of cellulose (Huang, 2013).

Hemicellulose comprises of branched heteropolysaccharides, mainly matrix polysaccharides, including monomers like glucose, mannose, galactose, xylose, and arabinose. Although similar enzymes are involved in the degradation of cellulose and hemicellulose, complete hemicellulose degradation requires more specific enzymes due to its greater chemical and structural heterogeneity (Malherbe and Cloete, 2002). Hemicellulose is degraded to monomeric sugars and acetic acid (Sánchez, 2009), which stand as the dominant methane precursor in AD (Wagner et al., 2018).

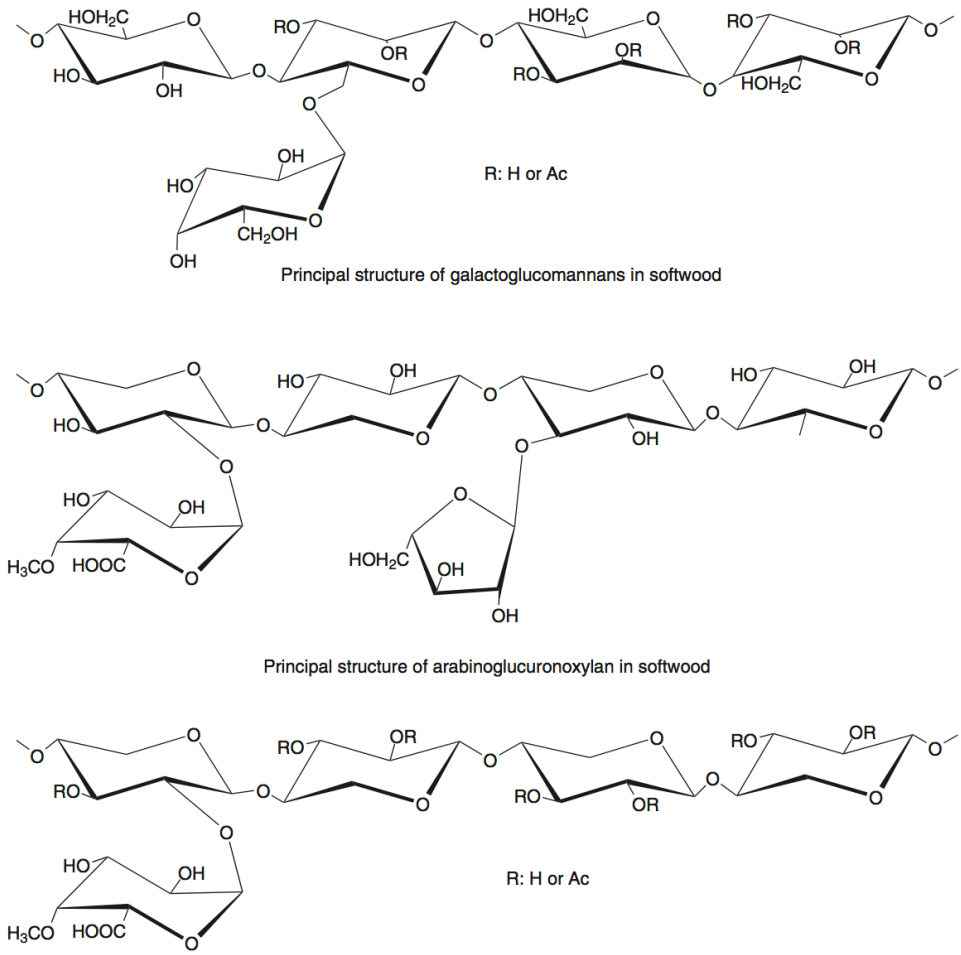


Figure 2.5. Principal polysaccharides in woody hemicellulose (Huang, 2013).

Lignin is an aromatic polymer synthesized of phenylpropanoid precursors. Cellulose and hemicellulose often come together with lignin to form lignocellulose, which acts as a physical seal, and is an impenetrable barrier in the plant cell wall structure. Free radicals released during the peroxide-mediated dehydrogenation of three phenylpropionic alcohols then synthesize lignin. The access to cellulose and hemicellulose is required to breakdown lignin, which only happens via co-metabolism (Sánchez, 2009).

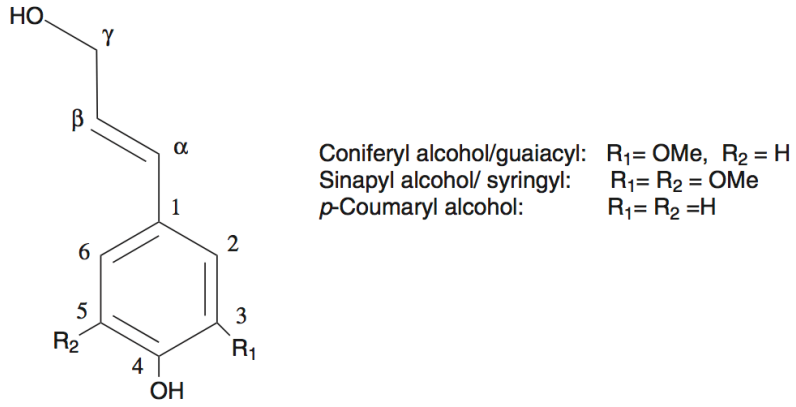


Figure 2.6. Three building blocks of lignin (Huang, 2013).

2.2.2. Insights into Microbiome of Lignocellulose-Based Anaerobic Digestion

Hydrolysis of lignocellulosic biomass often take place through the perfectly-coordinated action of phylogenetically diverse bacteria, acting as hydrolytic enzyme producers and end-product utilizers (Tsavkelova and Netrusov, 2012; van der Lelie et al., 2012). Fermentative bacteria usually refer to hydrolytic bacteria, along with acid-forming bacteria. They can either be facultative or obligate anaerobes, and a wide range of these phylogenetically diverse populations can be present in AD systems (Azman et al., 2015; Sun et al., 2013).

Anaerobic hydrolytic bacteria are mostly found within the phyla *Firmicutes* (genus: *Clostridium*, *Ruminococcus*, *Caldicellulosiruptor*, *Caldanaerobacter*, *Butyrivibrio*, *Acetivibrio*, *Halocella*, and *Eubacterium*), *Bacteroidetes*, *Fibrobacteres*, *Spirochaetes* (genus *Spirochaeta*), and *Thermotogae* (genus *Fervidobacterium* and *Thermotoga*) (Azman et al., 2015). In lignocellulose-based AD systems, lignocellulosic biomass degraders are mainly found in the phyla *Firmicutes* and *Bacteroidetes* (Sundberg et al., 2013), and bacteria of the class *Clostridia* are the principal group involved in lignocellulose hydrolysis (Rademacher et al., 2012; van der Lelie et al., 2012; Xia et al., 2014).

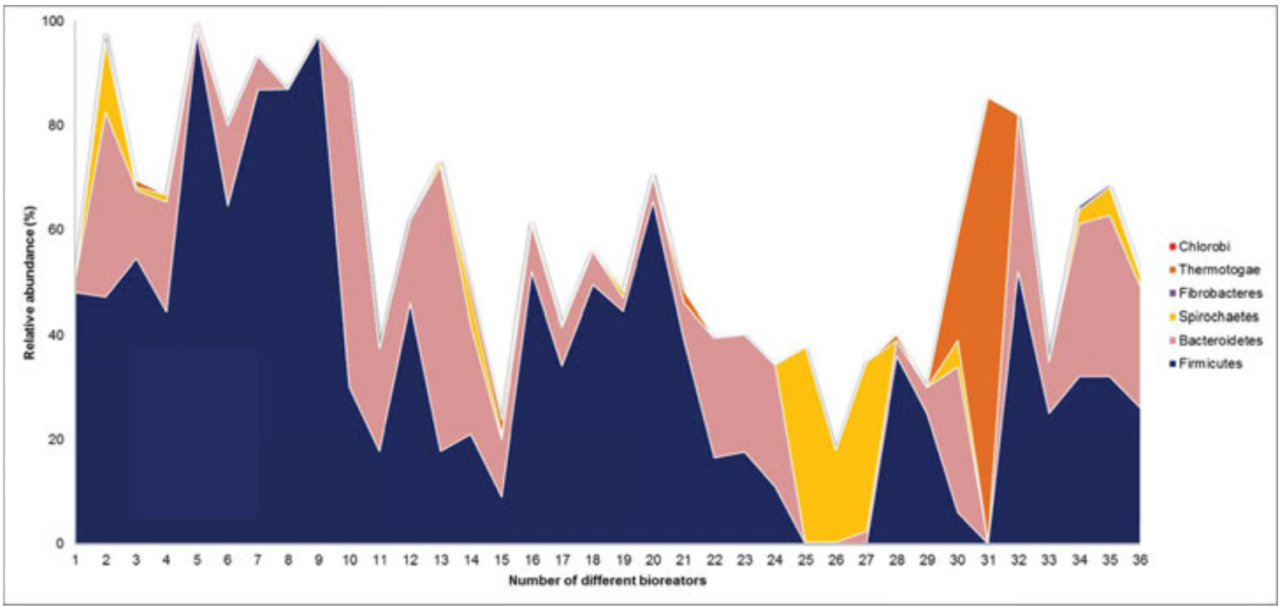


Figure 2.7. Stacked area graph showing the abundance of different phyla within 36 different anaerobic biogas reactors (Azman et al., 2015).

Microbial communities in AD systems have been investigated in the last decades usually by targeting phylogenetic marker genes (e.g., 16S rRNA gene), metabolic genes (e.g., *mcrA* gene), their transcripts and, more recently, by meta'omic approaches (i.e., metagenomics, meta- transcriptomics, metaproteomics, and metabolomics) (Bozan et al., 2017). While various molecular methods, primarily targeting 16S rRNA genes, have been employed since the 1990s to study the microbial community structure of AD systems (Talbot et al., 2008), the advances in next generation sequencing (NGS) methods, still primarily targeting 16S rRNA and a few metabolic genes (Ransom-Jones et al., 2012; Shrestha et al., 2017; Ziganshin et al., 2013), have allowed an increased resolution and identified low-abundance populations that were not previously recognized to be important in lignocellulose-based AD systems (e.g., *Fibrobacteres*). Moreover, metagenomics-based studies have allowed identification of microbial populations in AD systems down to the strain level and facilitated the study of metabolic potential of the microbial populations involved (Campanaro et al., 2016; Fontana et al., 2018; Xia et al., 2016).

There are well-established 6 orders of methanogenic archaea up to date, including *Methanobacteriales*, *Methanocellales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, and *Methanopyrales* (and also *Methanomassiliicoccales*, latter identified 7th *Euryarchaeal* order of methanogens) and placed in the phylum *Euryarchaeota*. This taxonomy is supported by comparative 16S rRNA gene sequence analysis and some distinct phenotypic properties, such as lipid concentration, substrate range, cell and shape (Felipe Sarmiento et al., 2011).

Methanobacteriales, *Methanococcales*, *Methanomicrobiales* and *Methanocellales* and *Methanopyrales* are hydrogenotroph and can reduce CO_2 to CH_4 using H_2 as the electron donor. In addition, a few representatives of the order *Methanosarcinales* are facultative hydrogenotrophs but primarily act as methylotrophs or acetotrophs, most studied and efficient methanogenic archaea are those belong to *Methanosarcina* sp. These species are also to perform all three metabolic pathways, namely acetoclastic, hydrogenotrophic and methylotrophic methanogenesis, making them versatile in methane generation (Nzila, 2017). Acetoclastic methanogenesis occurs in *Methanosarcinales* (e.g., *Methanosarcina* and *Methanosaeta*), in which *Methanosaeta* sp. can only do the acetoclastic methanogenesis (Fotidis et al., 2013). Some of acetoclastic *Methanosarcinales* and at least one member of *Methanomicrobiales* can also use methylotrophic pathway, in which methanol and methylamines serve as substrates. Methylyotrophic methanogenesis is mainly performed by the groups *Methanolobus* and *Methanococcus* (Nzila, 2017). A candidate methanogenic class, WSA2, has also been proposed and suggested to be restricted to methanogenesis through methylated thiol reduction (Nubo et al., 2016).

Activation of acetate to acetyl coenzyme A (acetyl-CoA) is required during acetate to methane conversion pathway. Acetolastic methanogen *Methanosaeta* contains high levels of acetyl-CoA synthetase which catalyzes the activation of acetyl-CoA. This activation is a one-step process that takes place in *Methanosaeta* and one of the five responsible enzymes is acetyl-CoA synthetase 1 (*Acs1*) (Akyol et al., 2015). Methyl-coenzyme M reductase alpha subunit encoding gene (*mcrA*) has also been frequently used as a perfect gene marker to track the distribution pattern of methylotrophic and hydrogenotrophic methanogens in anaerobic environments based on its comparatively conserved gene sequences (Zhou et al., 2014). mcrA catalyses the reduction of a methyl group bound to coenzyme-M, while releasing methane (Luton et al., 2002). Hence, the quantification of specific Acetyl CoA and/or *mcrA* genes and/or transcripts by quantitative polymerase chain reaction (qPCR) can be correlated with methane generation and could thus be used in AD studies (Maki et al., 2013).

2.2.3. Lignocellulolytic Enzymes in Anaerobic Digestion

Extracellular enzymes, such as cellulases and hemicellulases, are responsible for the hydrolysis of cellulose and hemicellulose into fermentable sugars during AD as summarized in Table 2.4. Cellulose-degrading enzymes have been defined as members of of glycoside hydrolases (GH), in which various GH families exist (i.e. GH 1, 3, 5, 6, 8, 9, 12, 44, 45, 48) (Berlemont and Martiny, 2013; Bernard et al., 2008). These GH families include at least three types of protein active on β-1,4 glycosidic bonds: (i) endocellulases, active on internal β-1,4 glucosidic bonds, (ii) exocellulases

degrading the polymer from its extremities and (iii) β-glucosidases producing glucose from cellobiose (Merlin et al., 2014). Of the hemicellulases, endo-1,4-β-xylanase cleaves the β-xylosidic bond between two β-xylopyranosyl residues, a crucial step in the breakdown of hemicellulose. The major enzymes responsible for the hydrolysis of xylan are xylanase (endo-1,4-β-d-xylanohydrolase) and β-xylosidase (1,4-β-d-xylohydrolase). Xylanase cleaves internal β-1,4 linkages of the xylan backbone. According to the Carbohydrate-Active Enzymes database (CAZy; www.cazy.org/) and on the basis of the sequence similarities of their catalytic domains, the majority of endo-1,4-β-xylanases are classified as GHs belonging to families 10 and 11, i.e., GH 10 and GH 11. Over the last years, numerous xylanases have been isolated and purified from typical rumen bacteria or from genome-wide sequencing of culturable ruminal bacteria, such as *Ruminococcus flavefaciens*, *Prevotella ruminicola*, *Clostridium thermocellum* , and *Fibrobacter succinogenes*. (Li et al., 2013).

Cellulases produced by some anaerobes assemble into a large multi-enzyme complex called cellulosome (Maki et al., 2009). Cellulosomes are mainly produced mainly by anaerobic bacteria belonging to the order *Clostridiales* (e.g., *Clostridium cellulolyticum*, *C. thermocellum*, *C. cellulovorans*) and *Ruminococcus* species. These enzyme systems may have evolved with respect to a more efficient cellulose degradation strategy, as they position cellulolytic cells at the site of hydrolysis, and also allow sufficient uptake of hydrolytic products by the cells producing these enzymes. Furthermore, it has been hypothesized that anaerobic environments may have contributed to the development of cellulosomes to counteract the low energy production of anaerobic fermentation; however, the nature of evolutionary drivers behind their formation is unclear (Fontes and Gilbert, 2010).

The resistance of lignin to biological degradation is one of the major obstacles for bioconversion of lignocellulosic biomass. As mentioned earlier, cellulases and hemicellulases do not facilitate degradation of lignin since lignin is a non-carbohydrate compound. It is generally believed that lignin cannot be degraded in anaerobic conditions since oxygen is required for enzymatic cleavage of the aromatic rings in lignin (Dollhofer et al., 2015). From this point of view, anaerobic microbes must be assisted in lignin removal by novel strategies so that cellulose and hemicellulose can be accessible to hydrolyse lignocellulosic biomass. Aerobic white-rot fungi and brown-rot fungi are famously known to possess enzymes involved in lignin biodegradation, such as peroxidases, laccase and other phenol-oxidizing enzymes, and H_2O_2 producing enzymes (Bugg et al., 2011). A few recent studies have also found evidence of lignin degradation in several anaerobic environments up to a certain limit (Billings et al., 2015; Shrestha et al., 2017).

Table 2.4. Enzymes involved in degradation of lignocellulosic biomass (Shrestha et al., 2017).

Substrates	Enzymes involved	Function	Products	Microorganisms
Cellulose	Endoglucanases	Cleave the cellulose chains at random internal amorphous sites	Glucose, cellobiose, cellotriose and other higher oligomers	Anaerobic bacteria in the genera *Acetivibrio, Anaerocellum, Butyrivibrio, Caldicellulosiruptor, Clostridium, Eubacterium, Fibrobacter, Flavobacterium, Fervidobacterium, Halocella, Ruminococcus, Spirochaeta*, and *Thermotoga*
	Exoglucanases	Attack the chain ends	Glucose, cellobiose	
	β-glucosidases	Cleave the last glycosidic bonds from the products of endoglucanases and exoglucanases	Glucose	
Hemicellulose	Endo-1,4-β-xylanase	Hydrolyse internal bonds in the xylan chain	Pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose, D-galactose)	
	1,4-β-D-xylosidase	Attack xylooligosaccharides		
	Endo-1,4-β-D-mannanase	Cleave internal bonds in mannan		
	1,4-β-D-mannosidase	Cleave mannoligosaccharieds		
	α-D-galactosidases, α-L-arabinofuranosidase, α-glucuronidases, acetyl xylan esterase	Remove side groups		
Lignin	Manganese peroxidase	Generates Mn^{3+}, which acts as a diffusible oxidizer on phenolic or non–phenolic lignin units via lipid-peroxidation reactions	β-arylether, di-arylether, biphenyl	White-rot fungi (e.g., *Phanerochaete chrysosporium, Phlebia radiata, Pleurotus* spp., *Trametes versicolor*) and brown-rot fungi (e.g., *Gloeophyllum trabeum, Laetiporus sulphureus, Serpula lacrimans*)
	Lignin peroxidase	Degrades non-phenolic lignin units		
	Laccase	Catalyze the oxidation of phenolic lignin units		
	Phenol-oxidizing enzymes, and H_2O_2 producing enzymes	Catalyze the degradation of lignin-derived compounds		

2.2.4. Pretreatment Methods for Anaerobic Digestion

For recalcitrant substrates such as lignocellulosic resources, conventional AD process cannot efficiently-undergo the substrate conversion into biogas. Agricultural wastes and/or by-products, such as cereal residues (straws, stalks and leaves), are lignocellulose-rich substrates that resist biodegradation and hence require pretreatment prior to AD (Mussoline et al., 2013). Animal manure, which is normally excreta of various types of livestock animals, also contains abundant lignocellulosic substances even though its content can be comparatively lower than cereal residues (Paudel et al., 2017; Tsapekos et al., 2016).

Pretreatment strategies commonly comprise physical, chemical, and biological methods (Zheng et al., 2014), and are applied in various fields of bioenergy and biofuel generation including biogas, bioethanol, biohydrogen, and hythane (H_2 + CH_4) production. Main factors for ecological and economical feasible application of these pretreatment strategies include low capital and energy investments, applicability over a wide variety of substrates, and high product yields to enhance revenues along with low waste treatment costs (Wyman et al., 2005).

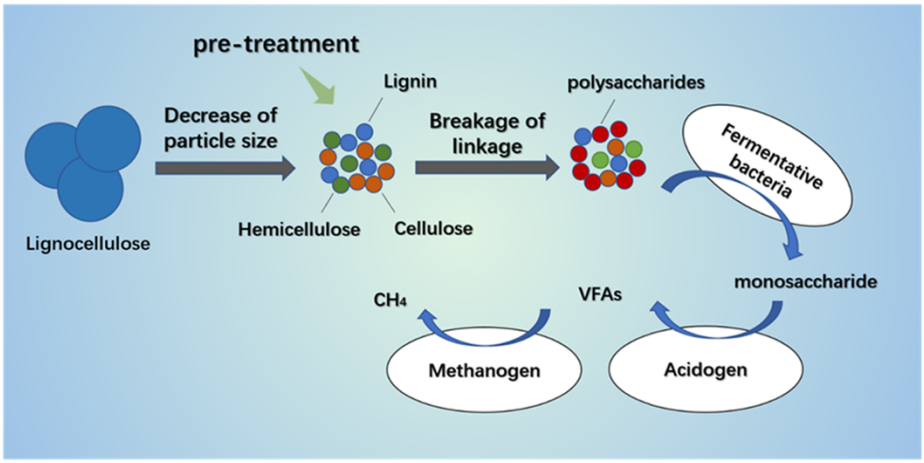


Figure 2.8. Schematic diagram of pretreatment enhancing methane production from lignocellulosic biomass (Li et al., 2019).

2.2.4.1. Mechanical pretreatment. Mechanical pretreatment refers to single and/or multiple application of various processes including grinding, milling, chipping, etc. The aim here is to disintegrate harsh soluble particles into smaller fractions; thus, the enlarged surface area is more accessible to anaerobic microbes and facilitates the AD process more efficiently (Elliott and Mahmood, 2012).

Table 2.5. Mechanisms and performance of various pretreatment methods used for AD of agricultural biomass (Paudel et al., 2017).

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

Table 2.5. Continued.

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

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

Several studies in literature have reported that particle size is inversely proportional to substrate utilization rate by microbes and consequently the methane production rate (Esposito et al., 2011). Reduction in particle size of crop residues increases the availability of substrate for hydrolysis (Ward et al., 2008). The selection of the process depends on the substrate characteristics as well as AD configuration. For instance, shearing instead of cutting of fibres was reported to be more effective on AD performance by increasing the surface area (Paudel et al., 2017).

2.2.4.2. Chemical pretreatment. Chemical pretreatment uses a variety of acids, alkalis, or oxidants to extract or break down organic fraction in biomass (Ariunbaatar et al., 2014). The initial purpose of most chemical pretreatment methods is the destruction of rigid organic biomass structures by cleaving the lignin-carbohydrate link and cellulose crystalline matrix, or the hydrolysis of hemicellulose. Strong acid treatment using H_2SO_4, HNO_3, H_3PO_4, and HCl at high severity, causes the inhibition of AD process through the production of unwanted by-products such as furfural and its derivatives (Mussoline et al., 2013). Furthermore, the use of such strong acids also causes an excessive degradation of the substrates resulting in loss of fermentable sugar. From an economic point of view, it negatively impacts AD operation by leading to corrosion and requiring additional chemicals for neutralization (Taherzadeh and Karimi, 2008). In this regard, dilute acids (< 4% w/w) are commonly used for acid pretreatment. Acid pretreatment is often coupled with high temperatures (> 100 °C), called thermo-chemical pretreatment (Agbor et al., 2011). Although acid pretreatment methods are available for enhancing the biodegradability of organic waste (Ariunbaatar et al., 2014), alkali treatment is more likely to be applied because alkali conditions provide a better environment for AD by preventing pH to drop (Li et al., 2012).

2.2.4.3. Thermal pretreatment. Treatment of organic compounds within a wide temperature range (50–250 °C) potentially enhances the anaerobic biodegradability. In addition to enhance biodegradability, the thermal pretreatment can also eliminate the pathogens from waste material. This is why thermal pretreatment is commonly preferred and applied on a large scale for the pretreatment of sewage sludge and organic fraction municipal solid wastes (OFMSWs), as well as agricultural biomass (Cesaro and Belgiorno, 2014). Hydrothermal, steam explosion, and microwave heating are some of the typical thermal pretreatment methods applied for enhancing the biodegradation of lignocellulosic biomass. Although thermal pretreatment has lots of advantages in terms of digestibility of organic substrates, extended pretreatment at high temperatures can unexpectedly trigger reactions to form complex recalcitrant or inhibitory substrates and eventually ceases the biogas production (Elliott and Mahmood, 2012; Hendriks and Zeeman, 2009). Therefore, the temperature and time of the thermal pretreatment should be thoroughly determined depending on the substrate

characteristics. In addition, hydrothermal pretreatment, steam pretreatment/the steam explosion method is also conducted to increase degradation of lignocellulosic biomass (Paudel et al., 2017).

2.2.4.4. Biological pretreatment. High energy input together with chemical requirements in most of mechanical, chemical and thermal pretreatment methods make them economically unfeasible, while severe temperature, pH, and inhibitory by-products cause environmentally unfavorable conditions which tend to thwart AD performance (Singh et al., 2008). On the other hand, biological pretreatment outshine these methods with its comparatively economical with reduced energy requirements and chemical expenses; it is also environmental-friendly as it does not produce inhibitory by-products. The net energy output through microbial pretreatment approaches is substantially more and relatively cost-effective compared to other established chemical and mechanical approaches. Industrial enzymes, lignolytic enzymes, and cellulase are often utilized to break down lignocellulosic biomass (Nichols et al., 2006). However, external addition of these commercial enzymes is not usually feasible due to high unit price and large required dose of each enzyme in bioreactors. Industrial enzymes, lignolytic enzymes, and cellulase can be employed to break down the lignocellulotic components (Nichols et al., 2006). External addition of these commercial enzymes is not economically advantageous due to high unit price and huge amounts of enzyme requirements. Hence, indirect addition of such enzymes via a biological route can be practically applied, for example the application of rot-fungi capable of secreting such extracellular lignocelluloytic enzymes (Sánchez, 2009). As microbial enzymes are capable of degrading lignocellulosic compounds, biological pretreatment is known to be suitable for lignocellulosic biomass even though its enzymatic reaction rate is very slow. The majority of studies conducting biological pretreatment of lignocellulosic biomass prior to AD uses various various types of fungi (Paudel et al., 2017).

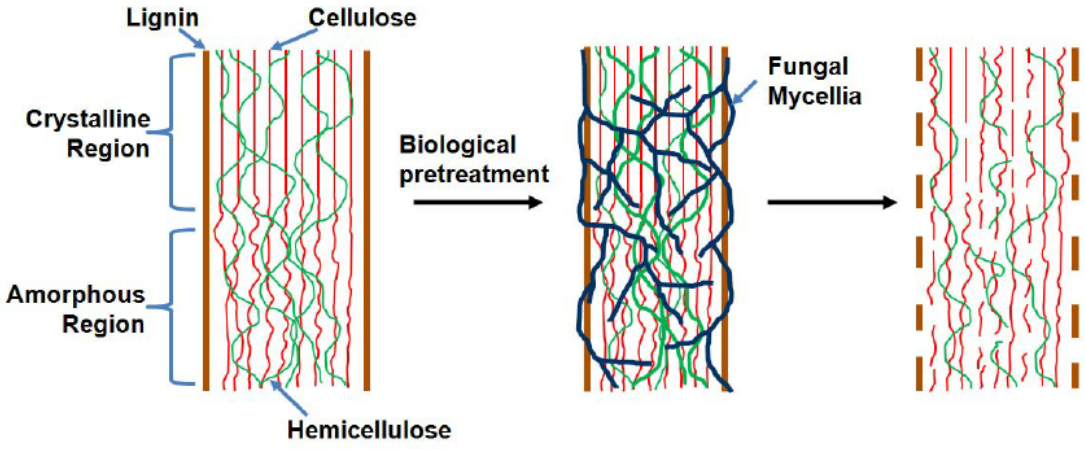


Figure 2.9. Effect of biological pretreatment on lignocellulosic structure.

Table 2.6. Enzymatic and microbial consortia pretreatment strategies applied to lignocellulosic biomass (adopted from Shrestha et al., 2017).

Substrate	Pretreatment	CH_4 yield (mL CH_4/g VS)	Methane improvement (%)
Sugar beet pulp and vinasse (3:1; w:w)	Enzymes (Celustar XL and Agropect pomace)	465	33
Switchgrass	Manganese peroxidase	223	34
Lignocellulose fraction of municipal solid wastes	Microbial consortium (*Clostridium straminisolvens* CSK1, *Clostridium* sp. FG4 b, Pseudoxanthomonas sp. strain M1-3, *Brevibacilus* sp. M1-5, and *Bordetella* sp. M1-6	221	126
Napier grass	Microbial consortium (Mainly composed of mesophilic anaerobic bacteria in the genera *Clostridium*, *Bacteroides*, *Alcaligenes*, and *Pseudomonas*)	278	50

Although it is not directly considered a pretreatment approach, bioaugmentation is widely applied in AD processes as an alternative biological treatment while it refers the addition of specific microorganisms to a reactor to enhance a particular microbial activity (Astals et al., 2016). This strategy has been considered to enhance the hydrolysis rate of AD of lignocellulosic biomass (Martin-Ryals et al., 2015; Peng et al., 2014). Anaerobic microbial consortia capable of degrading lignocellulosic biomass can be cultured from a wide range of sources such as the digestive tracts of ruminants (Shrestha et al., 2017).

2.3. Fungal Treatment Approaches for Lignocellulosic Biomass

In natural ecosystems, primarily fungi carry out the bioconversion of lignocellulosic residues and/or substrates. This mechanism occurs exocellularly or extracellulary due to the insolubility of cellulose, hemicellulose and lignin (Fliegerová et al., 2010). There are two types of fungal enzymes that break down lignocellulose: (i) the hydrolytic system that produces hydrolases responsible for polysaccharide degradation and (ii) a unique oxidative and extracellular ligninolytic system degrading lignin by opening phenyl rings (Dollhofer et al., 2015).

Cellulose degradation is predominantly carried out by aerobic order *Actinomycetales* (phylum *Actinobacteria*) and the anaerobic order *Clostridiales* (phylum *Firmicutes*) (Shrestha et al., 2017).

Mechanisms of bacterial decomposition differ significantly from those of their fungal counterparts. For instance, within cellulolytic *Clostridia*, the breakdown of cellulose is organized in the cellulosome, which is placed onto the cell surface, contains all necessary cellulolytic enzymes, and forms a bridge between the cell and the insoluble cellulose substances (Desvaux, 2005). In AD systems, cellulose-degrading bacteria play a crucial role with respect to the interaction between the existing microbiome, resulting in an almost-complete conversion into carbon dioxide, methane, and water (Wagner et al., 2018). However, due to the small amount of energy that can be preserved in anaerobic processes and the lower productivity of bacterial cellulases compared to fungal ones (Adney et al., 1991), the degradation of cellulose is much slower under anoxic conditions than oxic conditions.

A specialized group within the *Neocallimastigomycota* called "anaerobic fungi", commonly found in ruminants, is capable of degrading cellulose and hemicellulose under strictly anaerobic conditions. An earlier investigation was done by (Nakashimada et al., 2000) through methane production from cellulose as a substrate with defined mixed cultures using the cellulolytic *Neocallimastix frontalis* and methanogens. The use of anaerobic fungi for an enhanced AD was reported, e.g., by (Dollhofer et al., 2015; Nkemka et al., 2015; Procházka et al., 2012). In contrast to anaerobic fungi, the direct utilization of aerobic fungi in anaerobic systems is not possible due to their oxygen demand. Among fungi, there are a number of representatives, e.g., of the genera *Fusarium* and *Chaetomonium* that also target lignin-encrusted cellulose. In particular, white rot fungi can effectively degrade lignin using an oxidative process with phenol oxidases and laccase as the key enzymes (Rabinovich et al., 2004), including *Phanerochaete chrysosporium* and *Trametes versicolor*, representing the most extensively studied members (Rouches et al., 2016a). As the degradation of lignin is hardly possible under anoxic conditions (Brown and Chang, 2014), aerobic pretreatment prior to AD is of special interest in recent years (Hom-Diaz et al., 2016; Shirkavand et al., 2017).

2.3.1. Fungal Pretreatment with Aerobic Fungi

The natural wood decaying capacity of white-rot, brown-rot and soft-rot fungi paved the way for identifying them as potential groups for efficient biological pretreatment agents for lignocellulosic feedstocks (Ghosh et al., 2017; Martínez-Patiño et al., 2018; Shrestha et al., 2017). Aerobic fungi that are able to degrade lignocellulosic biomass can be sorted into brown-, white-, and soft-rot fungi. Brown-rot fungi, known as *Basidiomycetes*, are able to attack cellulose and hemicelluloses with leave the lignin content only with small modifications (Sánchez, 2009). Therefore, lignin degradation is limited upon a brown-rot fungi attack. The remaining lignin comprises a greater number of ring

hydroxyl groups and is demethylated on arylmethoxy groups (Wan and Li, 2012). In some cases, brown-rot fungi can be efficient for lignocellulosic substrate pretreatment; however, white-rot fungi are mostly preferred since these organisms are much more efficient in delignification (Mueller and Troesch, 1986; Sánchez, 2009). White-rot fungi have the capacity to attack phenolic structures and to transform lignin into CO_2 thanks to their unique enzymatic system. Degradation mechanisms by soft-rot fungi (*Asomycetes* and *Deuteromycetes*), on the other hand, are not well-known (Rouches et al., 2016a; Sánchez, 2009).

White-rot fungi use extra-cellular enzymes that form a hydrolytic (hydrolases) and a ligninolytic system while attacking lignocellulose (Rouches et al., 2016b). This unique system comprises three major oxidizing enzymes: lignin peroxidase (LiP or 'ligninase'), manganese peroxidase (MnP) and laccase (or phenoloxidases). Not all white-rot fungi produce all these enzymes. They can have either one or three of them or different combinations (Dashtban et al., 2010). Other peroxidases have also been reported: versatile peroxidase (VP) (Martínez et al., 2005), manganese independent peroxidase (MiP) (Moreira et al., 1997), dye- decolorizing peroxidase (DyP) and aromatic peroxygenase (APO) (Liers et al., 2011). Some concerns regarding these accessory oxidases are as follows: some of them lead to H_2O_2 generation that is used by peroxidases, while accessory oxidases affect several kinds of substrates such as glyoxal, aromatic alcohols, etc. (Rouches et al., 2016a) .

A quite number of researchers have conducted fungal pretreatment using white rot fungi (i.e. *Trametes versicolor*, *Leiotrametes menziesii*, *Flammulina velutipes*, *Ceriporiopsis subvermispora, Trichoderma reesei, Stereum hirsutum*) treating various lignocellulosic biomass (Canam et al., 2011; Hom-Diaz et al., 2016; Lalak et al., 2016; Mustafa et al., 2016; Rouches et al., 2016b; Shirkavand et al., 2017; Zhao et al., 2014). Among these studies, fungal pretreatment with *T. versicolor* was found to contribute remarkable losses in lignin, cellulose and hemicellulose (Shirkavand et al., 2017) and further achieve an increase in methane yield up to 74% during AD (Hom-Diaz et al., 2016). Even though pretreatment strategies have been extensively studied, most of them have been evaluated in batch experiments only or have used one microbial consortium for different substrates (Shirkavand et al., 2017; Tišma et al., 2018; Yuan et al., 2014b). Environmental and operating conditions greatly affect the growth of microorganisms. Hence, future studies should shift from lab-scale batch experiments to continuous reactors and focus on the frequency of inoculating microbial consortia (i.e. bacteria, fungi and/or enzymes) and its economic feasibility. The selection of a particular biological pretreatment approach should be based not only on product yield but also the inoculation frequency and cost associated with it (Shrestha et al., 2017).

Table 2.7. Comparison of different white-rot fungal pretreatment strategies for enhanced biogas production (adopted from Wagner et al., 2018).

White rot fungi	Substrate	Pretreatment conditions	AD conditions	Impact of pretreatment on substrate	Impact of pretreatment on biogas production
Phanerochaete chrysosporium	Corn stover silage	30 days, 28 ^{o}C	Batch, mesophilic	39% lignin removal of initial substrate, improved degradation of substrate cell wall components	19.6–32.6% increase in methane production
Fusarium sp.	Paddy straw	10 days, 30 ^{o}C , MC 70%	Batch, mesophilic	17.1% decrease in lignin content, 10.8% decrease in silica content compared with controls	53.8% increase in biogas production
Trametes versicolor	Corn silage	7 days, 27 ^{o}C, MC 70–80%	Continuous, mesophilic, co-digestion cow with manure	70% increase in lignin degradation compared with control approach	Increased pH stability and biogas productivity, enhanced anaerobic degradation
Ceriporiopsis subvermispora	Yard trimmings	30 days, 28 ^{o}C, MC: 60%	Batch mesophilic	20.9% degradation of initial lignin content	54% increase in methane production, increased cellulose degradation
Polyporus brumalis	Wheat straw	12.5 to 20 days, 20–30 ^{o}C	Batch, mesohpilic	-	Decrease in methane production compared with the control. Within fungal pretreatment, best methane production after 12.5 days incubation.

2.3.2. Fungal Bioaugmentation with Anaerobic Fungi

The microbiota of the fermentation chamber of the ruminant gut include prokaryotic (bacteria and archaea), and eukaryotic (protozoa and fungi) organisms (Bayané and Guiot, 2011) where a perfect symbiotic association takes place (Yue et al., 2013). Anaerobic fungi have significant role in the degradation of lignocellulosic substrates (Gruninger et al., 2014). They belong to the phylum *Neocallimastigomycota*, are the most basal lineage of the kingdom Fungi. The phylum contains only one order (*Neocallimastigales*) and one family (*Neocallimastigaceae*) in which 8 genera are described to date: The monocentric rhizoidal genera *Neocallimastix*, *Piromyces*, *Ontomyces* and *Buwchfawromyces*, the polycentric rhizoidal genera *Anaeromyces* and *Orpinomyces*, and the two bulbous genera, monocentric *Caecomyces* and polycentric *Cyllamyces*, respectively. These fungi exist in the digestive tracts of larger mammalian herbivores (or ruminants) and termites, where they play an important role as primary degraders of ingested fibers. Some of their most distinctive features based their obligatory anaerobic physiology include that mitochondria, cytochromes and other biochemical features of the oxidative phosphorylation pathway are absent. Energy generation occurs in hydrogenosomes where ATP is formed by malate decarboxylation to form acetate, CO_2, and H_2. *Neocallimastigales* do not require molecular oxygen for any of their physiological processes, so that the presence of oxygen is toxic. These anaerobic fungi have unique defence mechanism against the toxic effects of oxygen, for instance when degrading freshly ingested forage or during dispersal between host animals. Furthermore, their genomes are privileged for having the highest AT-content detected ever and a substantial expansion of important hydrolytic and cellulolytic gene families (Dollhofer et al., 2015).

In recent years, anaerobic fungi and their enzymes have been extensively studied as they can efficiently degrade crystalline cellulose (Morrison et al., 2016b). Anaerobic fungi can possess cellulases, including glycoside hydrolase 1 (GH1), GH3, GH5, GH6, GH8, GH9, GH16, GH31, GH45, and GH48 gene families (Grigoriev et al., 2016; Youssef et al., 2013). Anaerobic fungi have been reported to produce all xylanase, xylosidase, mannase, and β-glucanase enzymes to degrade xylans, mannans, and β-glucans, respectively, which can degrade hemicellulose to monosaccharides. Anaerobic fungi can produce a variety of hemicellulases including GH2, GH10, GH11, GH31, GH39, GH43, GH74 (Cheng et al., 2018; Grigoriev et al., 2016). For instance, the lignocellulolytic enzyme "cocktail" indicates rumen anaerobic fungi (i.e. *Orpinomyces* sp.) as a remarkable biomass degrader and, make them available as promising biological agents in AD (Couger et al., 2015; Youssef et al., 2013). Genome sequencing of *Orpinomyces* strain C1A revealed a highly diverse of these enzyme "cocktail" compared to aerobic fungi with a repertoire of 357 glycosyl hydrolases, 92 carbohydrate

esterases and 24 pectate lyases (Morrison et al., 2016b). One of the main reasons suggested for anaerobic fungi to have such robust and impressive cellulolytic and hemicellulolytic capability is horizontal gene transfer from bacteria. Furthermore, anaerobic fungi are the only fungi which possess cellulosomes. Description of GH39-family enzyme from anaerobic fungus *Orpinomyces* sp. strain C1A showed that the hydrolysis yields ranged between 65.0-77.4% using pretreated corn stover and switch grass as substrates(Morrison et al., 2016a).

Table 2.8. Examples for lignocellulosic residues degraded by anaerobic fungi (Dollhofer et al., 2016).

Lignocellulosic residue	Lignin content (%)	Organism
Wheat straw	16-21	*Neocallimastix frontalis*
Coastal Bermuda grass	6.4	*Piromyces* MC-1, *Orpinomyces* PC-1-3, *Neocallimastix* MC-2
Sugar cane bagasse	19-24	*Piromyces* strain E2
Hard wood	18-25	*Neocallimastix* sp.
Rice straw	18	*Piromyces* M014, *Orpinomyces* GSRI-001, *Neocallimastix* T010

Close association between anaerobic fungi and methanogens is well-known (Dollhofer et al., 2015), where hydrogen transfer within species leads to methane production as well as more efficient re-generation of oxidized nucleotides (NAD^+, $NADP^+$). Such syntrophic co-cultivation marked an increase in fungal growth rate, which further contributes to increased rates of cellulolysis and xylanolysis and organic matter reduction (Cheng et al., 2009). However, the interaction between anaerobic fungi and methanogens is more complicated than a simple cross-feeding mechanism. Hydrogen transfer is another parameter to influence fungal catabolic pathways and specific enzyme profiles, shifting fungal product formation away from more oxidized end products (lactate, ethanol) towards production of more reduced products (acetate, formate). This is in favour for AD since these are preferred growth substrates for methanogens (Nakashimada et al., 2000). Syntrophic interactions between acetogenic bacteria and methanogens are also well known to occur in the biogas microbiome (Angelidaki et al., 2011). Taking into account that anaerobic fungi show enhanced growth in the presence of methanogens, the idea of augmenting biogas reactors with this microbial group seems a promising alternative to enhance anaerobic biodegradability of lignocellulosic biomass (Dollhofer et al., 2015).

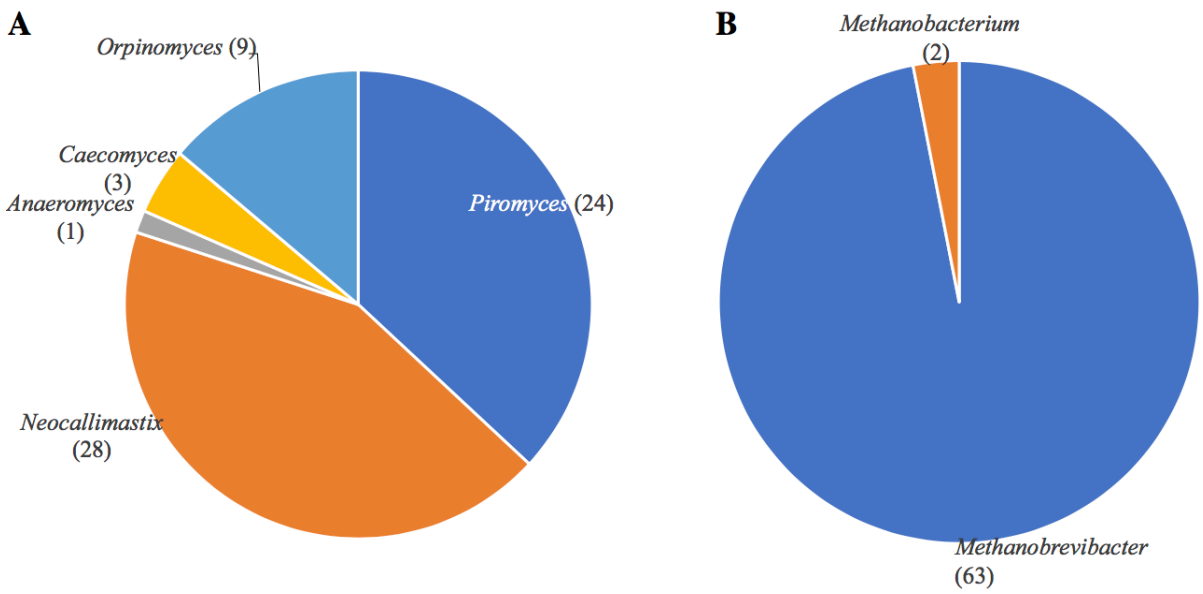


Figure 2.10. Anaerobic fungus and methanogen in the simple co- cultures from rumen and feces of herbivores. a) Anaerobic fungus b) Methanogen (Cheng et al., 2018).

2.4. Fungi-Assisted Anaerobic Digestion of Lignocellulosic Biomass

Biogas reactors are often designed mimicking natural ecosystems to promote AD of lignocellulosic biomass. Several important points are taken into account to ensure optimal growth and proliferation of appropriate microbial populations while studying these natural systems, such as, synergistic relationship between microorganisms, various physical–chemical mechanisms as well as nutritional requirements. Besides cost-effectiveness due to low chemical and energy requirements, the byproducts generated from biological pretreatment are slightly inhibitory or non-inhibitory to the subsequent stages of AD. This is attributed to the fact that biological pretreatment occurs under milder conditions and doesn't support the formation of more complex compounds (Sindhu et al., 2016).

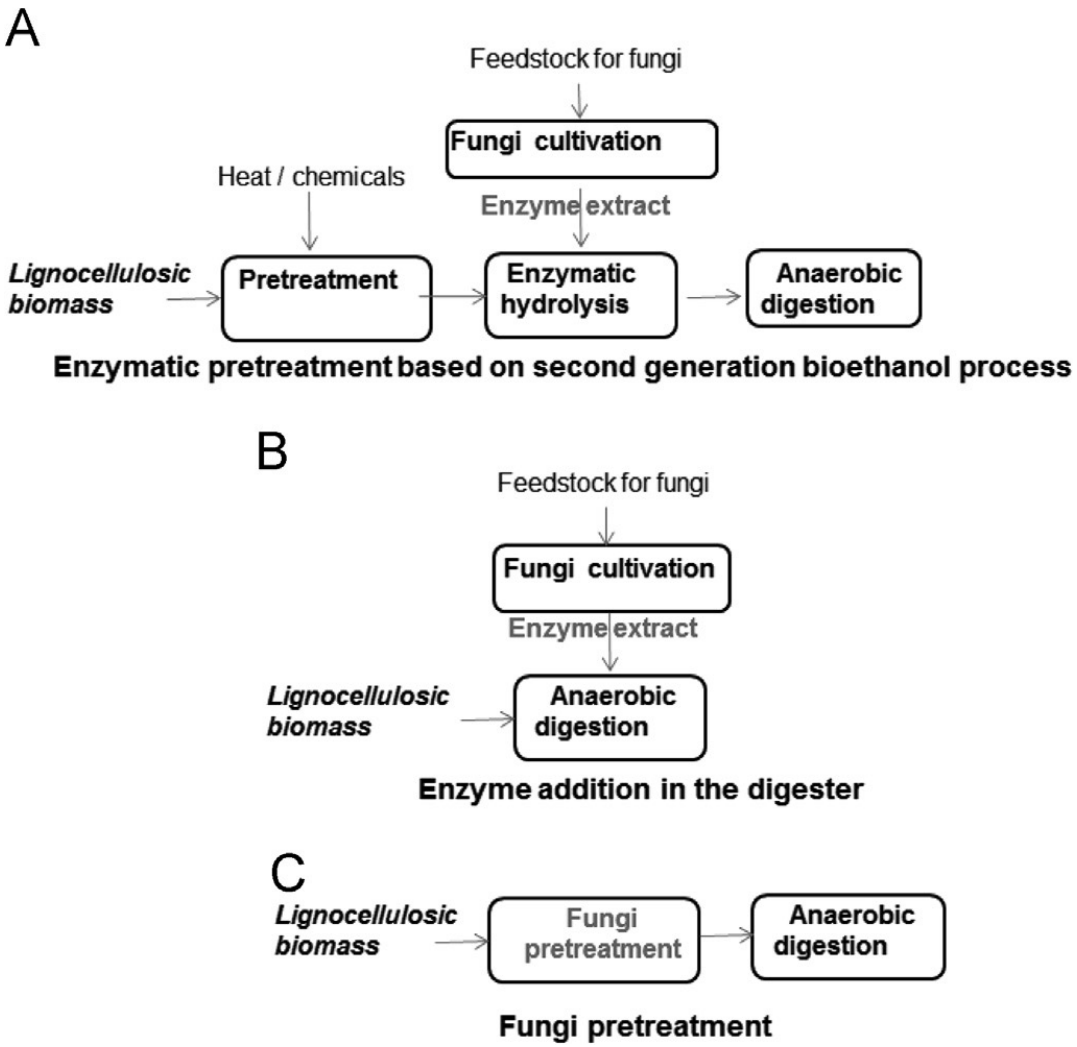


Figure 2.11. Strategies for the combination of enzymatic or fungal pretreatments and anaerobic digestion of lignocellulosic biomass (Rouches et al., 2016a).

In the previous section, affirmative effects of pretreatment using white rot fungi on the break down of lignocellulosic substrates were mentioned. However, only a few studies have highlighted the beneficial delignification effect of a pretreated lignocellulosic substrate for further methane production. On average, it only seems possible to improve the methane yield by 50% with a pretreatment period of 30 days; meanwhile, some authors observed not only an increase in the biogas production but also a higher methane content in the produced biogas. When the pretreatment increases the anaerobic degradability, the prior production of VFAs is sometimes increased naturally (Jalc et al., 2008; Rouches et al., 2016a). In another study, pretreatment of sisal leaf using *Trichoderma reesei* resulted in increases of biogas yields by 30–40% (Muthangya et al., 2009). (Yuan et al., 2014a) reported that biological pretreatment with three microbial consortia (fungi, *Coprinus cinereus* and *Ochrobactrum* sp.) supported saccharification and AD of Napier grass resulting 1.49 times greater methane yield than that of the untreated grass (Paudel et al., 2017).

Table 2.9. Comparison of white rot fungi pretreatments with other lignocellulosic biomass pretreatment techniques for AD (Rouches et al., 2016).

Pretreatment		Main impact on lignocellulosic biomass	Impact on methane yield	Additional remarks
Biological	White-rot fungi	Some selective lignin degradation but sugar losses may occur	0–600% increase in pretreated matter biodegradability but methane potential per initial TS may decrease	Able to decrease the concentration of certain inhibitors
	Ensiling (several weeks to several months)	Conversion of soluble sugars into acids, organic matter losses may occur	0–50% increase of pretreated matter biodegradability but loss of methane potential can occur	Widely used for crop storage
	Enzymatic (35 °C, few days)	Macromolecules hydrolysis	0–34 % increase	Enzymes may require a sterilization step or they can also be introduced in the digester
Mechanical	Grinding	Size reduction	Up to 80% improvement but may reduce methane yield	Used to ease full scale digester feeding
	Extrusion (60–90 °C, few MPa, few min)	Size reduction	Up to 70% improvement	Used in some full-scale plants
Thermal and thermo-chemical	Hydrothermal pretreatment/steam explosion (150–220 °C), few min, may be catalysed by acids	Hemicellulose solubilization and alteration of lignin structure	Up to 220% increase	Steam explosion is used in codigestion in some full-scale plants.
	Thermo-acid pretreatment (150–220 °C, few % acid, from few min to 1h	Hemicellulose and cellulose solubilization	Up to 200% increase	Need of heat energy (a part of biogas production)
	Thermo-alkali pretreatment (from room temperature to 50–70 °C, few % soda, or lime, higher dose of ammonium hydroxide, 0.5 to few days)	Lignin degradation	Up to 200% increase	Presence of soda is detrimental for digestate valorization, ammonium hydroxide must be recovered
	Wet oxidation (180–220 °C, few min) or oxidation with H_2O_2 (room temperature to 220 °C, few min to days)	Lignin degradation and hemicellulose solubilization	Up to 140% increase	-

Fungal pretreatment seems to be particularly efficient on wood biomass with a high lignin content (and low methane potential). The degree of enhancement in methane production should be thoroughly evaluated with respect to the loss of substrate during the pretreatment period. On one hand, if carbohydrate losses are too high, the pretreatment can negatively impact methane production (Verstichel et al., 2013). On the other hand, another possible benefit of fungal action would be the enhancement of AD kinetics (Mueller and Troesch, 1986). Despite being advantageous in several aspects, fungal pretreatment has several major drawbacks such as requiring careful growth conditions, larger space, longer treatment times, and loss of cellulose (Rouches et al., 2016a; Sindhu et al., 2016).

Microbial community analysis can be used to assess if the microorganisms used during fungal pretreatment remain present and continue to affect AD performance and microbiome. (Gagliano et al., 2015) observed that *Coprothermobacter* and *Methanobacteriales* were present at higher levels in the reactor fed with the pretreated waste activated sludge. The presence of these two populations was positively correlated with high concentrations of soluble protein in the pretreated feedstock. The pretreatment efficiency can also be affected by microbial contamination as observed by (Dhouib et al., 2006), who reported the overgrowth by bacteria suppressed the growth of fungi in a pretreatment reactor. In such cases, microbial community studies can help to assess synergisms or antagonisms taking place during pretreatment and further in biogas reactors (Shrestha et al., 2017).

While the use of aerobic fungi during pretreatment has been extensively studied, the use of anaerobic fungi has not been explored in detail. Only few studies that used anaerobic fungi exhibited improvements in biogas production via bioaugmentation approach, possibly due to out-competition of fungi by other microbial populations (Nkemka et al., 2015; Procházka et al., 2012). Bioaugmentation of lignocellulose-based AD processes has been successfully applied using a cellulolytic microorganisms, which achieved remarkable affirmation on methane yield (Nzila, 2017). Most of these studies focused on bacterial bioaugmentation using either single species (Öner et al., 2018; Tsapekos et al., 2017b) or mixed culture of bacteria (Martin-Ryals et al., 2015; Ozbayram et al., 2018), whereas few studies implemented methanogenic bioaugmentation (Fotidis et al., 2014) or fungal bioaugmentation (Ferraro et al., 2018; Kazda et al., 2014). One of the first attempts that augmented AD with anaerobic fungi to enhance methane production from energy crops was made by (Procházka et al., 2012). This approach was further combined using *Piromyces rhizinflata* YM600 with hydrogen and methane production in a two-stage system fed with corn silage and cattail (Nkemka et al., 2015). This method improved methane production and reduced digestion time, but only for a short time period, as the growth conditions became unfavourable for the fungi. Lately, mix culture of isolated anaerobic rumen fungi contributed 41% increase in methane yield in batch AD

tests (Aydin et al., 2017). Recently, enzyme activities of different strains of anaerobic fungi were analyzed and recommended to be considered for full scale applications (Dagar et al., 2018). In the comprehensive review paper of Shrestha and colleagues the need for future research was also highlighted to augment AD with anaerobic fungi and analyze tmicrobial community structure and (Shrestha et al., 2017). In another recent study, anaerobic fungi species (inclusing *Orpinomyces* sp.) was detected in full-scale agricultural biogas plants (Dollhofer et al., 2017). However, further investigation was recommended to develop efficient fungal bioaugmentation strategies. In this regard, further research should be considered for augmenting anaerobic fungi in AD and evaluate its use by analyzing the structure of the microbial community and the transcriptome.

2.5. Composting of Anaerobic Digestates

The residual sludge after AD, i.e., digestate, needs final 'polishing' in order to stabilize the remaining portion of organics to enhance their fertilizer value and applicability as a soil conditioner. Some undesirable characteristics of the digestate, such as odor, viscosity, high humidity and residual VFAs, may restrict its direct application to agricultural soils without any treatment (Abdullahi et al., 2008). Also, digestates can contain pathogens, such as pathogenic bacteria (e.g. *Salmonellae*, *Enterobacter*), parasites (e.g., *Ascaris*, *Giardia*), viruses (e.g. norovirus, enterovirus,) and fungi (*Candida*, *Aspergillus*), if waste materials are subjected to AD and not conducted under thermophilic conditions (Walker et al., 2009). One option is to separate the digestate into a liquid and a solid fraction, the latter being composted in order to obtain valuable and ready-to-use end-products for agriculture (Holm-Nielsen et al., 2009). After subjected to composting, the digestate can be used on land applications as a high-quality soil conditioner (Bustamante et al., 2013).

Composting refers to biological decomposition of organic matter under controlled aerobic conditions to form a stable, humus-like end-product. A highly diverse group of microbes is responsible for this process, whose dynamics vary temporally and spatially, and usually create thermophilic temperatures as a result of biologically produced heat (Farrell and Jones, 2009). Microbial growth highly requires nutrients such as carbon, nitrogen, phosphorous, and potassium which carbon and nitrogen play a crucial role. Carbon to nitrogen (C:N) ratio essentially represents the nutritional balance and the optimum ratio for composting is between 25-35 (Bernal et al., 2009). During the early stages of composting, depletion of oxygen is very high and then it decreases as the process is completed. If necessary amount of oxygen is not supplied to the system, organic matter cannot be degraded sufficiently, anaerobic zones and potential odor can be faced (Ince et al., 2016). Another important parameter, moisture, provides an optimal environment for biochemical reactions.

If moisture content is lower than 40% composting process cannot be accelerated. On the other hand, if moisture content is higher than 65% then it prevents air transport within compost pile. Thus, sufficient moisture content should be maintained in the system to maintain optimum microbial activity and decomposition rate (Bernal et al., 2009; Bustamante et al., 2013).

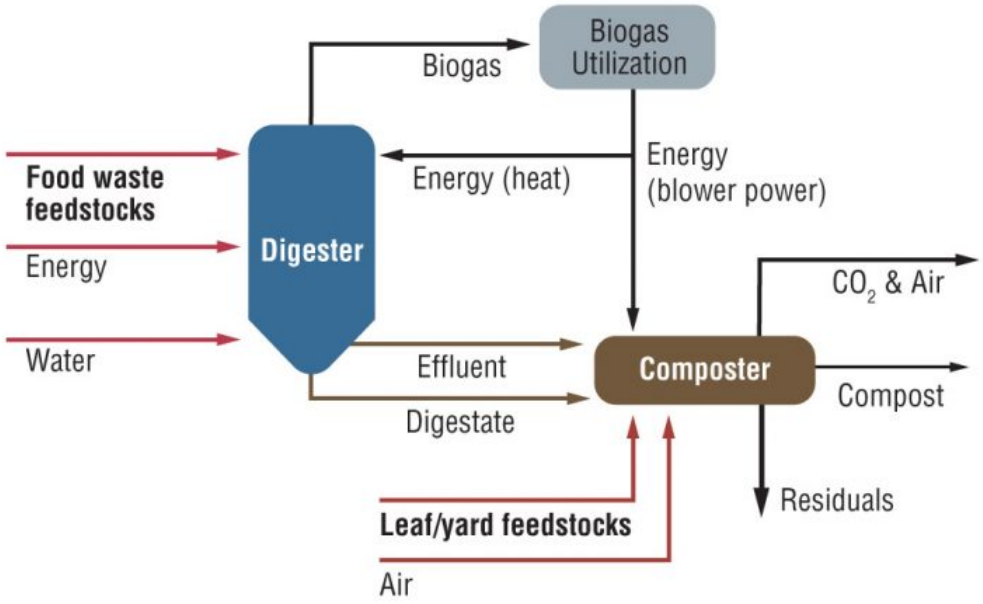


Figure 2.12. Integrating AD with composting (Arab and McCartney, 2017).

Anaerobic digestates are often characterized by very low TS content and this make their composting process differ from other common substrates (e.g. yard waste, kitchen waste) (Franke-Whittle et al., 2014). Several operational difficulties are faced regarding the sludge composting, mainly due to high water content and/or low organic matter (Feng et al., 2015; Wu et al., 2015). Thus, dry materials, such as straw and sawdust, are usually mixed with digestates to act as bulking agents and adjust the moisture content together with C/N ratio (Banegas et al., 2007; Zhang et al., 2018).

There are several studies that implemented aerobic post-treatments of anaerobic digestates, such (Abdullahi et al., 2008; Bortone, 2009). However, there is not enough information about the composting of anaerobic digestates especially regarding the link between microbial characterization and substrate degradation (Bustamante et al., 2013). Most of the sludge residue obtained from AD is alkaline. It has a high moisture content and low C/N ratio, together with low biodegradability (Zeng et al., 2016). These characteristics make the composting process of anaerobic digestate different from that of fresh materials (Zeng et al., 2016). In this regard, it is necessary to evaluate the performance of composting of anaerobic digestates and the resulting compost quality, together with microbial diversity specific to related substrates (Wang et al., 2017).

During composting, microorganisms and above-mentioned environmental factors play significant role in mineralization of organic matter as illustrated in Figure 2.13 (Song et al., 2014). The succession of microorganisms, from bacteria to fungi, during mesophilic and thermophilic temperatures, depends on several factors that directly or indirectly influence the process (Boulter et al., 2000; Coelho et al., 2013). The active microbial population changes from a predominantly mesophilic one in the early stages to predominantly thermophilic population at the maximum temperatur. Each stage is characterized by different populations of bacteria, actinomycetes and fungi (Steger et al., 2007).

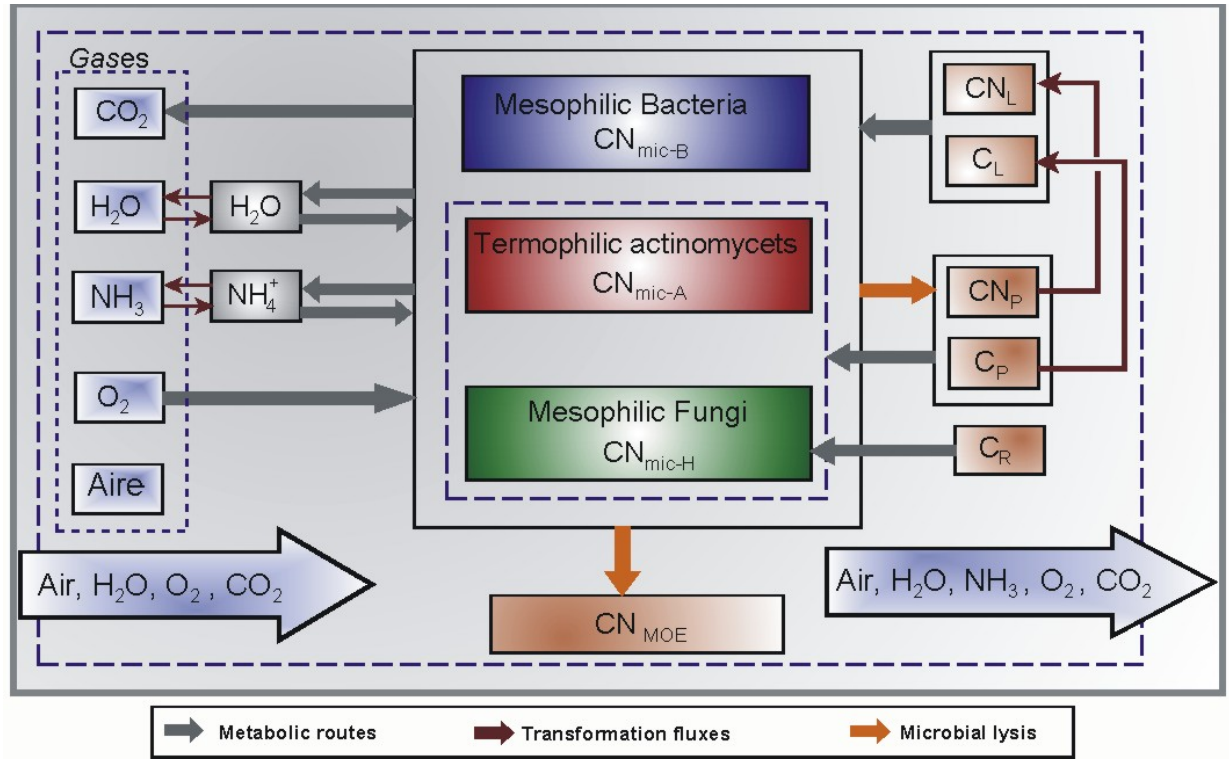


Figure 2.3. Metabolic routes, transformation fluxes and microbial lysis in composting process (Gras et al., 2008).

In compost environments of 25-30 °C and a pH between 5 and 9, all of these organisms are the most represented group (Coelho et al., 2013). During the thermophilic phase achieved in the early stages, mesophilic bacteria are found comparatively in lower abundance prevailing thermophilic bacteria. Furthermore, the optimal temperature range for fungi is between 22.5 and 45 °C. Limiting temperature for fungal activity appears to be 60 °C since above this temperature, fungi die or form spores (Boulter et al., 2000). Fungi reach maximum population values after 7–10 days of composting and are mostly favored in acidic environments (pH < 5). However, their development is restricted by lower moisture levels. Once the temperature falls below 40 °C, there is a restocking by the mesophilic bacteria (Coelho et al., 2013). Actinomycetes, although can be affected by acidic conditions, are

common in many environments and can resist to extreme conditions by forming spores. Actinomycetes colonize slower than fungi and bacteria, remaining mainly at about 15 cm of the surface in an adequately ventilated pile. In poorly ventilated piles, the colonization is limited. Mesophilic temperatures are ideal for actinomycetes growth; however, some species can survive high temperatures, and become more active and even when the nutrient levels are low. Actinomycetes play an important role in composting by degrading macromolecules, such as lignocellulosic materials and chitin, which are very important for the release of inorganic nutrients and humus formation (Coelho et al., 2013; Steger et al., 2007). Taken together, the investigation of relative abundance and microbial dynamics during the composting is of great importance to understand the overall biodegradation mechanism of organic wastes. Among all molecular techniques, high-throughput sequencing has been used as an efficient tool to unveil the detailed inside corresponding to microbial dynamics with more accuracy (Awasthi et al., 2018).

2.6. Soil Amendment of Composts

Organic agriculture has some key elements regarding the nutrient cycle, together with recycling and local production of renewable energy. In the case of biogas, this implies that animal manure together with additional crop residues and energy crops are anaerobically digested and the digestates are applied to the soil, preferentially right after composting, as fertilizer with high levels of plant-available nutrients (Lehtomäki and Björnsson, 2006). Soil mineral dynamics are complex and depend on various factors such as assimilation in the plant and microbial biomass, immobilization on clay particles, and also loss by leaching to groundwater or via gaseous emissions (NH_3, N_2 and N_2O) (Johansen et al., 2013)

Organic matter contents of soils can be raised with sustainable planning in order to make improvements in physical, chemical and biological properties of soils and to increase soil fertility. For this purpose, the amendment of composts originated from agricultural wastes to agricultural soils is an economically attractive waste management strategy while being a safe disposal methods and a valuable source of organics and nutrients (Kayikcioglu, 2013).

The addition of compost to soils has been shown to improve soil function by increasing water-holding capacity, porosity, and surface area (Cogger, 2005). Such organic amendments can help to provide a fruitful soil environment that allow the growth of healthy root systems. They also supply nutrients to growing plants and increase the concentrations of plant-available nutrients in soils (De Lucia and Cristiano, 2014). For example, (Ingelmo et al., 1998) reported an increase in soil mineral

nitrogen concentration in field soils amended with compost products of sewage sludge or municipal solids. The addition of composts can also affect other soil chemical properties, such as pH and electrical conductivity (EC). Soil EC and pH tends to increase in compost-amended soils (Wright et al., 2013) after addition of organic amendments compared with unamended soils (De Lucia and Cristiano, 2014).

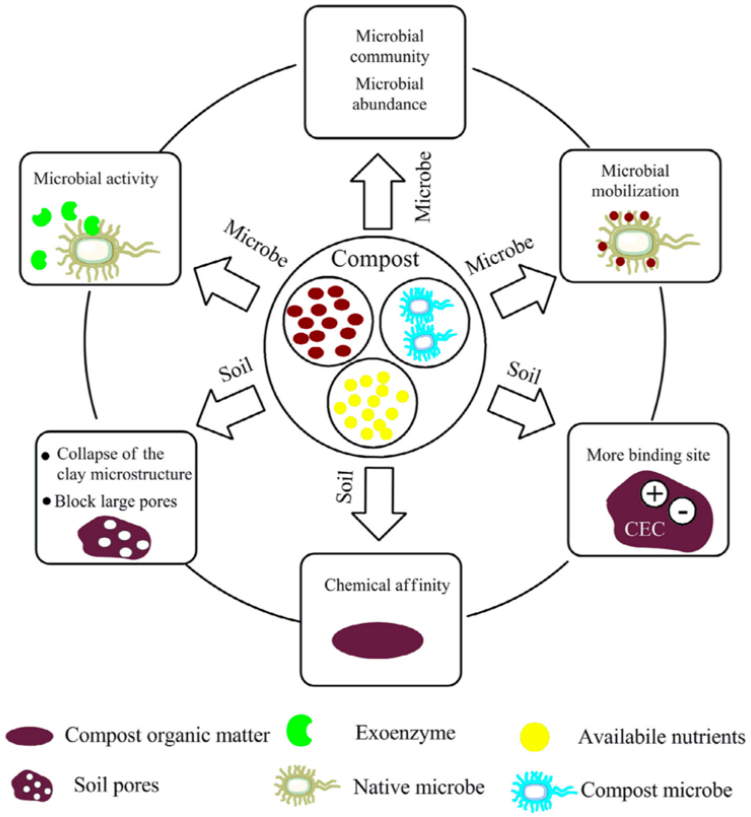


Figure 2.14. Effects of compost amendment on soil microbiome and soil physicochemical characteristics (Ren et al., 2018).

Besides biochemical affirmations, the amendment of compost products also introduces new microbes to the soils which can affect the existing soil microbiome in the long term (Ren et al., 2018). Different compost characteristics lead varying changes in the diversity and abundance of the soil microbiome by causing a shift to different classes of microbes (Cozzolino et al., 2015). The replacement of C source from recalcitrant to easily degradable dissolved organic C was found to cause variations in the soil microbiome including fungi and Gram(+) and Gram(−) bacteria (Bardgett et al., 2007). This modification directly stimulates the growth of soil bacterial communities (Lazcano et al., 2013). Different carbon sources determines the specific metabolic strategies of Gram(+) and Gram(−) bacteria (Kramer and Gleixner 2006). In fact, Gram(+) bacteria are capable of using more recalcitrant C components in soil, while Gram(−) bacteria are known to utilize easily degradable carbon substrates

(Lazcano et al., 2013; Treonis et al., 2004). Hence, recent high-throughput sequencing methods can be used to monitor such variations in the long term.

3. AIM OF THE STUDY

The main objective of this PhD study was to improve the sustainability of lignocellulose-based biogas production by implementing different fungal treatment methods on differently-harvested cereal crops and cow manure. In this regard, pretreatment with aerobic fungus *T. versicolor* and bioaugmentation with anaerobic fungus *Orpinomyces* sp. were elucidated as possible solutions to increase anaerobic biodegradability of lignocellulosic biomass and eventually improve biogas production. Furthermore, anaerobic digestates were collected at the end of AD tests with respect to crop materials and further composted to achieve a ready-to-use soil conditioner. Hence, final compost products were amended to soils where cereal crops were cultivated. In each step, microbial diversity was analyzed by 16S rRNA gene amplicon sequencing.

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

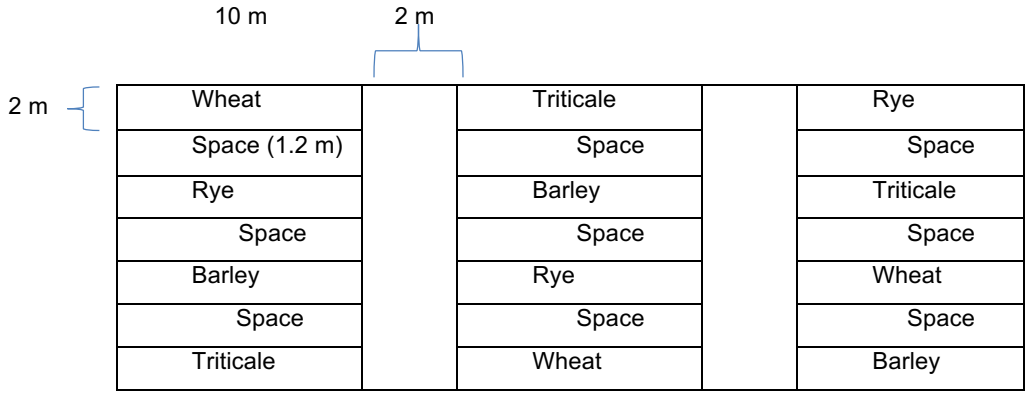
- Determination of the effect of different crop harvesting rotations on the lignocellulosic structures of cereal crops and their harvesting residuals
- Enhancement of hydrolysis and acidification rates by the application of aerobic and anaerobic fungal treatment during AD of lignocellulosic biomass
- Increased understanding of the use of fungi species for improved biogas production in AD processes
- Determination of most promising crop materials with respect to different fungal treatment methods
- Assessment of the biochemical methane potentials (BMP) and biogas microbiome during the AD of cow manure and cereal crops
- Elucidation of the relation between fungal treatment approaches and selected lignocellulolytic enzymes expression levels together with microbial community dynamics in AD tests
- Composting practice of the anaerobic digestates based on crop materials and determination of bacterial and fungal diversity
- Comparison of final compost products' applicability in agricultural applications
- Soil amendment of final compost products as soil conditioner and assessment bacterial diversity changes

4. MATERIALS AND METHODS

4.1. Sample Collection and Characterization

4.1.1. Feedstock

Cereal crops (i.e. wheat, rye, barley and triticale) were cultivated in the fields of Faculty of Agriculture, Uludağ University, Bursa, Turkey (Scheme 4.1). Crop materials of barley and triticale were considered with respect to different harvesting stages, namely early harvest (grain in the milk stage) and late harvest (maturity complete stage) (Amon et al., 2007) and also harvesting residues (straw parts); meanwhile, only harvesting residues of wheat and rye were used. The cereal crops were harvested between May-July of 2016.



Scheme 4.1. Crop planting conditions in the field.

Cow manure was obtained from a healthy, non-medicated cow that was kept at the barn of Veterinary Faculty of Istanbul University, Istanbul, Turkey. The main characteristics of crop materials and cow manure are presented in Table 4.1.

4.1.2. Anaerobic Seed Sludge

The inoculum was obtained from a mesophilic anaerobic digester of a full-scale biogas plant treating cattle manure and other organic process residues, located in Bursa, Turkey. Physicochemical and microbial characterization of the anaerobic seed sludge are given in Table 4.1 and Figure 4.1, respectively.

Table 4.1. Initial characterization of the crop materials, cow manure and seed sludge (n=2).

Harvesting stage	Crop	pH	TS (%)	VS (%)	VS/TS (%)	Alkalinity (mg $CaCO_3$/L)	sCOD (mg/L)	TKN (mg/L)	C:N	Cellulose (% TS)	Hemicellulose (% TS)	Lignin (% TS)
Grain in the milk (early harvest)	Wheat	5.15	44.0	36.6	83	1,000	18,250	803	22:1	18.24	18.39	2.78
	Triticale	5.96	48.8	40.0	82	800	13,460	1081	21:1	19.28	14.15	2.43
	Rye	5.60	41.0	35.2	86	1,200	20,790	915	33:1	18.01	18.34	2.60
	Barley	4.82	40.6	37.9	93	960	10,500	905	23:1	12.03	17.56	1.15
Maturity complete (late harvest)	Wheat	4.61	90.6	69.6	77	325	7,960	1600	27:1	12.85	12.13	1.68
	Triticale	4.60	90.5	71.8	79	500	11,290	1595	22:1	11.67	14.35	2.09
	Rye	4.60	90.7	73.1	81	500	13,860	1993	27:1	12.65	19.03	1.64
	Barley	4.58	90.5	69.8	77	400	6,850	1186	28:1	11.25	27.79	0.93
Harvesting residues (straws)	Wheat	6.59	89.6	69.5	78	1,050	7,210	182	81:1	51.39	20.64	7.61
	Triticale	6.30	89.0	68.9	77	800	6,630	223	74:1	50.39	23.37	7.75
	Rye	6.71	89.4	68.3	76	1,125	6,210	565	85:1	50.76	23.63	7.65
	Barley	6.59	88.2	68.6	78	800	5,960	454	87:1	48.86	24.5	7.67
-	Cow manure	7.48	14.3	11.5	80	2,825	11,500	135	25:1	35.90	17.19	14.52
-	Seed sludge	8.35	7.20	4.50	62	19,000	31,875	1797	11:1	-	-	-

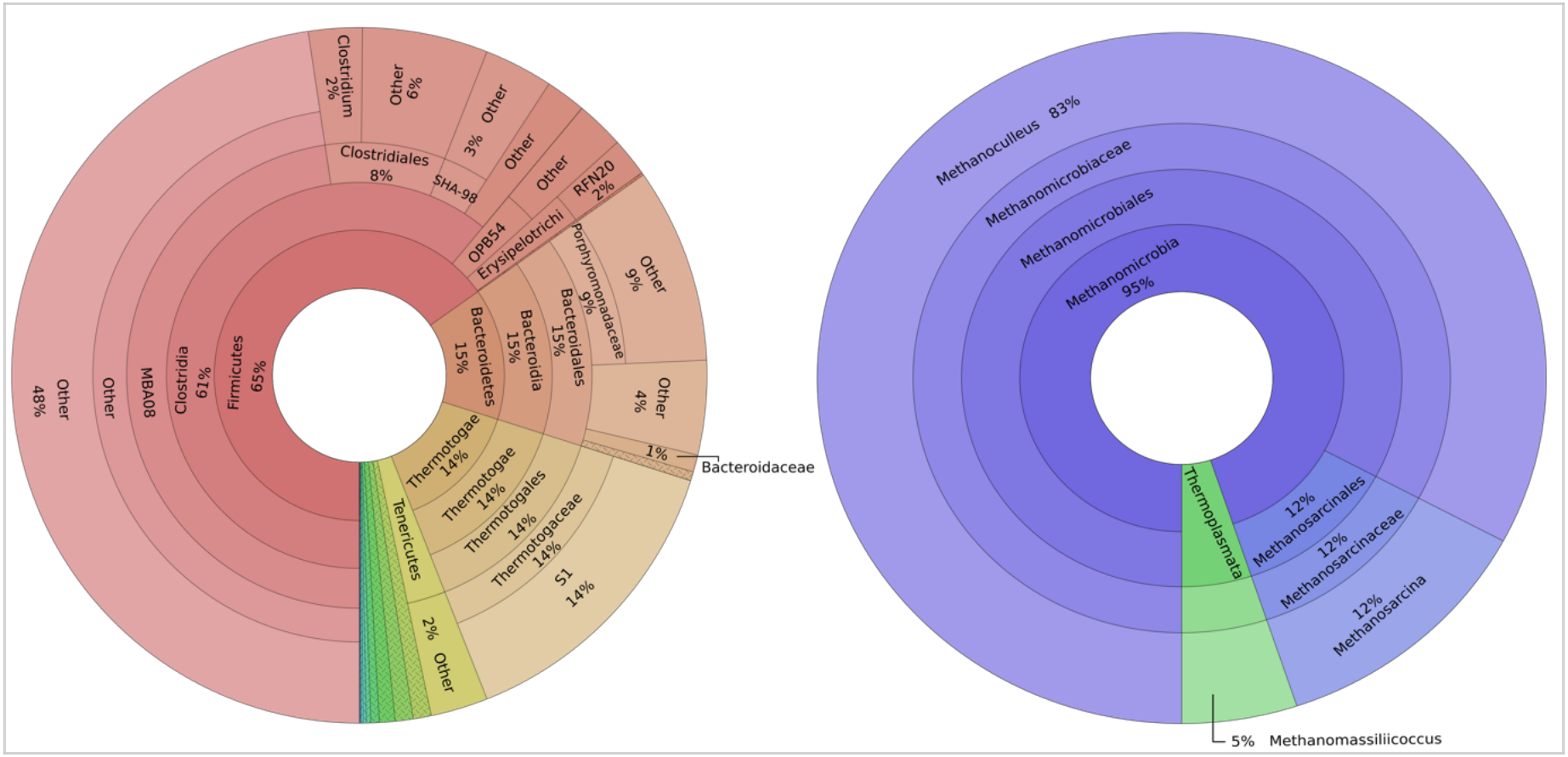


Figure 4.1. Microbial characterization of the anaerobic seed sludge.

4.1.3. Collection of Anaerobic Digestates

At the end of AD tests, digestates were collected based on each crop type, namely anaerobic digestate of barley (AD_B), anaerobic digestate of triticale (AD_T), anaerobic digestate of wheat (AD_W) and anaerobic digestate of rye (AD_R), and stored at 4 ^{o}C for further composting. Physicochemical properties and metal content of raw digestates are given in Table 4.2 and Table 4.3, respectively.

Table 4.2. Characterization of anaerobic digestates and bulking agent.

	TS (%)	VS (%)	VS/TS (%)	C:N	TKN (mg/L)	pH	Alkalinity (mg $CaCO_3$/L)	EC (dS/m)
AD_B	5.4	4.0	74	16:1	8327	8.1	12625	3.2
AD_T	5.0	3.6	72	17:1	5687	8.1	14000	3.6
AD_W	5.8	4.2	72	14:1	5395	8.1	9750	2.9
AD_R	5.4	4.0	74	15:1	5658	8.4	12000	3.1
Wheat straw	92.7	82.3	89	86:1	979	4.5	250	-

Table 4.3. Metal content of anaerobic digestates (mg/kg).

	Cr	Ni	Cu	Zn	Cd	Pb
AD_B	3	7	113	676	ND*	4
AD_T	13	25	121	587	ND	3
AD_W	ND	5	101	651	ND	4
AD_R	2	5	144	704	ND	5

**ND: Not detected*

4.1.4. Soil Sampling

Soil samples were taken from the fields of Uludağ University, Bursa, where the crops were planted. Hence, 4 soil samples namely barley soil (BS), triticale soil (TS), wheat soil (WS) and rye soil (RS) were taken on Day 0 prior to compost amendment for initial NGS analysis. Each crop-specific compost products were amended to related fields, and after 3 months, samples (i.e. BS.3, TS.3, WS.3, RS.3) were collected for further NGS analysis.

4.2. Experimental Set-up

4.2.1. Fungal Pretreatment

The strain *T. versicolor* ATCC 42530 was acquired from the American Type Culture Collection. *T. versicolor* was cultivated on 2% malt extract agar plates at 25 ^{o}C. The medium was sterilized at 120 ^{o}C for 30 min after adjusting its pH to 4.5 with 0.5 M NaOH or 0.5 M HCl. A mycelia suspension of *T. versicolor* was obtained by inoculating 0.5 cm diameter plugs from the growing zone of fungi on malt agar, transferred in 250 mL malt extract medium (2%) in a 1 L Erlenmeyer flask. Flasks were closed with cotton stoppers and incubated in an incubating shaker (135 rpm) at 25 °C. A thick mycelial mass was formed after 6 d. This suspension was then used to produce pellets by inoculating 1 mL of the suspension in 250 mL 2% malt extract medium a 500 mL Erlenmeyer flask. The flasks were incubated in an incubating shaker (135 rpm) at 25 °C for 14 d to investigate the time course of laccase production and determine maximum laccase production. Samples were taken from the culture medium daily to assay laccase activity. Culture medium was centrifuged at 10000 rpm at 4 ^{o}C for 10 min in a refrigerated centrifuge (Allegra 64R, Benchtop Centrifuge, Beckman Coulter) and the supernatant was used for enzyme assay. Enzyme assay was performed according to a modified protocol described elsewhere (Kocyigit et al., 2012). Laccase activity was performed spectrophotometrically (UV-160A UV-Visible Recording Spectrophotometer, Shimadzu) by measuring the oxidation of ABTS at 420 nm. The reaction mixture contained 0.2 mL of 5 mmol/L ABTS and 0.6 ml 0.1 mol/L glycine-HCl buffer (pH 3.0) and 0.4 mL aliquots of appropriately diluted culture fluid. Unit of one laccase activity was defined as the amount of enzyme that oxidized 1 μmol ABTS per min under reaction conditions. The activities were expressed in U/L. Following the fungal pretreatment in predetermined conditions, pretreated biomass was mixed with the anaerobic seed sludge and AD tests were established.

4.2.2. Fungal Bioaugmentation

Pure culture of anaerobic rumen fungus *Orpinomyces* sp. was obtained from the fungi culture collection of Department of Zootechnics, Kahramanmaraş Sütçü İmam University, Kahramanmaraş, Turkey. Anaerobic basal media was prepared based on the protocol (Orpin, 1976) with some modifications. Basal media contained following ingredients: 150 mL/L clarified rumen fluid - supernatant of centrifuged rumen fluid, 150 mL/L Mineral Solution I (0.3% K_2HPO_4), 150 mL/L Mineral Solution II (0.3% KH_2PO_4, 0.6% NaCl, 0.6% $(NH_4)_2SO_4$, 0.06% $CaCl_2$, and 0.06% $MgSO_4$) 6 g/L $NaHCO_3$, 2.5 g/L yeast extract, 10 g/L peptone, and 1 mg/L resazurin. This mixture was then

stirred and boiled for an hour to remove oxygen. Afterwards, 5 g/L cellobiose and 1 g/L L-cystein.HCl were added to mixture for the final reduction. Hungate tubes and serum bottles were filled with the final media, tightly sealed and autoclave-sterilized at 121 ^{o}C for 15 min. Isolation of *Orpinomyces* sp. was done according to (Theodorou et al., 1993). Briefly, 10 g animal manure was added to 100 mL basal media. 1 mL of mixture was then transferred to Hungate tubes that contained 9 mL basal media and wheat straw; and then serial dilution was applied up to 10^3. Antibiotics (Cloramphenicol: 100 μg/mL, Ampicillin: 100 μg/mL, Streptomycin: 140 μg/mL, Erythromycin: 200 μg/mL) were also added to the media to prevent any possible bacterial contamination. Whole procedure was carried out under anaerobic conditions by supplying CO_2 to the samples continuously. The diluted samples were incubated at 39 ^{o}C for 3-10 days.

Roll Tube method (Joblin, 1981) was then applied to achieve single colonies out of the diluted samples. 0.5 mL of the diluted sample was injected to melted basal anaerobic agar media and rolling method was applied during solidification of media. After incubation at 39 ^{o}C for 3-4 days, single colonies were selected and transferred to basal broth media for further morphological analyzing under Olympus BX51 light microscopy.

Isolated *Orpinomyces* sp. was maintained and subcultured by utilizing cellobiose-containing basal media. Prior to AD tests, 80 mL of media in 120 mL serum bottles was inoculated with *Orpinomyces* sp. spent medium and incubated for 5 days at 39 ^{o}C. Following the final incubation, the fungus and spent medium were directly utilized for the bioaugmentation of anaerobic digesters (10% v/v of *Orpinomyces* sp. and spent medium) (Nkemka et al., 2015) and AD tests were established.

4.2.3. Anaerobic Digestion Tests

Biochemical methane potentials (BMP) of the cereal crops and cow manure were determined in anaerobic batch tests using 1 L glass reactors with a working volume of 750 mL. These anaerobic co-digestion trials were conducted subjecting cow manure and early-harvested barley (EB) and triticale (ET), late-harvested barley (LB) and triticale (LT) as well as harvest residues of barley (RB), triticale (RT), rye (RR) and wheat (RW) for pretreatment (P), bioaugmentation (B) and control (C) set-ups. Biomass was mixed with the anaerobic seed sludge at the inoculum to substrate ratio of 1:1 (VS basis). Cow manure was used as the co-substrate in AD tests to balance C/N ratio and nutrient content. Final TS in the anaerobic digesters corresponds to 8-9%. The control digesters (without fungal treatment) were performed following the same conditions. The experiments were conducted in

duplicates. The anaerobic digesters were operated in an incubating shaker at 37 ± 1 ^{o}C at a mixing speed of 100 rpm. No pH adjustment was made, and the digesters were flushed with N_2 to maintain anaerobic conditions. Blank digesters were also operated only with anaerobic seed sludge and background biogas production was subtracted from the experimental digesters. The experiments were monitored until there was no significant change in biogas production ($p < 0.05$). Methane yield was expressed as mL CH_4/g VS_{added}.

4.2.4. Composting

The composting experiments were carried out by a tumbler system having two compartments with the dimensions of 200 x 90 x 150 cm (length x width x height). 4 different anaerobic digestates, collected from the AD tests, were used in the composing systems namely barley composting (BC), triticale composting (TC), wheat composting (WC) and rye composting (RC). Each digestate was mixed with wheat straw as bulking agent to fix the moisture content around 50%. Exhausted coffee waste was also added to each composting pile to adjust C:N ratio between 25:1-35:1.

The systems were operated as a batch reactor in which digestates were fed once, and then the systems were allowed to start composting process. The tumblers were turned periodically three times a day throughout the process to homogenize the mixture and maintain adequate O_2 levels.

4.2.5. Soil Amendment

Following the composting study, each crop-specific final compost products were collected and taken to cereal crop fields of Uludağ University, Bursa, Turkey. After the initial sampling of soils), compost products were amended to soil approximately 5 t/ha as the common agricultural practice.

4.3. Analytical Methods

Total solids (TS), volatile solids (VS), soluble Chemical Oxygen Demand (sCOD), Total Kjeldahl Nitrogen (TKN) and alkalinity were measured according to Standard Methods (APHA/AWWA/WEF, 2012). pH was measured by benchtop pH meter (FEP20, Mettler Toledo). Carbon to nitrogen (C:N) ratio was determined using Elemental Combustion System (Costech, CHNSO, USA) with dried samples. Cellulose, hemicellulose and lignin contents of crop materials and cow manure were analyzed according to Standard Forage Analysis (Goering and Van Soest, 1970). Reduction in cellulose content in anaerobic digesters was determined according to a method

explained elsewhere (Siegert and Banks, 2005). Reducing sugar concentration of pre-treatment samples was measured with dinitrosalicylic (DNS) colorimetric method.

Biogas generation in anaerobic digesters was recorded cumulatively by Milligascounter (MGC-1, Ritter Bochum, Germany). Gas composition and VFA concentration were determined using HP Agilent 6850 Gas Chromatograph and Perkin Elmer Clarus 600 Gas Chromotograph, respectively, as described in a previous study (Akyol et al., 2016).

In composting, representative samples of each pile were collected randomly to monitor temperature, pH and moisture content (MC). pH and electrical conductivity were measured in 1:10 (w:v; compost:water) aqueous suspension that was placed on a shaker for 30 min, by a pH probe (Hanna HI 221 Microprocessor, Italy) and a conductivity probe (WTW LF 320, Germany), respectively. Temperature was measured by a glass thermometer.

4.4. Enzyme Expression Assays

Slurry samples were taken on every 10 day of AD for RNA extraction. A PureLink RNA extraction kit (Invitrogen, UK) was used in accordance with recommended procedures to isolate the total RNAs from 500 mL slurry sample. Total RNA was stored at 80 ^{o}C until cDNA synthesis. cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions.

Changes in the quantity of selected specific enzymes in the digester samples within time was determined using qPCR. Primer sets were employed to assess enzyme quantification through the use of the template cDNAs. ABI Prism 7000 Real Time PCR System (Applied Biosystems, USA) was used to analyse the quantification of the cDNAs. Power SYBR Green kit (Applied Biosystems, USA) was used for the qPCR assays. Triplicate PCR reactions were carried out to a final volume of 20 µL. The mixed qPCR solution contained 10 µL PCR master mix, 1 µL of each primer, and 2 µL template, all diluted to the final volume of the reaction mixture with DNase/RNase free water. qPCR assays was performed using specific primers and the qPCR conditions was carried out at a temperature profile of 10 min initial denaturation at 95 ^{o}C, followed by 40 cycles each of denaturation at 95 ^{o}C for 0.5 min, annealing at 57 ^{o}C for 1 min, and extension at 72 ^{o}C for 0.5 min. The standard curves for qPCR was constructed from the cDNA of previous slurry samples with specific annealing temperature for each primer set as given in Table 4.4. and Table 4.5. Deionized water and DNase-treated *E. coli* was used as negative controls.

Table 4.4. Primer information on the selected lignocellulolytic enzymes.

Target	Primer	Sequence (5'-3')	Annealing temperature (^{o}C)	Reference
GH5	cel5_392F	GAGCATGGGCTGGAAYHTNGGNAA	52	(Pereyra et al., 2010)
	cel5_754R	CATCATAATCTTTGAAGTGGTTTGCAATYTGDKTCCA		
GH6	cell2F	ACCTGCCCGRCCGYGACT	64	(Merlin et al., 2014)
	cell2R	GAGSGARTCSGGCTCRAT		
GH48	cel48_490F	TNATGGTTGAAGCTCCDGAYTAYGG	56	(Pereyra et al., 2010)
	cel48_920R	CCAAANCCRTACCAGTTRTCAACRTC		
Xylanase	xynA-F	CTTCCGCCAGTCGCCTCTCTACAAGATTG	60	(Li et al., 2013)
	xynA-R	CTGGCTCTTCACGGGGTCGCACTC		
Xylanase	xynB-F	TCGCCAGAGCGCTATGTACCG	60	(Li et al., 2013)
	xynB-R	CAAAGTGATGGCAGAGTCGAGACGAG		
Xylanase	xynC-F	CCAGGCTCCCTATATCCAGACAC	60	(Li et al., 2013)
	xynC-R	AGTGAGCGCTTAGCCTTGAAGTTC		
Xylanase	xynD-F	GTTCGAGTTCGTCGATGGCAAG	60	(Li et al., 2013)
	xynD-R	GTGATGGCCTTTTCGAGCTGC		
Xylanase	xynE-F	CTGTCGTCTGGTGAAAAACCTGAAG	60	(Li et al., 2013)
	xynE-R	GCTGCAACTCATAGTTCTGGCTG		
Xylanase	xynF-F	TCCTGCACCCGTCACCAGACCGATG	60	(Li et al., 2013)
	xynF-R	TACCATCTCGTCGTGGCTCTTGGGAGTG		
Laccase	Cu1AF	ACM WCB GTY CAY TGG CAY GG	48	(Ausec et al., 2011)
	Cu4R	TGC TCV AGB AKR TGG CAG TG		

Table 4.5. Primer information on the selected biomethanation enzymes.

Target	Primer	Sequence (5'-3')	Annealing tempereture (^{o}C)	Reference
Acs1	MSaeta_Aco-A_f	TAATCCGCCAAAAGAGTTGG	56	(Ince et al., 2011)
	MSaeta_Aco-A_r	TCTTCTGGACTGGCTGGTCT		
mcrA	M13F	TGTAAAACGACGGCCAGTGGTGGTGTMGGATTCACACARTAYGCWACAGC	55	(Luton et al., 2002)
	M13R	CAGGAAACAGCTATGACCTTCATTGCRTAGTTWGGRTAGTT		

4.5. Metagenomic Analysis

Triplicate subsamples were collected and total genomic DNA was extracted from 500 µL/µg samples using MN NucleoSpin Soil DNA isolation kit (Macherey-Nagel, Germany) and a ribolyser (Fast PrepTM FP120 Bio 101 Thermo Electron Corporation, Belgium) according to manufacturer's protocol. The DNA was quantified by absorbance measurement on the NanoPhotometer P-Class (Implen, Germany). Purity of the DNA samples was determined by measuring the 260/280 nm absorbance ratio. Extracted DNA samples for each AD system were then pooled to have a representative sample and stored at -20 °C for further analyses.

Microbial community compositions were processed and analyzed with the ZymoBIOMICS™ Service - Targeted Metagenomic Sequencing (Zymo Research, Irvine, CA). 16S and 18S ribosomal RNA gene targeted sequencing was performed with Quick-16S™ NGS Library Preparation Kit (Zymo Research, Irvine, CA) for bacterial and/or methanogenic archaeal and fungal communities, respectively. The final library was sequenced on Illumina® MiSeq™ using a V3 reagent kit. Details of high throughput gene amplicon sequencing can be found in ZymoBIOMICS™ Service website.

4.6. Bioinformatics

Amplicon sequences were inferred from raw reads using the Dada2 pipeline (Callahan et al., 2016). Chimeric sequences were also removed with the Dada2 pipeline. Taxonomy was assigned using Uclust from Qiime v.1.9.1 with Greengenes 16S database as reference. Taxonomy assignment, alpha-diversity and beta-diversity analyses were performed with Qiime v.1.9.1 (Caporaso et al., 2010). Taxa that have an abundance significantly different among groups were identified by LefSe (Segata et al., 2011) with default settings if applicable. Data visualization was performed with in-house scripts.

4.7. 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. FUNGAL PRETREATMENT WITH *Trametes versicolor* TO ENHANCE METHANE PRODUCTION FROM LIGNOCELLULOSIC BIOMASS

There is a remarkable number of research on fungal pretreatment using white rot fungi prior to AD; however, these studies remained limited to investigate the indigenous microbial community changes in anaerobic digesters upon fungal pretreatment. A lack of research and knowledge leads the authors unaware of any studies regarding the synergistic effects of fungal pretreatment on biogas microbiome. In this particular study, it was therefore aimed to investigate the influence of harvesting stage on lignocellulosic structure of selected cereal crop materials and determine BMP of cereal crops and cow manure in anaerobic co-digestion trials with respect to varying combinations. Affirmation in methane yield by fungal pretreatment with *T. versicolor* together with VFA profiles and cellulose degradation were further elucidated. A further and deeper insight was gained and discussed with the 16S rRNA gene amplicon sequencing analysis of the anaerobic digesters to obtain clear differences in the key players during the biodegradation of lignocellulosic feedstock upon the application of fungal pretreatment.

5.1. Fungal Broth Production and Aerobic Pretreatment

To determine the maximum laccase production in the medium, time course of laccase production was investigated for 14 days (Figure 5.1a). Laccase activity was measured daily in shaken flask cultures at 25 ^{o}C at 135 rpm mixing speed. Enzyme activity increased through 9 days and the maximum laccase activity with 2070 U/L was achieved on the 9th day of cultivation. Similar laccase activity behavior by *T. versicolor* was reported by other authors (Borchert and Libra, 2001). In the following aerobic pretreatment assays, *T versicolor* broth was added to flasks on the 9th d of its cultivation.

Optimum incubation time for the aerobic pretreatment with *T. versicolor* was determined using representative lignocellulosic biomass (i.e. wheat straw and cow manure). Substrates were inoculated with *T. versicolor* at an initial enzyme activity of 500 U/L and incubated for 10 days at 25 ^{o}C at the mixing speed of 135 rpm. Laccase activity and reducing sugar concentrations were measured daily and given in Figure 5.2b. The results pointed out the 6th d as the best possible pretreatment time to further consider in pretreatment assays prior to AD tests. Laccase concentration decreased meanwhile reducing sugar concentrations increased until 6th day regarding the degradation of lignocellulosic

content. After the 7th day of the incubation, re-growth of *T. versicolor* was observed which started to produce laccase and caused a more bulk composition due to excessive cell growth.

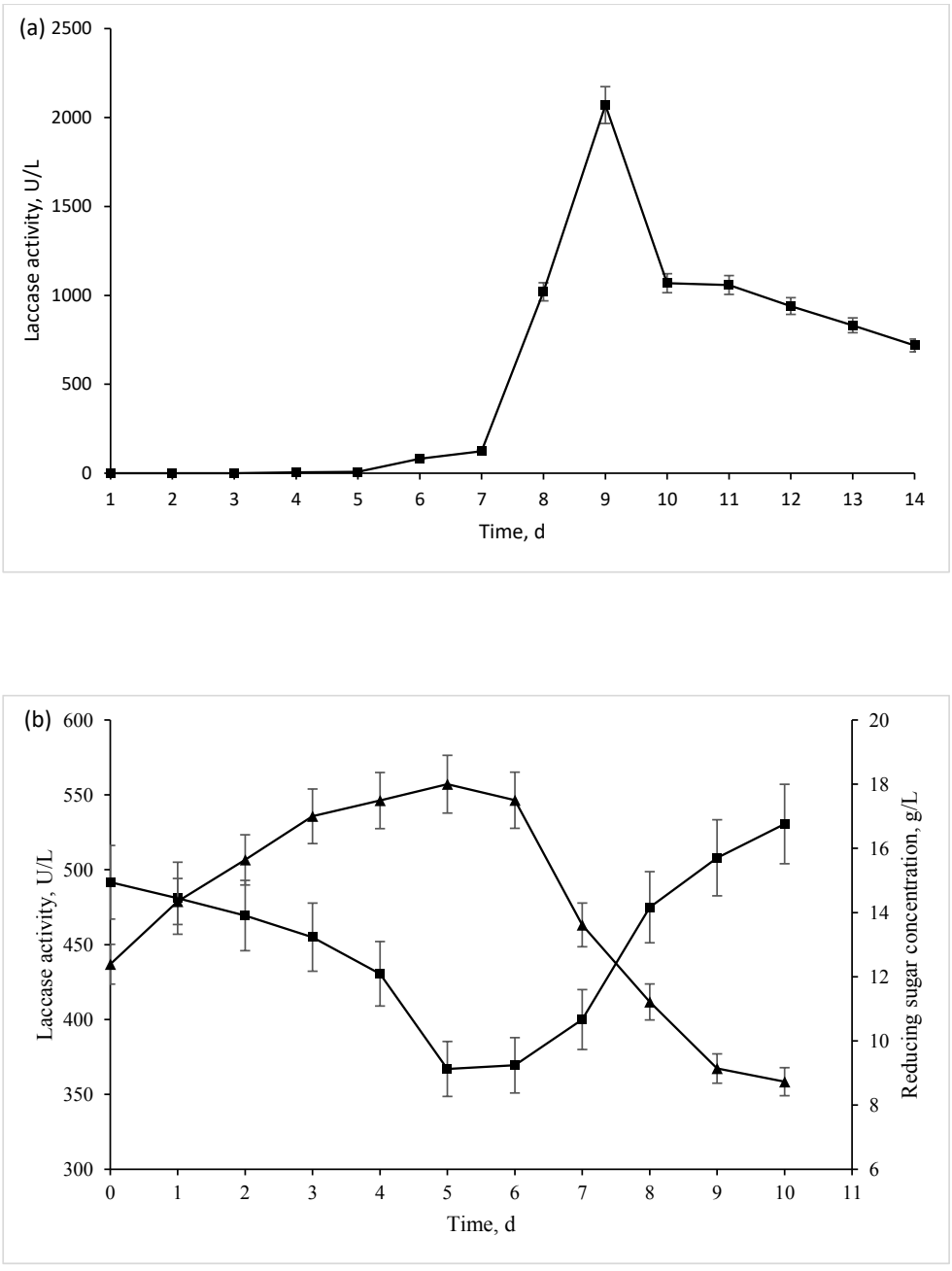


Figure 5.1. (a) Time course for extracellular laccase production by *Trametes versicolor* (b) Laccase activity (■) and reducing sugar concentrations (▲) by *Trametes versicolor* during the optimization of aerobic pretreatment.

5.2. Anaerobic Co-Digestion Trials

5.2.1. Methane Production

To assess the influence of harvesting cut as well as fungal pretreatment on methane production, the methane yields of the anaerobic digesters were calculated by keeping a record of cumulative methane production. Among the pretreated digesters, anaerobic co-digestion of cow manure and early-harvested barley (EB_P) performed best with the highest methane yield of 386 mL CH_4/g VS (Fig. 5.2), which was not a significant increase (2%) in comparison to its control digester (EB_C) (378 mL CH_4/g VS) ($p > 0.05$). Anaerobic co-digestion of cow manure and late-harvested barley (LB_P) achieved the second-highest methane yield as 232 mL CH_4/g VS with 15% increase compared to its control (LB_C, 202 mL CH_4/g VS).

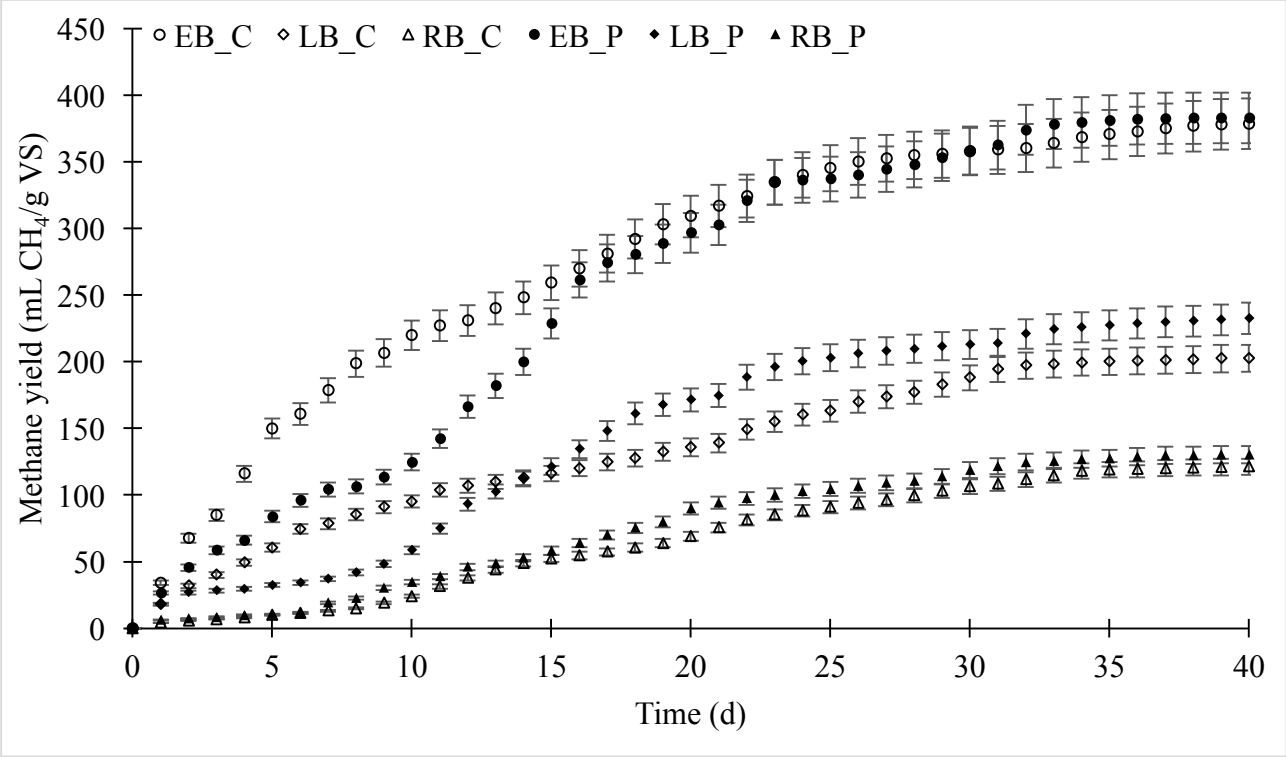


Figure 5.2. Methane yields in control and pretreated trials during anaerobic co-digestion of cow manure and barley at different harvesting stages.

Anaerobic co-digestion of cow manure and triticale showed similar methane production and increase rate with respect to the different harvesting stages both with and without fungal pretreatment as shown in Fig. 5.3. Accordingly, methane yields of LT_C and ET_C were calculated as 193 mL CH_4/g VS and 192 mL CH_4/g VS, respectively; whereas their fungal-pretreated digesters, namely

LT_P and ET_P, acquired approximately 18% higher methane yield as 228 mL CH_4/g VS and 225 mL CH_4/g VS, respectively ($p < 0.05$).

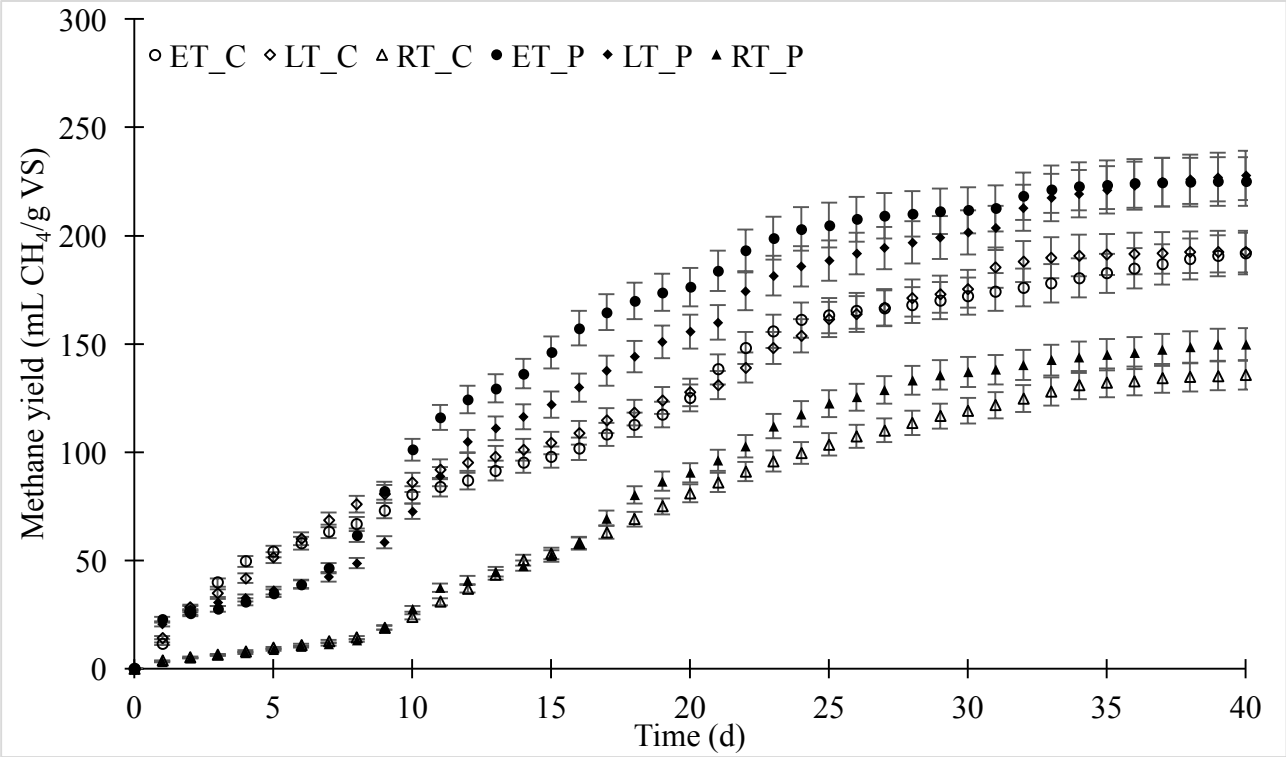


Figure 5.3. Methane yields in control and pretreated trials during anaerobic co-digestion of cow manure and triticale at different harvesting stages.

Among the pretreated crop residues (Figure 5.4.), triticale performed a higher methane yield (150 mL CH_4/g VS) during the anaerobic co-digestion of cow manure and triticale straw (RT_P), 10% increase compared to its control digester RT_C (136 mL CH_4/g VS). Similar methane yields were attained during the anaerobic co-digestion of cow manure and residual barley straw (RB_C, 121 mL CH_4/g VS), wheat straw (RW_C, 118 mL CH_4/g VS) and rye straw (115 mL CH_4/g VS), in which fungal treatment caused similar increase rates on methane yields (10-14%) as 133 mL CH_4/g VS, 130 mL CH_4/g VS and 132 mL CH_4/g VS in RB_P, RW_P and RR_P, respectively.

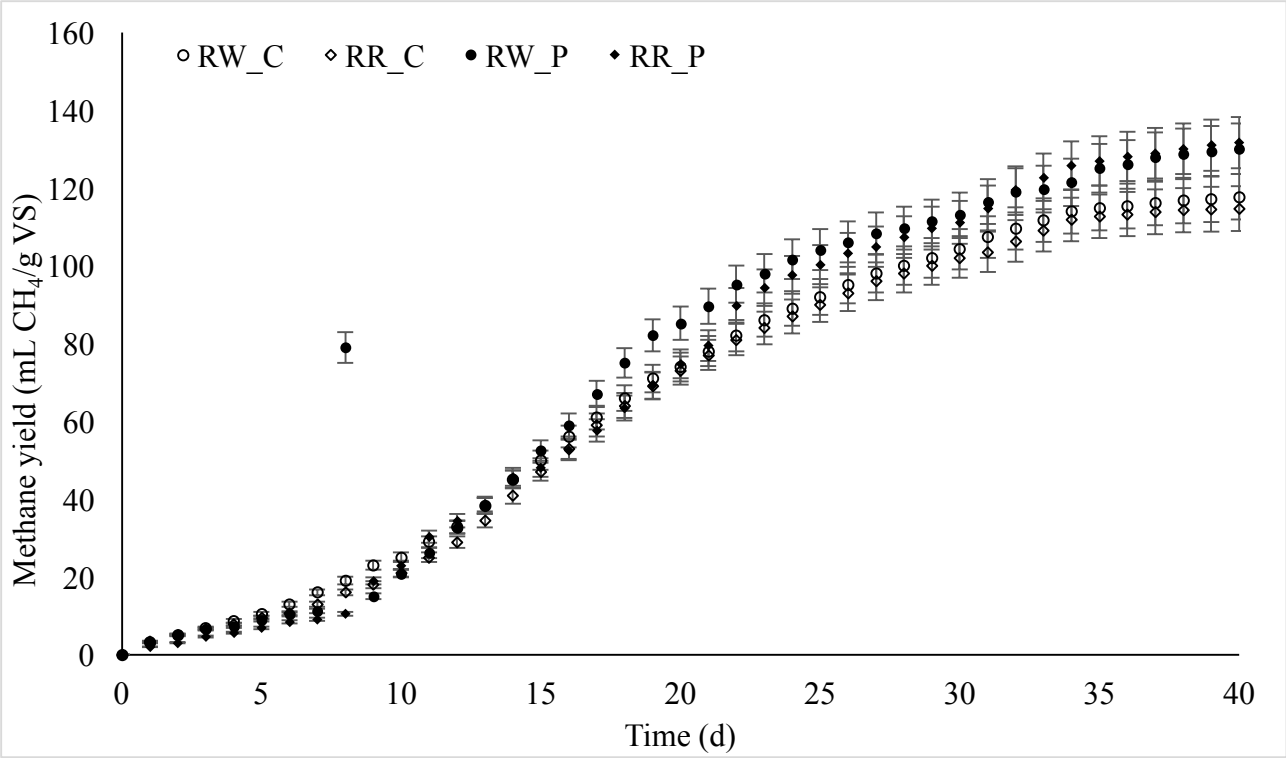


Figure 5.4. Methane yields in control and pretreated trials during anaerobic co-digestion of cow manure and wheat straw and rye straw.

Feedstock composition is an important factor in AD affecting both CH_4 yield and digestion stability; which in turn is governed by plant species, geographical location, and biomass maturity (Sawatdeenarunat et al., 2015). As investigated by (Amon et al., 2007), harvesting time also greatly influence methane potential of crops and harvesting time between ‘‘grain in the milk stage’’ to ‘‘grain in the dough stage’’ was mostly suggested for cereal crops. Similarly, in this particular study, grain in the milk stage (early harvest) of barley yielded significantly higher methane yields in anaerobic co-digestion of cow manure and barley both in the control and pretreatment trials compared to the maturity complete stage (late harvest). This can be attributed to comparatively high organic matter content and low lignin content of the early-harvested barley (see Table 4.1) which might provide a more easily biodegradable and organic-accessible environment for the biogas microbes. The choice of using barley at different harvesting stages, namely early and late harvest, as well as harvesting residues, is of great importance here. Hence, subjecting the EB in anaerobic co-digestion with cow manure resulted 87% and 212% higher methane yields compared to LB and RB, respectively. Similarly, leaves fraction of wheat straw showed higher methane potential as stem structures, such as internodes and nodes, had lower BMP (Motte et al., 2014). Although fungal pretreatment with *T. versicolor* did not have a significant effect of methane yield in the EB digesters, methane yield of LB was further increased by 15% with the aid of *T. versicolor*. No remarkable effect of harvesting stage on methane yield was recorded in the anaerobic digesters fed with cow manure and triticale. However,

the choice between using the whole crop (both ET and LT) and crop residues (RT) resulted 42% variation on methane yield. As mentioned earlier, fungal pretreatment contributed same amount of methane increase (18%) in ET_P and LT_P digesters. As another prominent outcome of the BMP tests, RT-containing anaerobic digesters always achieved higher methane yields comparing to other crop residues with and without fungal pretreatment. One of the reasons for obtaining higher methane yields by RT could be its comparatively lower C/N ratio than other crop residues (see Table 4.1). Hence, C/N ratio is stated as the main parameter to go for anaerobic co-digestion applications to compensate the carbon deficiency of animal manure with agricultural residues (Neshat et al., 2017). Wheat winter harvested at medium-milk stage was reported to achieve a higher methane yield (360 mL CH_4/ g VS) than later-harvested material (311 mL CH_4/ g VS) (Rincón et al., 2010). Overall, methane yields achieved within our study are in good accordance with the values stated in the literature (Amon et al., 2007; Ward et al., 2008).

The influence of fungal pretreatment on methane yield is widely stated in the literature with respect to different fungi species and feedstocks (Ghosh et al., 2017). In a very recent study, 41% methane increase was achieved (from 167 mL CH_4/ g VS to 236 mL CH_4/ g VS) when anaerobic digesters were fed with corn-silage pretreated with *T. versicolor* (Tišma et al., 2018). Even up to 74% increase in methane yield was reported by the fungal broth pretreatment with *T. versicolor* prior to AD of microalgae (Hom-Diaz et al., 2016). Improved saccharification of canola straw together with enhanced lignin removal were also reported by Canam and colleagues (Canam et al., 2011) during biological pretreatment with a cellobiose dehydrogenase-deficient strain of *T. versicolor*. The authors further highlighted the potential biofuel enhancement in this wise. Differentiations in operating and environmental conditions selected for aerobic pretreatment as well as AD eventually reveal varying methane yield affirmations (Zhao et al., 2014). The choice of fungi species in aerobic pretreatment is no doubt another major parameter affecting the lignin degradation and methane yield.

5.2.2. VFA Production and Cellulose Degradation

Total VFA production as well as VFA speciation throughout the AD operation is shown in Figure 5.5. In the control digesters, propionic acid was the predominant VFA species, followed by acetic acid, isovaleric acid and isobutyric acid. Other VFA species were measured at minor concentrations. Propionic acid was measured between 3000-6000 mg/L during the first 20 days of the AD, which further ceased and exhibited no accumulation at the end. Acetic acid concentrations were comparatively higher in the control digesters fed with cow manure and harvesting residues up to 4000 mg/L. In the pretreated digesters, VFA profiles were completely different.

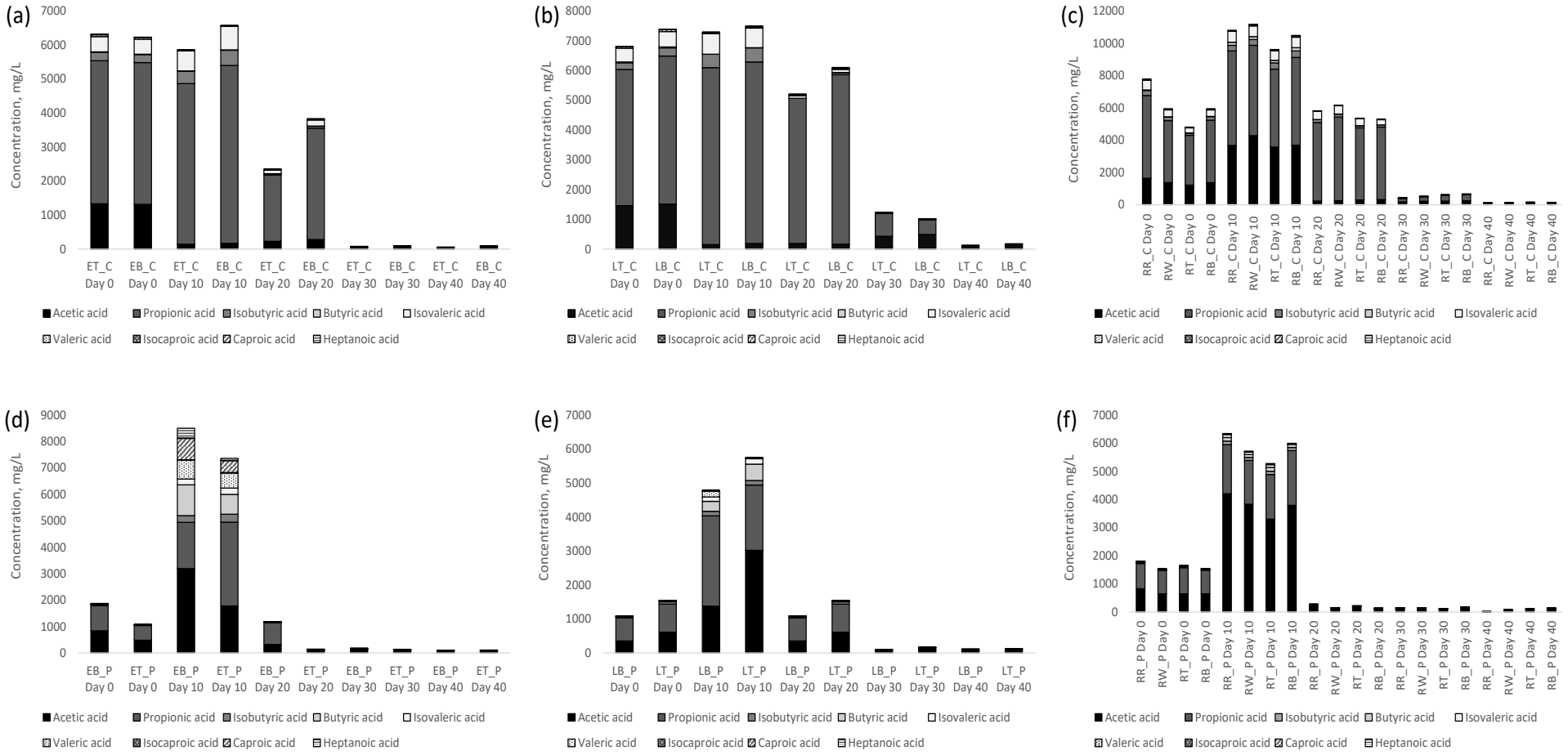


Figure 5.5. VFA profiles during anaerobic co-digestion of cow manure and (a) early-harvested barley and triticale in control trials (b) late-harvested barley and triticale in control trials (c) wheat straw and rye straw in control trials (d) early-harvested barley and triticale in pretreated trials (e) late-harvested barley and triticale in pretreated trials (f) wheat straw and rye straw in pretreated trials.

Major VFA species were acetic acid, propionic acid, butyric acid and valeric acid; followed by caproic acid, isovaleric acid and isobutyric acid. VFA concentrations significantly increased on the 10^{th} day in the pretreated digesters ($p < 0.05$), which similarly showed no accumulation at the end. Highest VFA speciation was observed in EB_P and ET_P digesters. Up to 1161 mg/L butyric acid, 803 mg/L caproic acid and 714 mg/L valeric acid were measured in EB_P on the 10th day of AD. Despite the high concentrations of VFAs in the anaerobic digesters, pH values were in the range of 7.0-7.5 and never dropped below 7 due to excess alkalinity of the seed sludge (see Table 4.1.).

Cellulose degradation in the anaerobic digesters are shown in Figure 5.6. Higher amount of cellulose degradation was recorded in the pretreated digesters compared to the controls. In the control digesters fed with cow manure and harvest residues, namely RB_C, RT_C, RW_C, RR_C, cellulose reduction was determined in the range of 36-44%. In the pretreated digesters, cellulose reduction almost doubled and reached to 75%, 76%, 77% and 80% in RB_P, RT_P, RW_P, RR_P, respectively. In the control digesters fed with cow manure and early-harvested crops, EB_C and ET_C, cellulose reduction was 44% and 47%, which was increased to 52% and 56% in EB_P and ET_P after the fungal pretreatment. Meanwhile, reduction in cellulose content of anaerobic digesters treating cow manure and late-harvested crops were 56% and 57% in LB_C and LT_C and slightly increased to 64% and 59% in the pretreated digesters LB_P and LT_P, respectively.

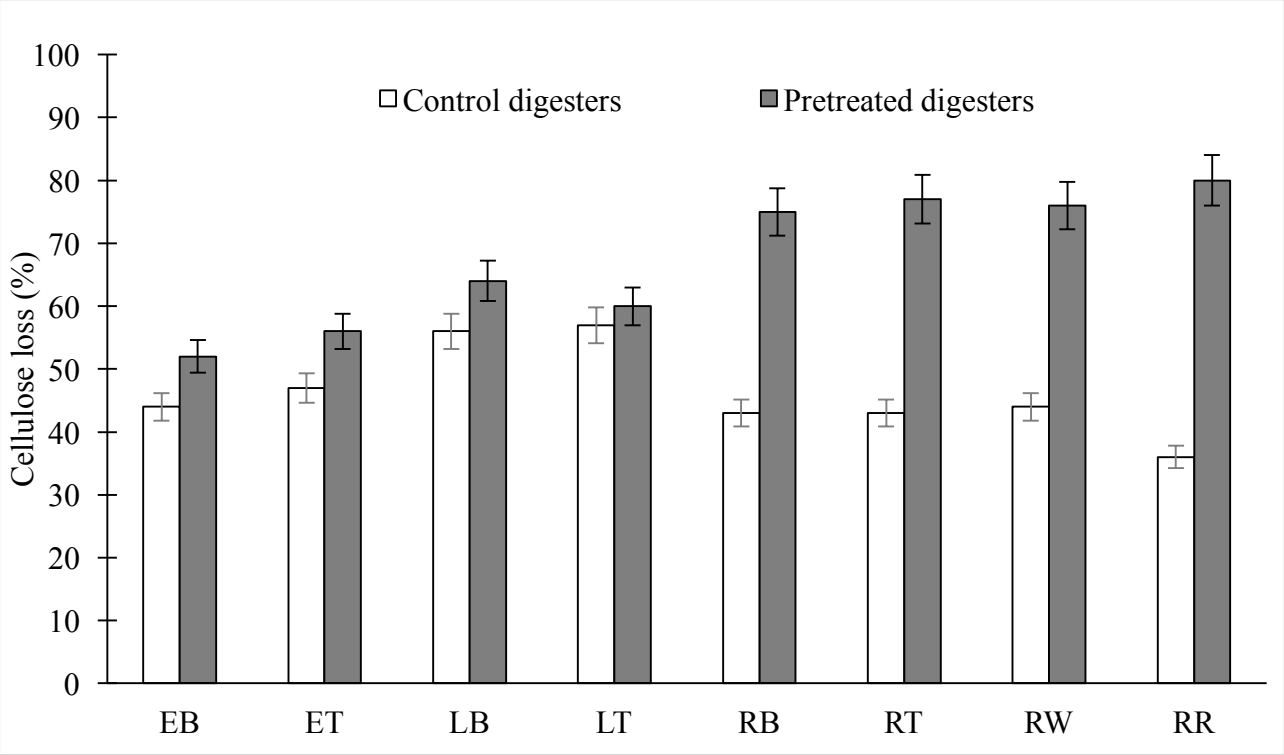


Figure 5.6. Cellulose loss in control and pretreated digesters.

Pretreatment of radiate pine using two different species of white rot fungi, namely *Stereum hirsutum* and *T. versicolor* (Shirkavand et al., 2017), the authors reported better result in selective lignin degradation by *T. versicolor* but more cellulose degradation by *S. hirsatum*. Different lignocellulose degradation and eventually varying methane yields are reported with respect to selected white rot fungi species (Lalak et al., 2016; Mustafa et al., 2016).

VFA production in anaerobic digesters upon fungal pretreatment is another lack of information stated in the literature. In this particular study, VFA profiles showed major variations between the control digesters and pretreated digesters in terms of major VFA species and concentrations. Propionic acid was found to be the most prevalent VFA product in the control digesters; meanwhile acetic acid mostly dominated the pretreated digesters. It is well-known that high concentrations of propionic acid can be a good indicator of process upset, as it tends to build up during digestion imbalance (Tsapekos et al., 2017a). Although no VFA accumulation was recorded at the end of the digestion period, we can clearly say that pretreated trials went through a more stable digestion in terms of VFA profiles. Furthermore, the effect of harvesting time on VFA production was also another remarkable outcome. As mentioned earlier, VFA speciation was much higher in pretreated anaerobic digesters fed with cow manure and early harvested barley (EB_P) and triticale (ET_P) comparing to LB_P and LT_P.

Without degrading or solubilizing the lignin, cellulose and hemicellulose are not accessible at all by the cellulases and hemicellulases, respectively (Ghosh et al., 2017; Zikeli et al., 2014). As more lignin is degraded during fungal pretreatment, more cellulose is exposed to anaerobic bacteria bacteria in the AD step. In this regard, taking advantage from the ligninolytic potentials of these microorganisms via fungal pretreatment creates a more favorable environment for the biogas microbiome in anaerobic digesters.

5.3. Enzyme Expression Assays

Comparison of expression levels for the selected key functional groups responsible for cellulose degradation in AD possessing genes encoding GH families 5 (*cel5*), 6 (*cel2*) and 48 (*cel48*) are presented in Figure 5.7. There was not a significant difference in GH5 expression level between control and pretreated digesters, On the other hand, GH6 gene was expressed higher in pretreated digesters in comparison to the control digesters. Similarly, the gene copy numbers of GH48 was slightly higher pretreated digesters, respectively.

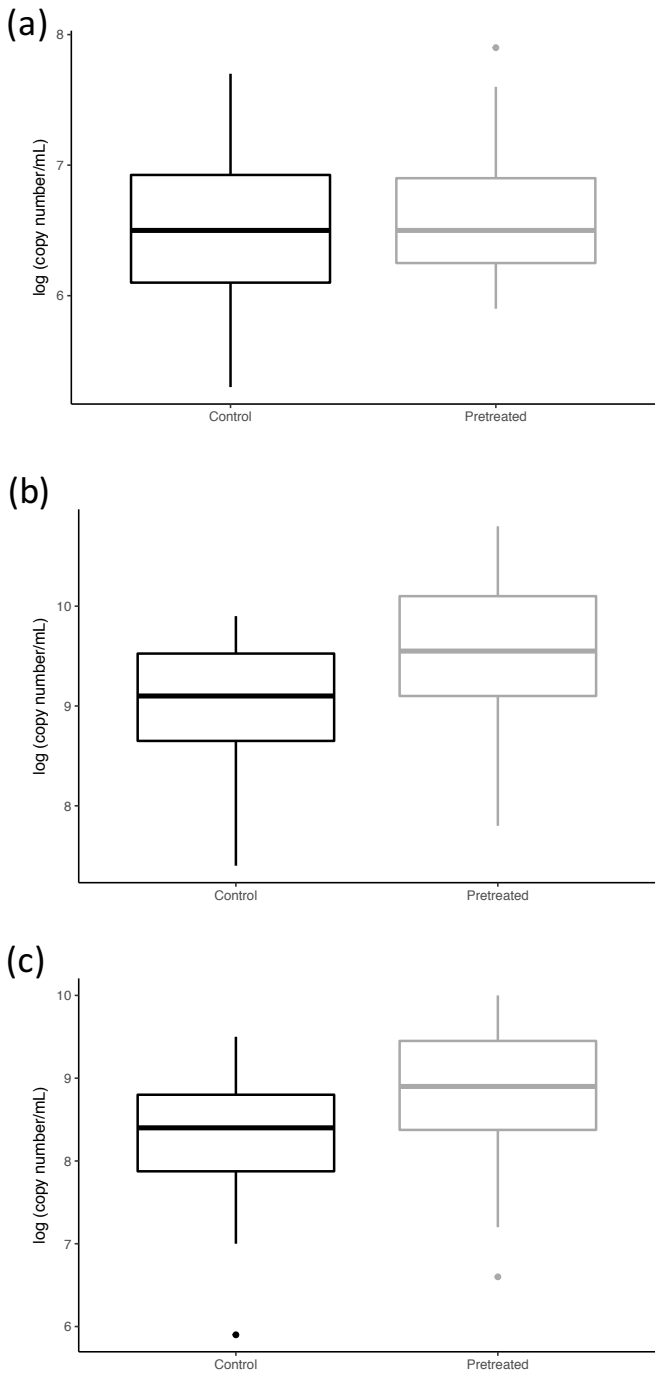


Figure 5.7. Expression levels of target cellulase genes for (a) GH5 family (b) GH6 family (c) GH48 family in control and pretreated digesters.

Expression levels of 6 representative xylanase genes (*xynA*, *xynB*, *xynC*, *xynD*, *xynE*, *xynF*) responsible for the breakdown of hemicellulose in AD are given in Figure 5.8. In all examined gene expressions, there was not a significant difference between the control and pretreated digesters and were slightly higher in pretreated digesters.

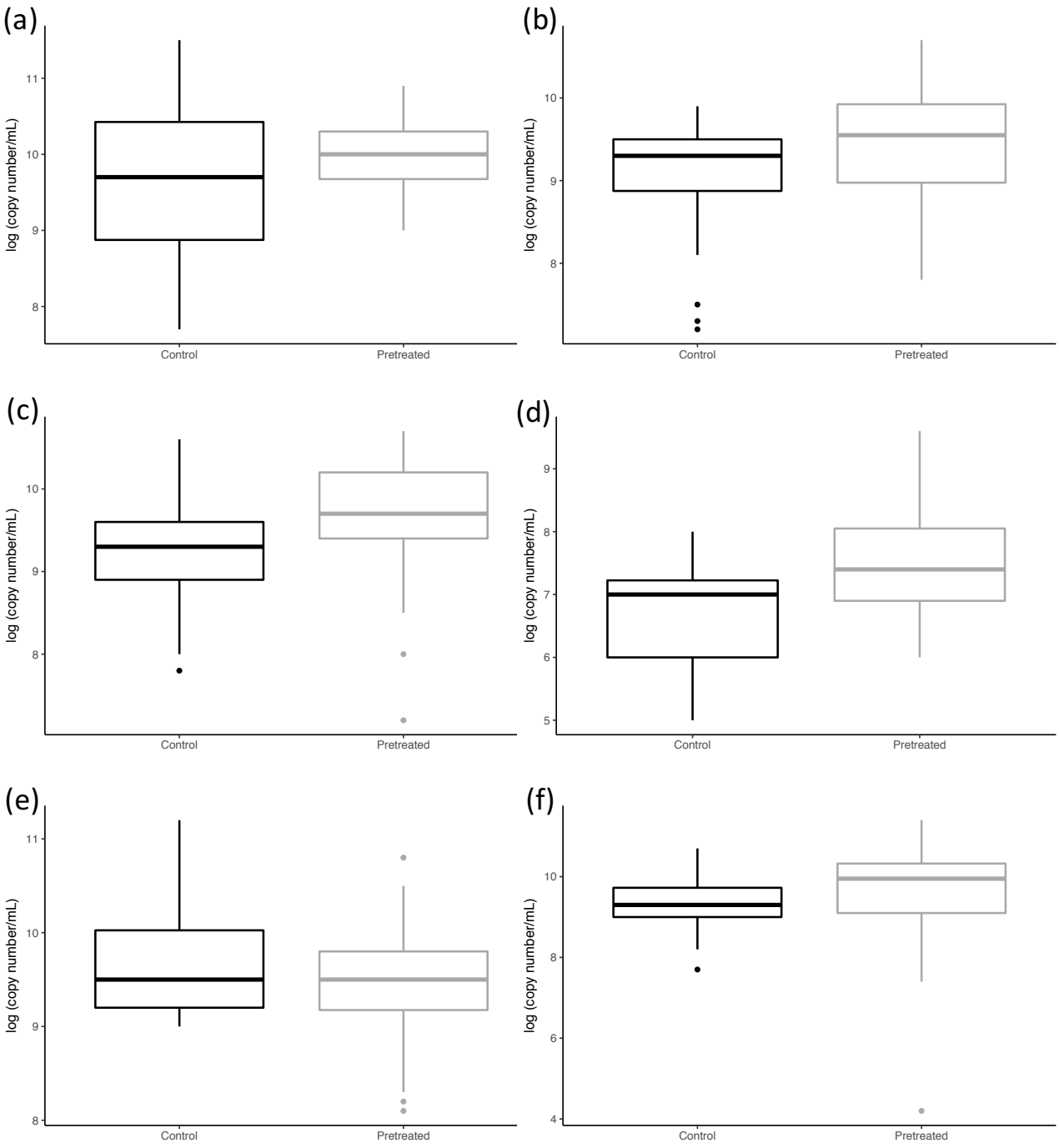


Figure 5.8. Expression levels of target xylanese genes for (a) *xynA* (b) *xynB* (c) *xynC* (d) *xynD* (e) *xynE* (f) *xynF* in control and pretreated digesters.

Figure 5.9 represents the expression levels of target laccase gene to compare lignin degradation in the digesters. There was an increase on the level of this gene expression due to fungal pretreatment.

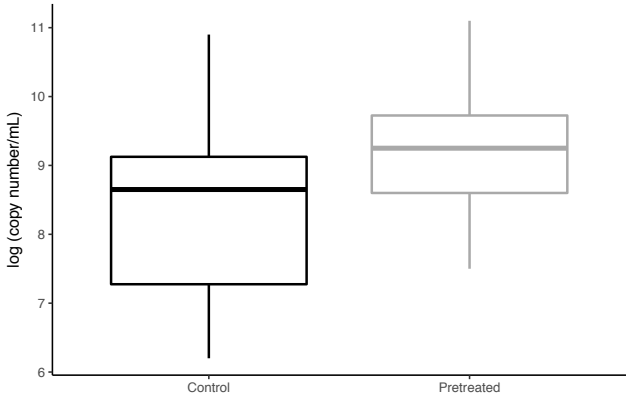


Figure 5.9. Expression level of target laccase gene in control and pretreated digesters.

The quantification of the expression levels of the key enzymes of acetoclastic and hydrogenotrophic methanogenesis, *Acs1* and *mcrA* genes, respectively, are shown in Figure 5.10 and Figure 5.11. There was not a significant difference in terms of *Acs1* expression level between control and pretreated digesters. Meanwhile, *mcrA* gene copy number in the pretreated digesters were slightly higher than that of the controls.

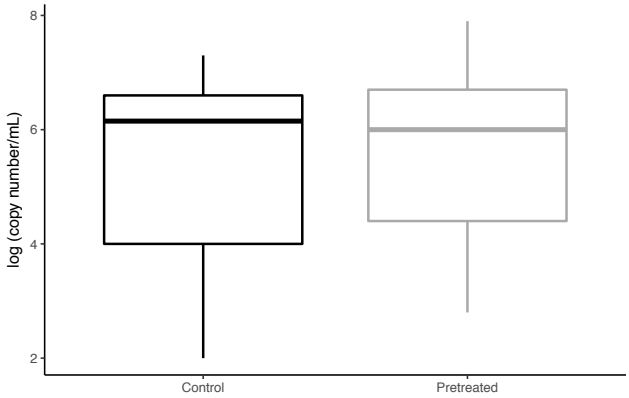


Figure 5.10. Expression level of target acetyl-CoA gene (*Asc1*) in control and pretreated digesters.

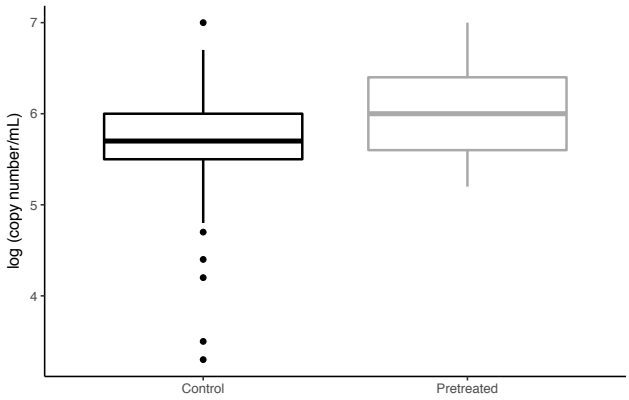


Figure 5.11. Expression level of target methyl coenzyme M reductase gene (*mcrA*) in control and pretreated digesters.

Overall, the results showed that there was quite a difference in the expression levels of selected lignocellulolytic enzymes, which marked a more efficient breakdown of lignocellulose (Biswas et al., 2014). While there was not a significant difference in *Acs1*copy gene numbers, higher expression levels of *mcrA* gene pointed out the superiority of hydrogenotrophic methanogenesis over acetoclastic methanogenesis in the pretreated digesters.

5.4. Microbial Community Dynamics

The principal coordinate analysis did show a distinct clustering between microbial communities in the control and pretreated anaerobic digesters as shown in Figure 5.12. Beta diversity analysis introduced a significant crop type and pretreatment effect in which each type of samples clustered together ($p < 0.05$).

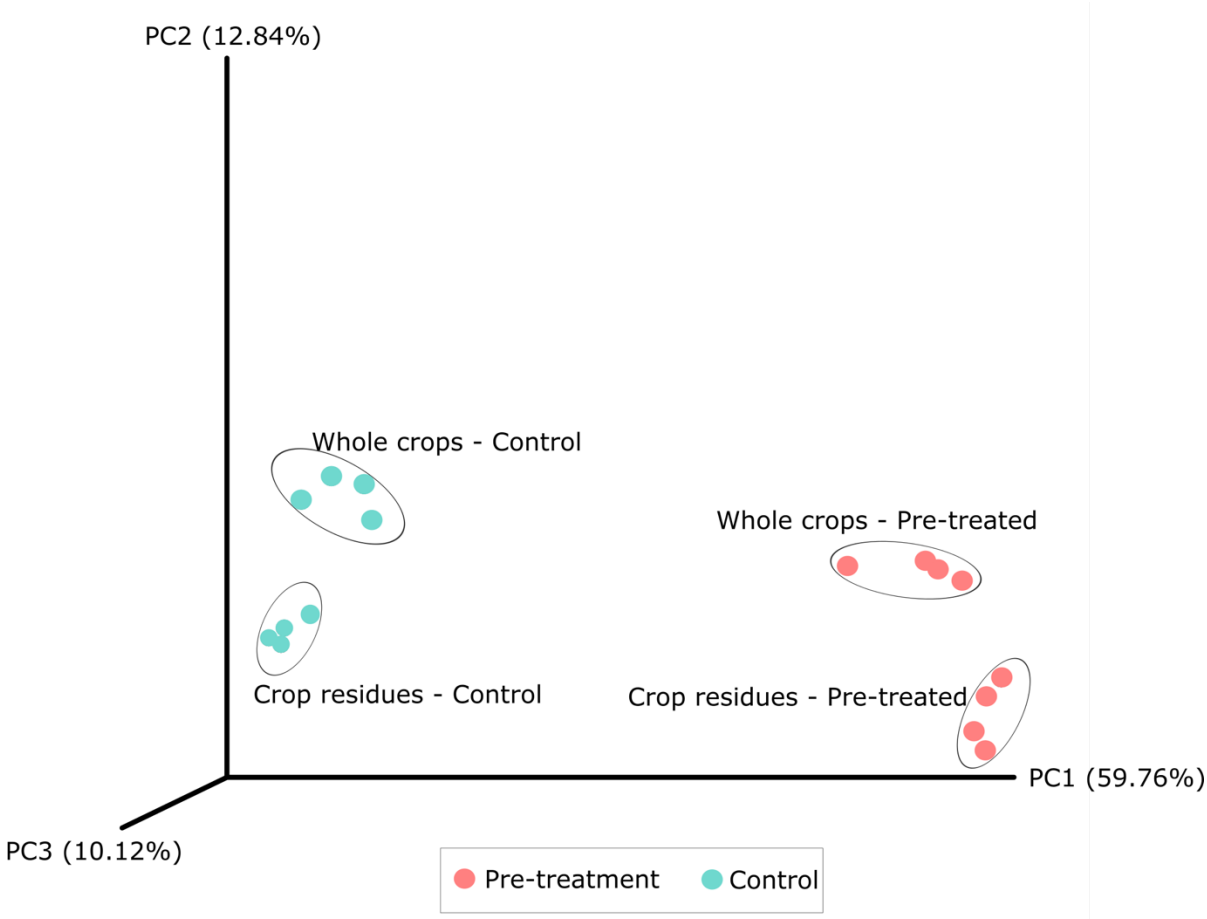


Figure 5.12. Two dimensional plot of a 3D principal component analysis based on the beta diversity of microbial communities in control and pretreated digesters.

Table 5.1 displays the estimated richness and evenness of the microbial communities in the control and pretreated digesters. The estimated richness based on Chao1 index ranged between 174-

337 and 145-313 in the control and pretreated digesters, respectively, in which highest values were calculated for RW digesters. Compared to the pretreated digesters, higher diversity, evenness and richness indices were calculated for the control digesters.

Table 5.1. Summary of the estimated richness and evenness of the microbial communities in the control and pretreated digesters.

Sample	Shannon	Simpson	Chao1	Pielou's evenness
EB_C	4.31	0.98	196	0.82
ET_C	4.14	0.97	191	0.79
LB_C	4.08	0.96	187	0.78
LT_C	4.24	0.97	238	0.77
RB_C	3.95	0.96	174	0.76
RT_C	4.24	0.97	253	0.77
RW_C	4.34	0.97	337	0.75
RR_C	4.18	0.97	240	0.76
EB_P	3.67	0.94	150	0.73
ET_P	3.65	0.93	178	0.70
LB_P	3.47	0.93	145	0.70
LT_P	3.59	0.93	190	0.69
RB_P	3.91	0.95	228	0.72
RT_P	3.81	0.94	241	0.69
RW_P	3.93	0.95	313	0.68
RR_P	3.89	0.94	265	0.70

Taxonomic profiling revealed that, although relative abundances differed among the samples at the phylum level, *Firmicutes* (55-75%) and *Bacteroidetes* (15-41%) dominated the bacterial communities in digesters regardless of the crop type and pretreatment (Figure 5.13). Whereas, *Synergistetes* and *Thermotogae* were determined in very minor abundances in the control digesters, the species of these phyla got advantage and became abundant in the pretreated digesters. The relative abundances of *Synergistetes and Thermotogae* were determined between 6-8% and 1-3% in the fungal-pretreated digesters, respectively.

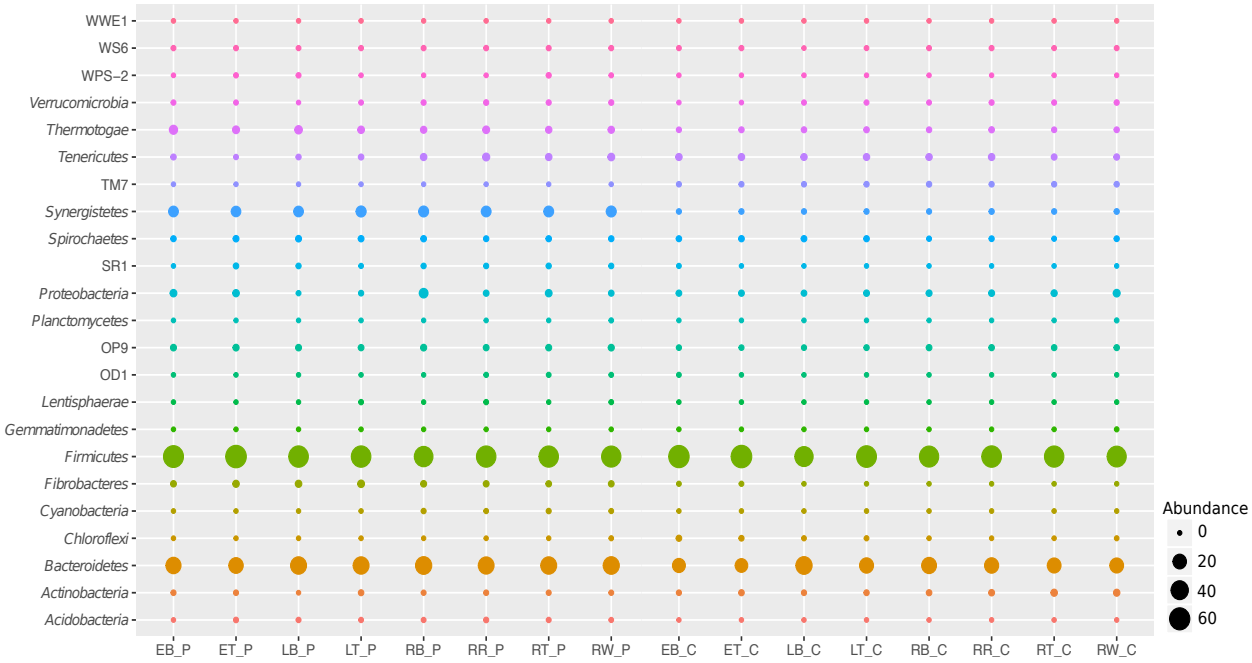


Figure 5.13. The relative abundances of bacterial phyla in control and pretreated digesters with a proportion of at least 0.1% in one sample.

At the family level, by far the greatest portion of the sequences belonged to *Porphyromonadaceae* (phylum *Bacteroidetes*) as shown in Figure 5.14. The abundances of *Firmicutes* families *Caldicoprobacteraceae* and *Clostridiaceae* were doubled in the control digesters compared to the pretreated sets. On the other hand, there is not a distinct difference in the abundances of *Ruminococcaceae* and *Syntrophomonadaceae* between the control and pretreated digesters. Differently, whereas *Tissierellaceae* and *Erysipelotrichaceae* families were more abundant in the control digesters, *Anaerobaculaceae* and *Thermotogaceae* had higher abundances in the pretreated digesters.

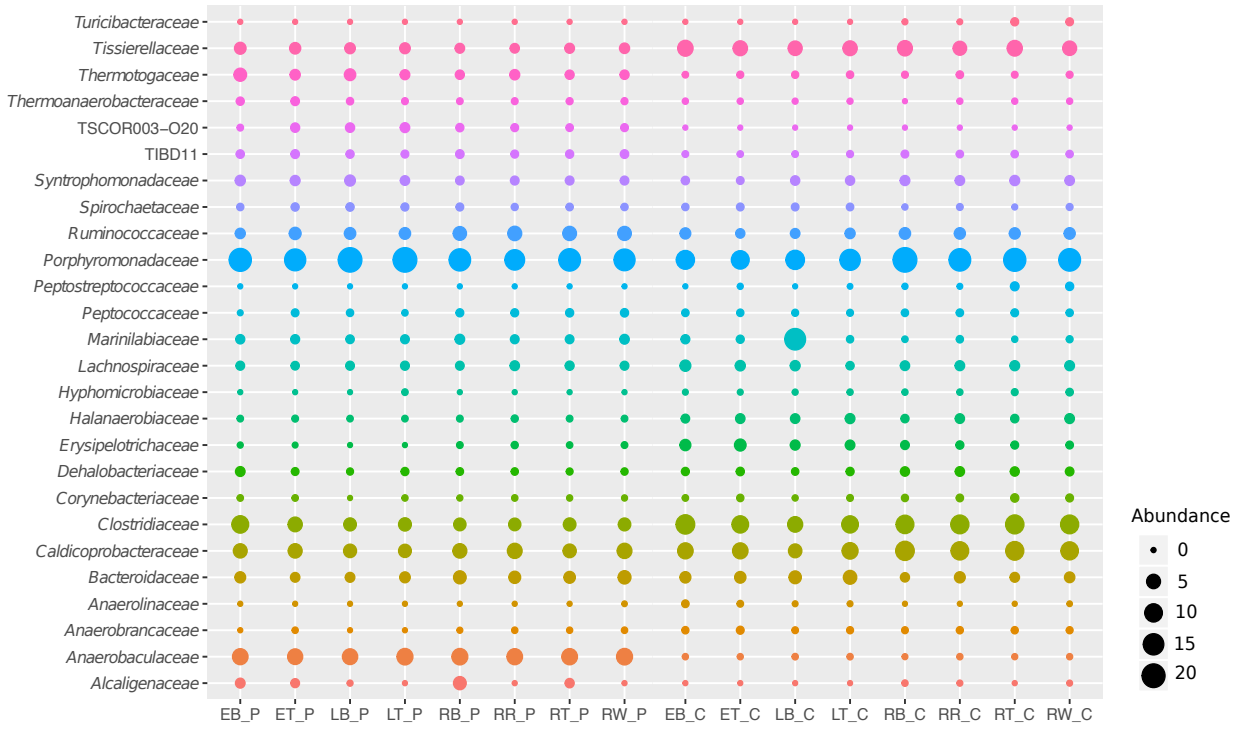


Figure 5.14. The relative abundances of bacterial families in control and pretreated digesters with a proportion of at least 0.1% in one sample.

At the genus level, the control digesters were dominated by *Caldicoprobacter*, *Clostridium* and *Sedimentibacter* species (Figure 5.15). On the other hand, *Anaerobaculum* was the most dominant genus in the pretreated digesters (except EB_P digester). *Caldicoprobacter*, *Clostridium* and *Sedimentibacter* were also detected in the pretreated digesters at relatively low abundances compared to the control digesters.

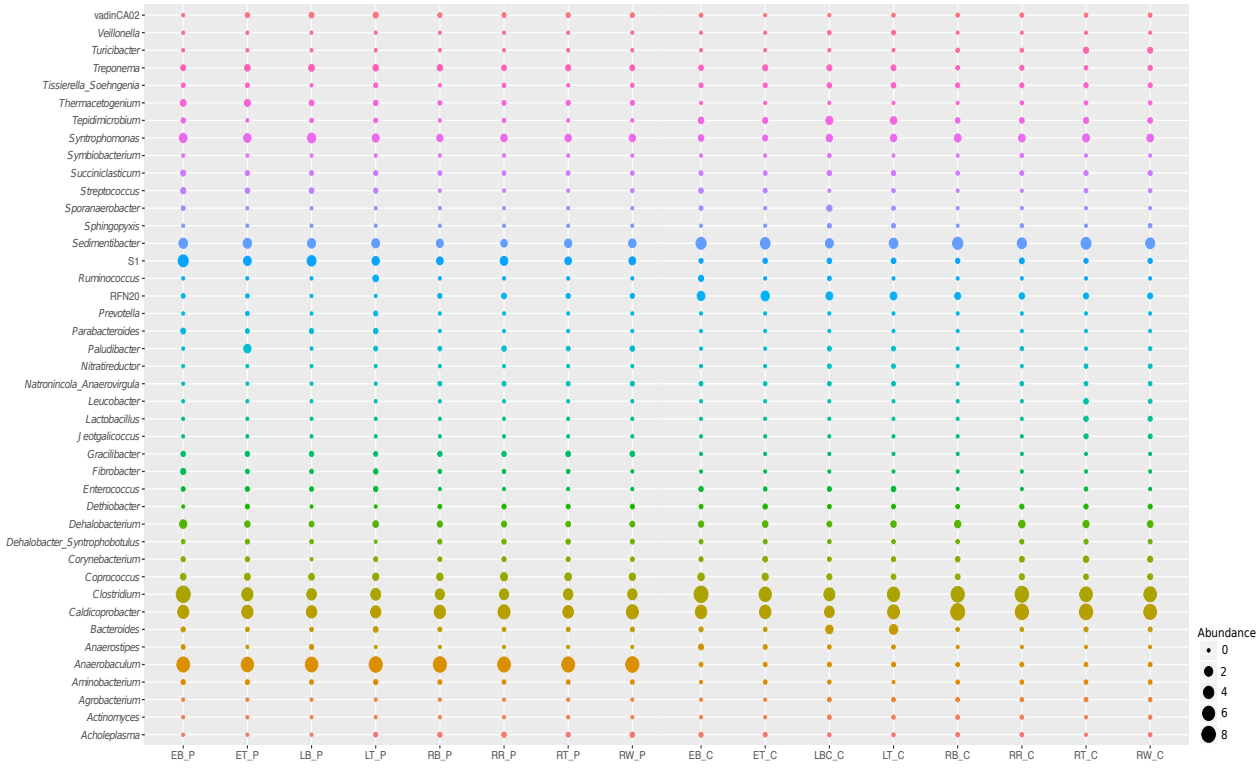


Figure 5.15. The relative abundances of bacterial genera in control and pretreated digesters with a proportion of at least 0.1% in one sample.

Figure 5.16 display the relative abundances of methanogenic families in the control and the pretreated digesters. The families, *Methanobacteriaceae*, *Methanomicrobiaceae* and *Methanosarcinaceae* were determined in the pretreated digester samples. As it is clear from Figure 5.17, almost all of the reads belong to *Methanosarcina* in the control digesters except RT_C and RW_C digesters at the genus level. The pretreated digesters harbored a more diverse methanogenic community in which *Methanoculleus* and *Methanosarcina* were also the predominant genera. Additionally, *Methanobacterium* was only detected in the pretreated digesters. This can be attributed to VFA production and speciation in pretreated digesters as mentioned earlier. Acetic acid was the main VFA product in the pretreated digesters meanwhile higher VFA speciation was observed. This might have caused a more favorable environment for the methanogens.

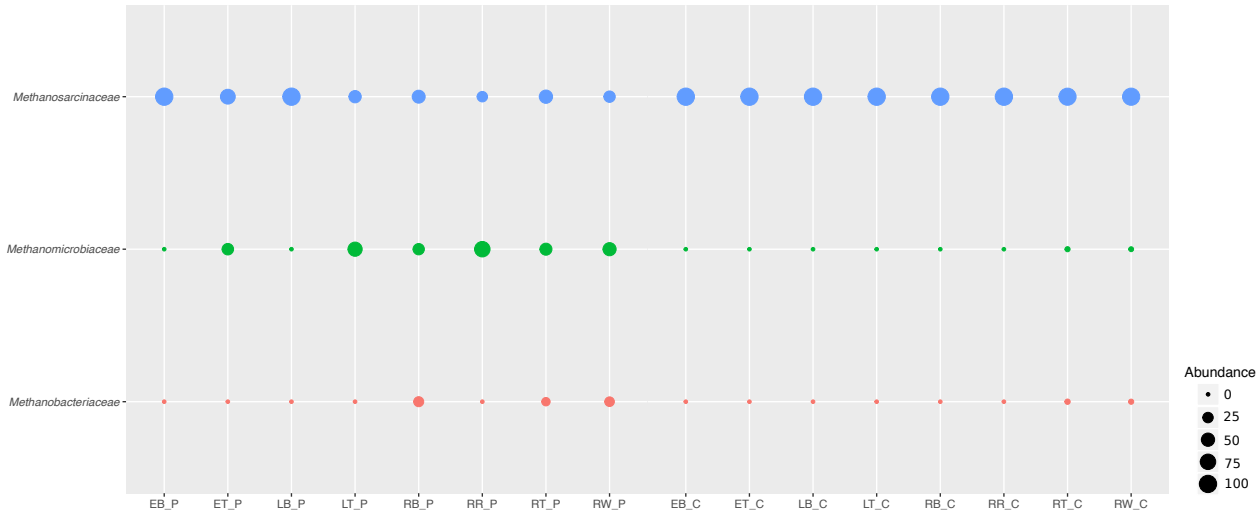


Figure 5.16. The relative abundances of methanogenic families in control and pretreated digesters with a proportion of at least 0.1% in one sample.

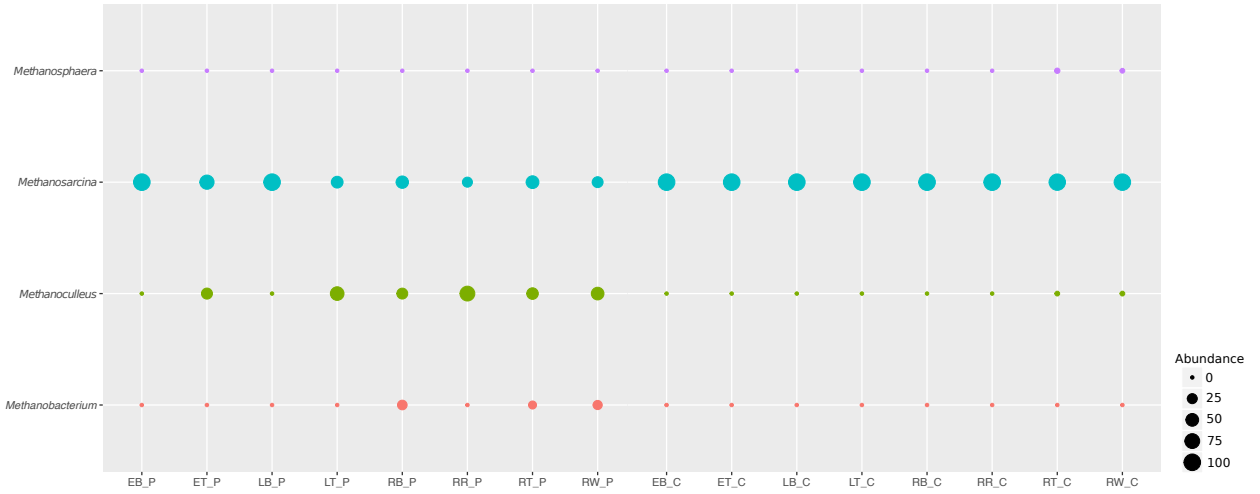


Figure 5.17. The relative abundances of methanogenic genera in control and pretreated digesters with a proportion of at least 0.1% in one sample.

According to the clustering of OTUs as given in Figure 5.18, two main groups and four subgroups were formed. Bacterial communities were clustered with respect to the implementation of fungal pretreatment and further formed two main groups based on the use of whole crop or straw.

An exclusive finding in the pretreated digesters was the phylum *Synergistetes*, which was mainly assigned to the genus *Anaerobaculum*. The members of this genus which can ferment various sugars to mainly acetate and H_2 (Maune and Tanner, 2012), increased their relative abundance after the pretreatment with *T. versicolor*. These species are most probably the main contributors of the

fermentation and the activity of these species may explain the higher acetic acid concentrations in the pretreated digesters.

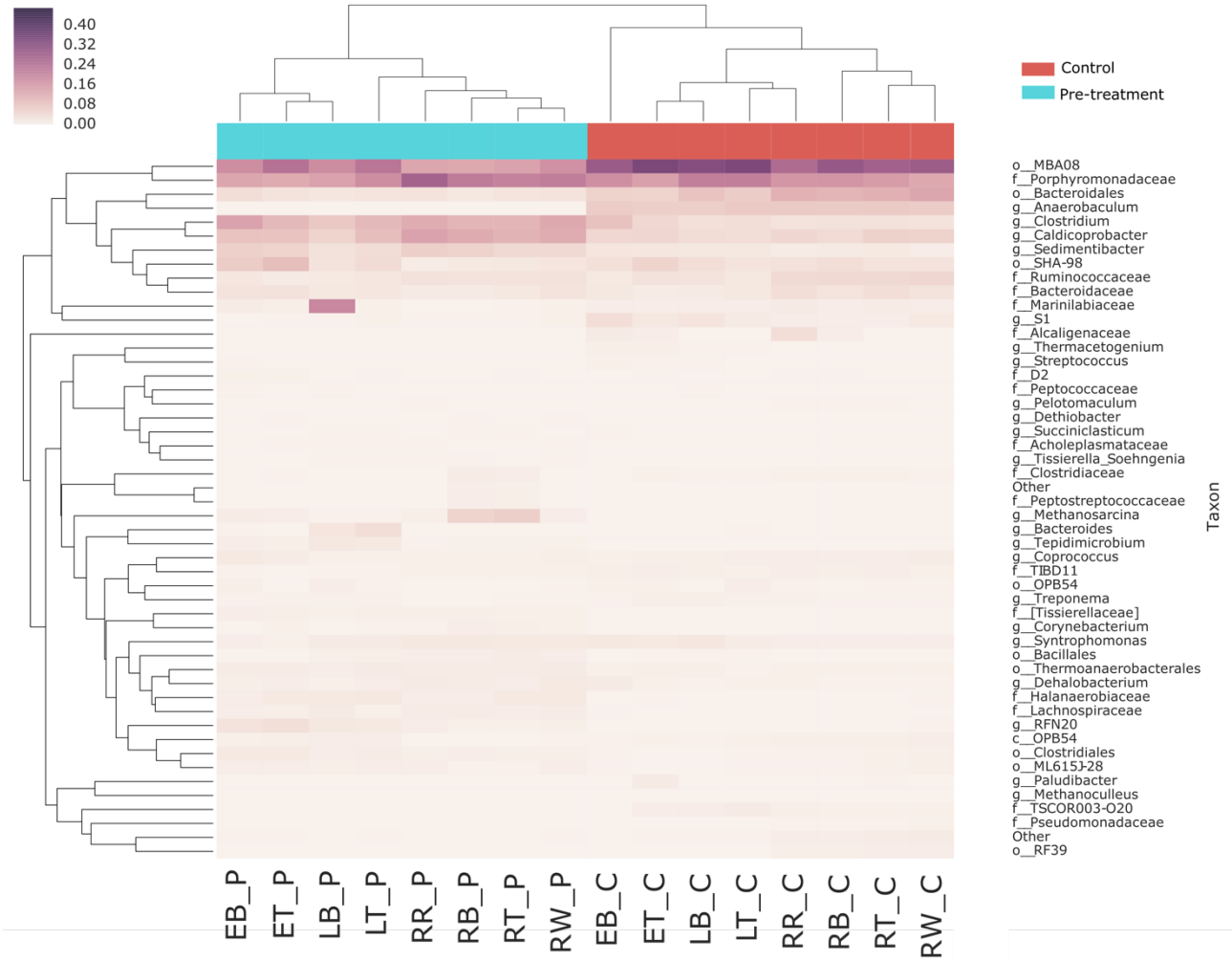


Figure 5.18. Heatmap displaying the unique sequence abundance of microbial communities in control and pretreated digesters.

Caldicoprobacter, *Clostridium* and *Sedimentibacter* species were observed in higher abundances in the control digesters. Similarly, Sun and colleagues (Sun et al., 2015) also determined *Sedimentibacter* in high abundance in the digesters treating lignocellulosic substrate, wheat straw. It was not an expected outcome since the members of this genus cannot use carbohydrate and just utilize amino-acids (Imachi et al., 2016). Whereas, *Clostridia* species are considered as cellulosic-degrading bacteria (Wiegel et al., 2006), *Caldicoprobacter* can degrade various sugars such as fructose, galactose, lactose, mannose, xylose, and cellobiose.

Fungal pretreatment with *T. versicolor* also contributed a more diverse methanogenic community, which in return achieved higher methane yields. *Methanosarcina* almost completely

dominated the control digesters, except for RT_C and RT_W. On the other hand, the predominant genera were *Methanoculleus* and *Methanobacterium* in the pretreated digesters along with *Methanosarcina*. *Methanosarcina* species are among the most-detected methanogenic archaea in anaerobic digesters (Bozan et al., 2017) which typically grow on acetate (Vavilin et al., 2008); meanwhile *Methanoculleus* and *Methanobacterium* are *hydrogenotrophic* methanogens (Franke-whittle et al., 2014). *Methanoculleus* was previously reported in anaerobic digesters fed with cattle manure (Goberna et al., 2009) and cattle manure with wheat straw as the co-substrate (Öner et al., 2018), both indicated a more diverse group of methanogenic archaea (Franke-whittle et al., 2014). It is known that high concentrations of VFAs inhibit methane production, known as substrate inhibition, both viable for acetic acid and propionic acid (Dang et al., 2016; Vavilin et al., 2008). We assume that comparatively higher concentrations of VFAs (mostly propionic acid) might have limited the methanogenic activity in the control digesters. The presence of hydrogenotrophic methanogen community would indicate the syntrophic relationships between acetate oxidizers and hydrogenotrophic methanogens which marks the main pathway for acetate degradation and methanogenesis (Franke-whittle et al., 2014). In case of less production of propionic acid, butyric acid and hydrogen, the conversion of fermentable substrates primarily to acetate and carbon dioxide with electron transfer to *Methanosarcina* would be expected which limits the need for syntrophic metabolism of the VFAs (Dang et al., 2016). This could be contributed to the fact that among the pretreated digesters, EB_P and LB_P were also dominated by *Methanorsarcina*. Furthermore, no significant increase in the methane yield was observed between EB_C and EB_P. Further elucidation of which functional genes take place during lignocellulose degradation as well as methanogenesis will be imported to improve our knowledge on biogas microbiome.

6. FUNGAL BIOAUGMENTATION WITH *Orpinomyces* sp. TO ENHANCE METHANE PRODUCTION FROM LIGNOCELLULOSIC BIOMASS

Limited information exists regarding the microbial community dynamics in AD upon fungal bioaugmentation. Existing studies reported that indigenous biogas microbiome remarkably changed via fungal bioaugmentation with a mix culture of anaerobic fungi. It is well known that the bioaugmentation culture as well as the type of feedstock greatly affects the efficiency of bioaugmentation and biomethanation in AD. To the best of our knowledge, no research was conducted to investigate fungal bioaugmentation of cereal crops in AD and evaluate changes in biogas microbiome. This study therefore aimed to examine the effect of fungal bioaugmentation with *Orpinomyces* sp. on methane production from selected cereal crops and cow manure under pre-mentioned AD conditions. The link between the harvesting stage of the crops, the application of fungal bioaugmentation, VFA and methane production together with cellulose degradation was thoroughly assessed. Bacterial and methanogenic archaeal populations were characterized using high throughput 16s rRNA gene amplicon sequencing. Since cereal crops and straws are perfect candidates for biogas production, this aprticular study provided the investigation of the key players in lignocellulose degradation and reveal the impacts of fungal bioaugmentation approach on biogas microbiome as the presence and activity of anaerobic fungi in agricultural biogas plants were recently revealed.

6.1. Anaerobic Co-Digestion Trials

6.1.1. Methane Production

Bioaugmentation of the anaerobic digesters with *Orpinomyces* sp. significantly increased ($p<0.05$) the methane production in almost all AD trials. Early-harvested barley (EB_B) yielded highest methane as 430 mL CH_4/g VS upon fungal bioaugmentation, an increment by 14% comparing to its control (EB_C) (Figure 6.1). On the other hand, the impact of fungal bioaugmentation was more apparent on late-harvested barley (LB) trials, since 22% of increase in methane yield was observed in LB_B compared to LB_C. Similarly, fungal bioaugmentation of barley residues (RB_B) digesters achieved 23% higher methane yield than RB_C.

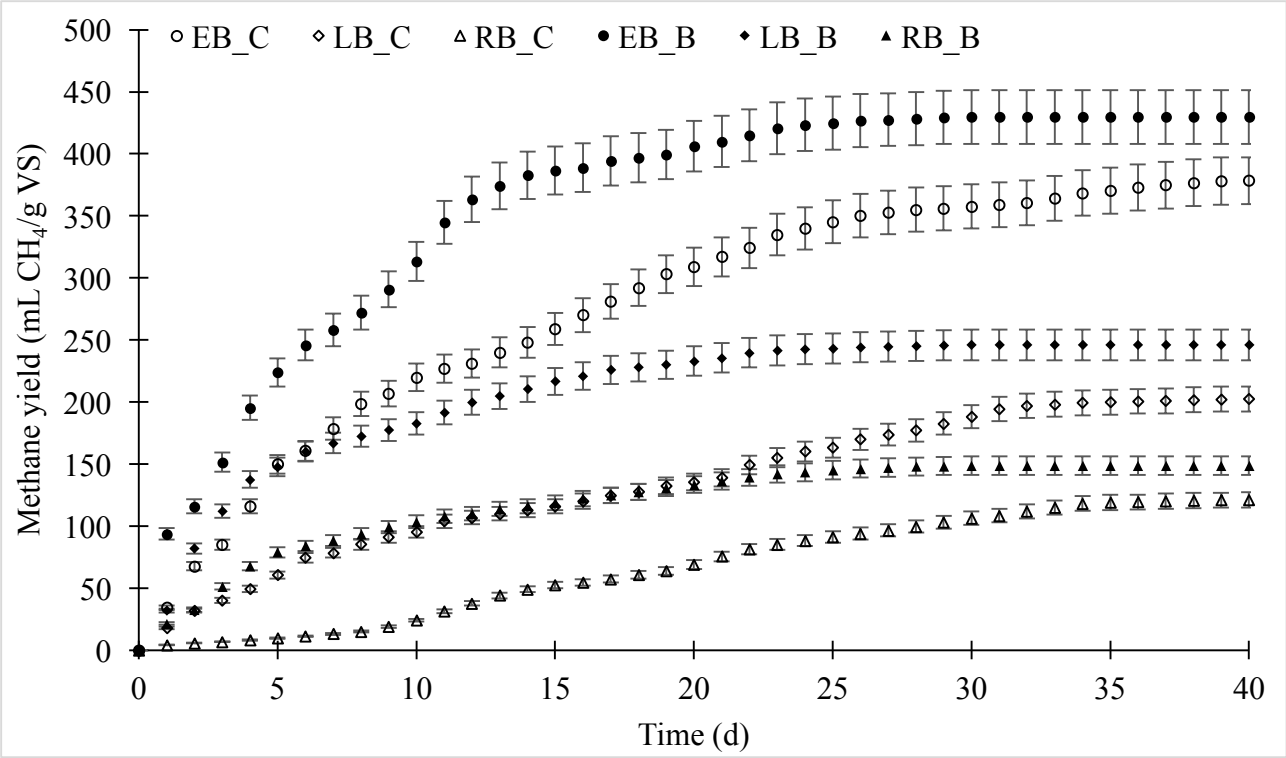


Figure 6.1. Methane yields in control and bioaugmented trials during anaerobic co-digestion of cow manure and barley at different harvesting stages.

The effect of bioaugmentation with *Orpinomyces* sp. was observed at the highest when the co-substrate of cow manure was selected as triticale (Figure 6.2). Fungal bioaugmentation of the digesters fed with early-harvested (ET_B) and late-harvested triticale (LT_B) boosted the methane yield up to 254 mL CH_4/g VS and 244 mL CH_4/g VS, performing 33% and 26% higher than their controls, ET_C and LT_C, respectively. Meanwhile, methane yields in the anaerobic digesters fed with triticale residues (RT) were 161 mL CH_4/g VS and 136 mL CH_4/g VS in bioaugmented (RT_B) and non-bioaugmented (RT_C) set-ups, respectively. Methane yields obtained from the anaerobic co-digestion of cow manure and wheat straw (RW) and rye straw (RR) are also given in Figure 6.3. This time, there was no significant difference was observed between these straws ($p > 0.05$) yielding 118 mL CH_4/g VS and 115 mL CH_4/g VS in RW_C and RR_C, respectively. However, fungal bioaugmentation contributed to an increase in methane yields by 15% and 17% in RW_B and RR_B comparing to their controls, respectively.

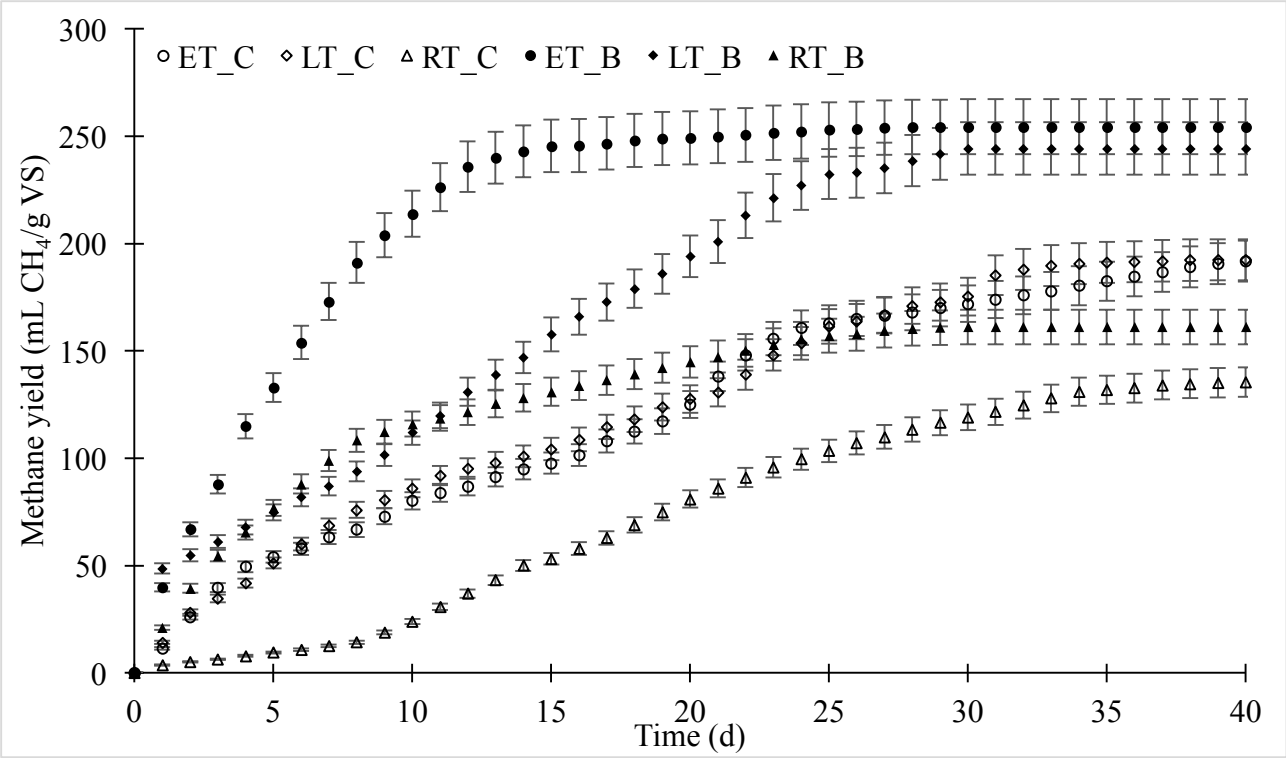


Figure 6.2. Methane yields in control and bioaugmented trials during anaerobic co-digestion of cow manure and triticale at different harvesting stages.

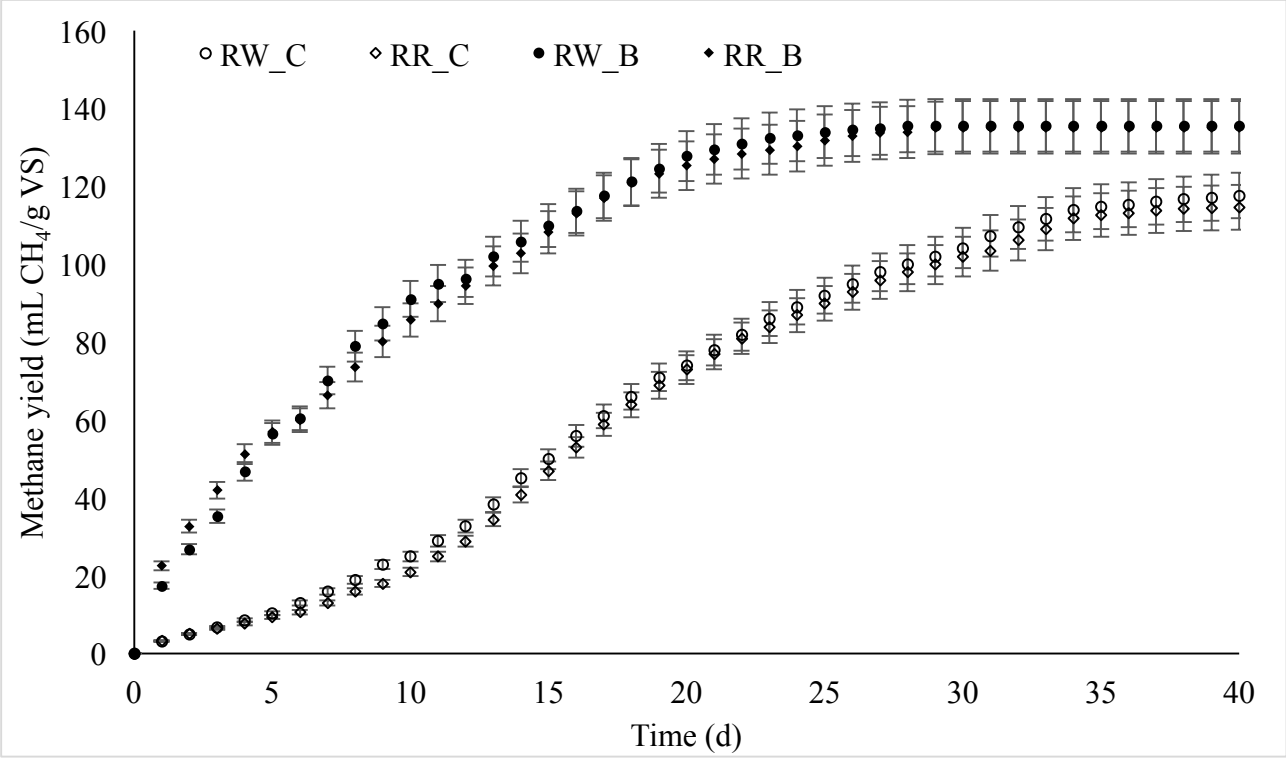


Figure 6.3. Methane yields in control and bioaugmented trials during anaerobic co-digestion of cow manure and wheat straw and rye straw.

Based on the results obtained from fungal bioaugmentation of AD tests, 15-33% increase in methane yields was achieved. Selection of co-substrate type as well as its harvesting stage was a critical factor to effect methane production together with the degree of enhancement by bioaugmentation. In all conditions, early-harvested barley achieved the highest methane yield most probably due to its comparatively lower lignin content and/or higher organic content (see Table 4.1). On the other hand, fungal bioaugmentation worked best on triticale-fed digesters. Straw parts of the cereal crops produced lower methane as expected, due to their high lignocellulose content compared to main crop. However, bioaugmentation showed remarkable methane yield increase in these digesters.

Another important contribution of fungal bioaugmentation was that it contributed to shorter microbial lag phase during anaerobic biodegradation, which eventually brought reduced retention time of anaerobic digesters. (Nkemka et al., 2015) also reported affirmative effects (3-10% increase in methane yield) of fungal bioaugmentation with another anerobic fungus *P. rhizinflata* YM600 in a two-stage (leach bed-UASB reactor) system using corn sillage and cattail as the substrates. Recently, combined bioaugmentation with anaerobic ruminal fungi (i.e. *Neocallimastix* sp. and *Orpynomyces* sp.) and fermentative bacteria (a fermenting-acidogenic component, consisting of a hydrogen-producing bacterial pool (F210)) was used to enhance biogas production from wheat straw and mushroom spent straw.

6.1.2. VFA Production and Cellulose Degradation

VFA production in the bioaugmented digesters are given in Figure 6.4. Accordingly, the highest VFA production was measured on Day 10 in all trials. Although acetic acid was the main product on Day 0, propionic acid dominated the VFAs on Day 10. Other VFA species were measured at minor concentrations. When compared to their control digesters (given in Figure 5.5), much lower VFA production was observed. As can be seen, highest VFA concentration was measured in RW_B and RR_B (approximately 2500 mg propionic acid/L), followed by EB_B (1830 mg propionic acid/L). It was most probable that the peak VFA production was missed some time between Day 0 and Day 10. As mentioned earlier, fungal bioaugmentation caused faster hydrolysis and therefore acidification occurred earlier than expected. Nevertheless, similar to fungal pretreatment experiments, VFA speciation was richer than the control digesters. Furthermore, most part of the produced VFAs were consumed and not accumulated at the end of the AD tests. pH was also in the range of 7.0-7.5 during the digestion thanks to excess alkalinity of the anaerobic seed sludge.

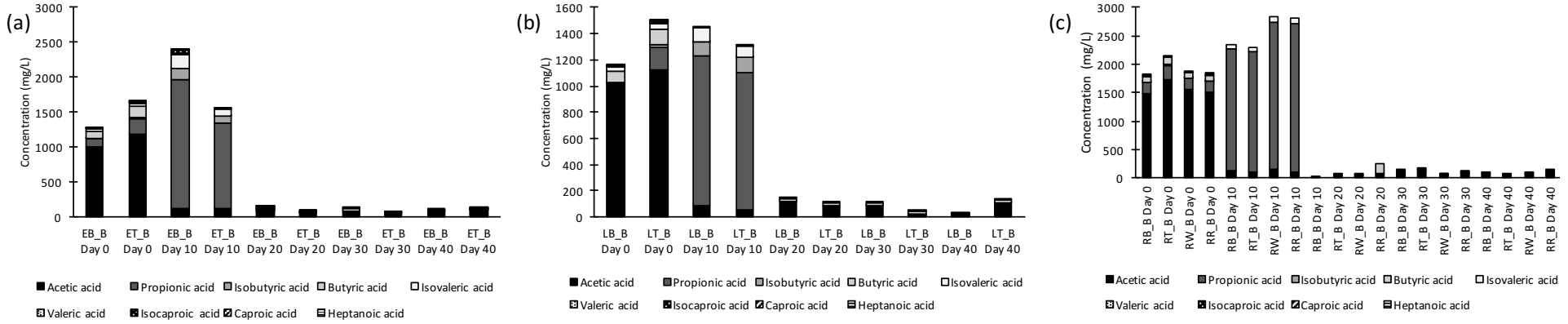


Figure 6.4. VFA profiles during anaerobic co-digestion of cow manure and (a) early-harvested barley and triticale in bioaugmented trials (b) late-harvested barley and triticale in bioaugmented trials (c) wheat straw and rye straw in bioaugmented trials.

Cellulose reduction in the anaerobic digesters are shown in Figure 6.5. Fungal bioaugmentation resulted in significantly higher cellulose degradation ($p < 0.05$) of 72%, 69%, 66% and 76% in EB_B, ET_B, LB_B and LT_B, respectively. Cellulose degradation in bioaugmented straw-fed digesters reached up to 80%, 79%, 82% and 86% in RB_B, RT_B, RW_B and RR_B, respectively. It can be easily said that fungal bioaugmentation contributed the highest cellulose degradation among all AD set-ups. This is because anaerobic fungus *Orpinomyces* posses a wide range of enzymes that are capable of cellulose degradation.

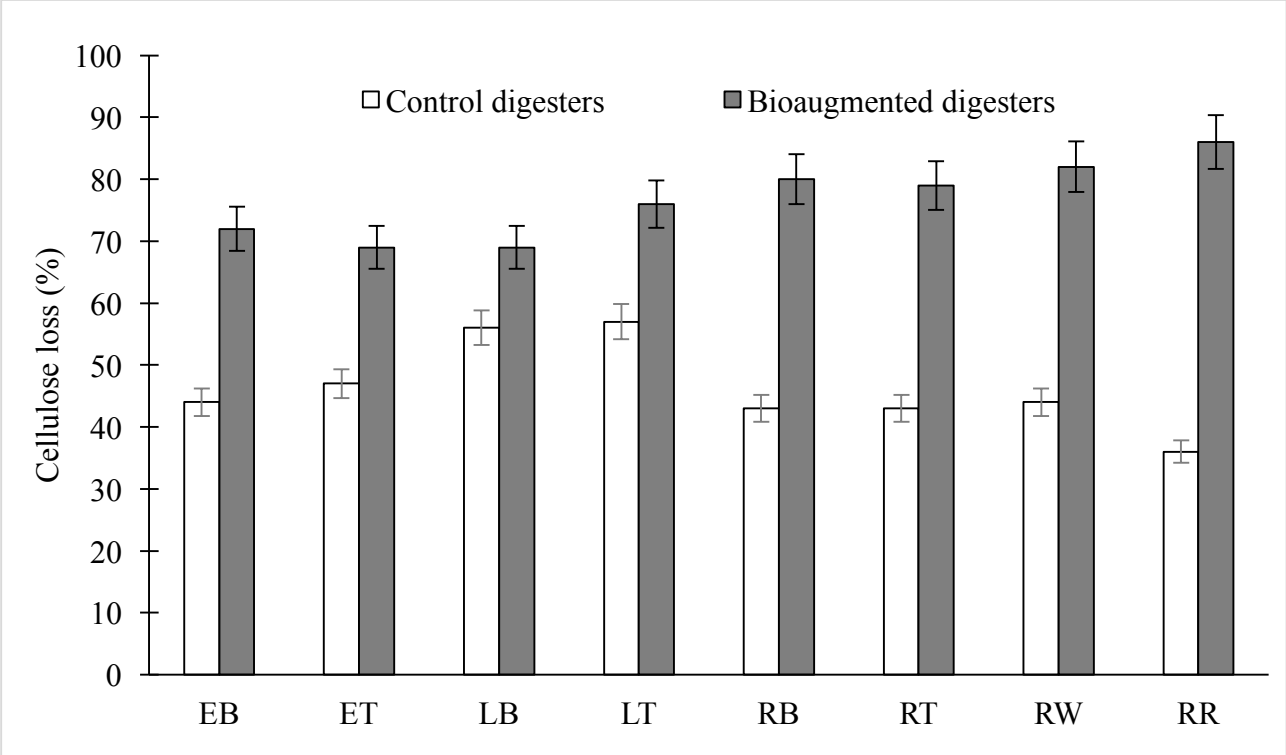


Figure 6.5. Cellulose loss in control and bioaugmented digesters.

In the study of (Nkemka et al., 2015), fungal bioaugmentation was reported to lead to an increased degradation of VFA, which was concluded to be beneficial to avoid VFA accumulation and inhibition of AD. However, much lower pH values were also reported in the bioaugmented digesters, which could be problematic for AD stability. Similarly, acetic acid, butyric acid and propionic acid were found to be the main VFA products. In the study of (Aydin et al., 2017), acetic acid was the dominant VFA species in the early days of AD (600-700 mg/L), which latter dominated by propionic acid and consumed completely at the end upon fungal bioaugmentation of microalgae-fed anaerobic digesters.

6.2. Enzyme Expression Assays

Expression levels of the representative genes for GH families 5, 6 and 48 are shown in Figure 6.6. There was a clear difference in GH5 and GH6 expression level between control and bioaugmented digesters. On the other hand, GH48 gene was expressed on the same level in all digesters.

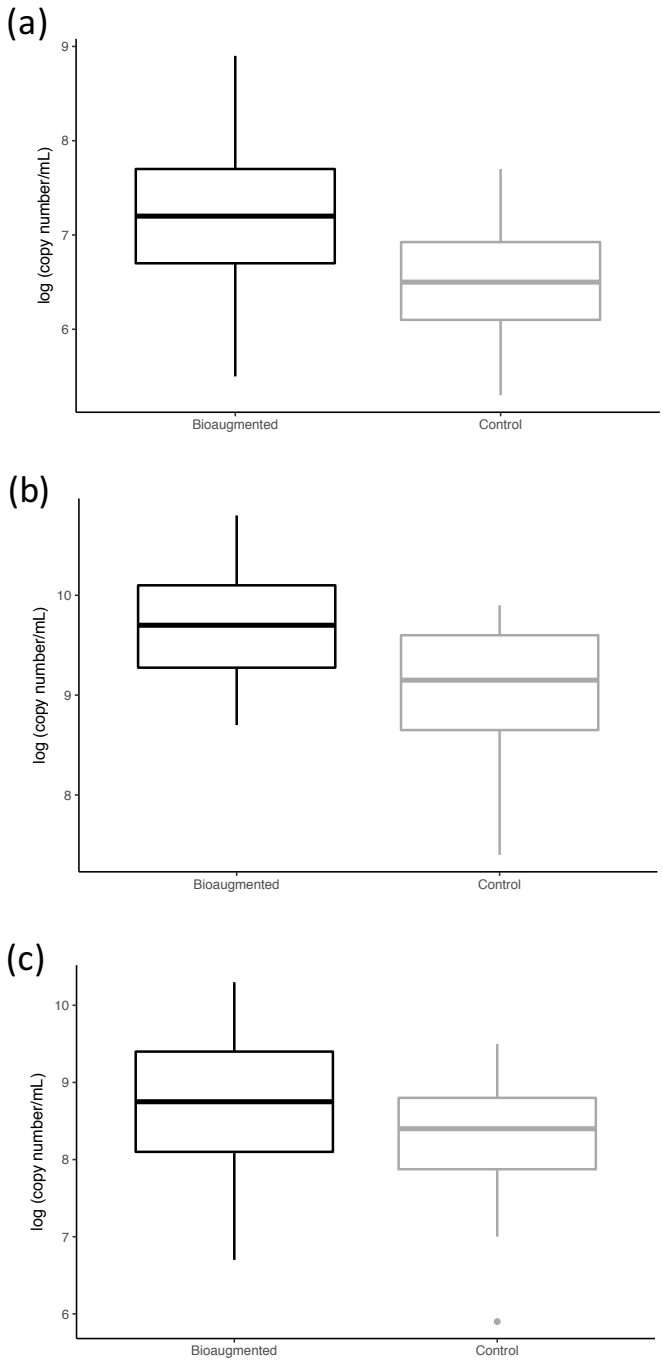


Figure 6.6. Expression levels of target cellulase genes for (a) GH5 family (b) GH6 family (c) GH48 family in control and bioaugmented digesters.

Expression levels of 6 representative xylanase genes (*xynA*, *xynB*, *xynC*, *xynD*, *xynE*, *xynF*) in the control and bioaugmented digesters are given in Figure 6.7. *xynD* and *xynF* genes were expressed significantly higher ($p<0.05$) in the bioaugmented digesters compared to the controls. Other xylanese genes, on the other hand, were expressed slightly higher in the bioaugmented digesters. Accordingly, the effect fungal bioaugmentation was most apparent on hemicellulose key enzymes.

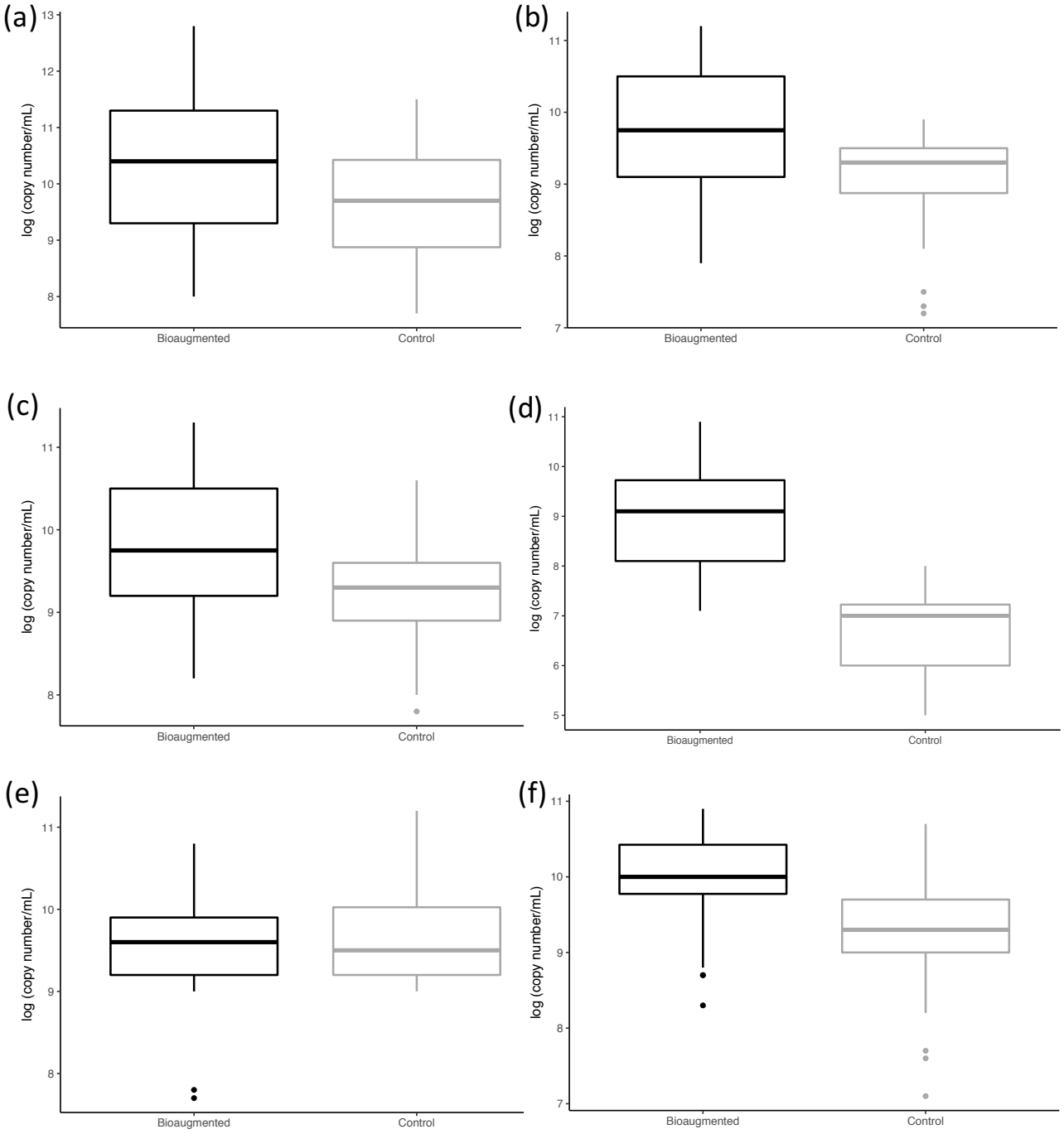


Figure 6.7. Expression levels of target xylanese genes for (a) *xynA* (b) *xynB* (c) *xynC* (d) *xynD* (e) *xynE* (f) *xynF* in control and bioaugmented digesters.

As shown in Figure 6.8., the level of target laccase gene expression was at the same level between in control and bioaugmented digesters. This is most probably due to the fact that anaerobic fungus *Orpinomyces* sp. possess cellulolytic enzymes specific for cellulose and hemicellulose degradation but not effective for the breakdown of lignin.

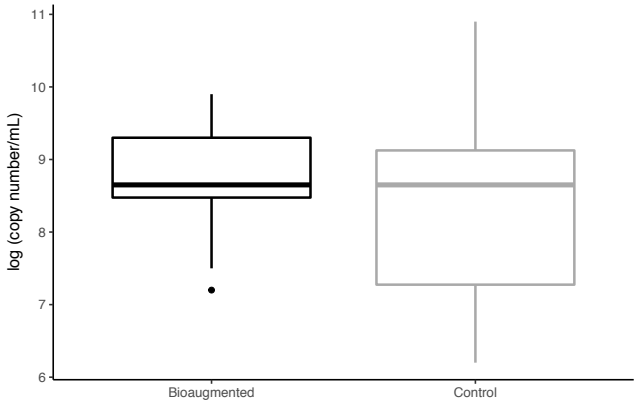


Figure 6.8. Expression level of target laccase gene in control and bioaugmented digesters.

The quantification of *Acs1* and *mcrA* genes are shown in Figure 6.9 and Figure 6.10, respectively. Similar to the fungal pretreatment set-up, there was not a significant difference in terms of *Acs1* expression level between control and bioaugmented digesters. On the other hand, *mcrA* gene copy numbers were significantly different in the bioaugmented digesters and almost one-fold higher than that of the controls.

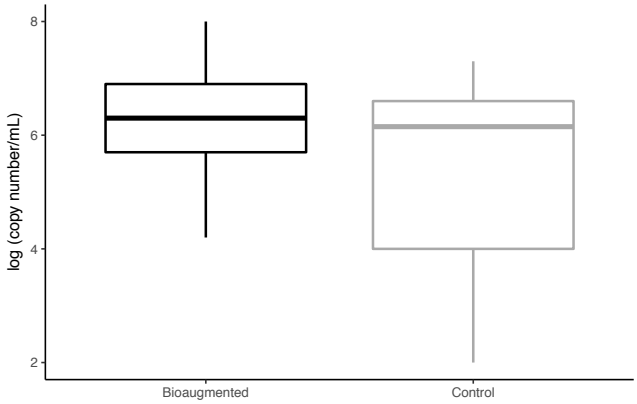


Figure 6.9. Expression level of target acetyl-CoA gene (*Asc1*) in control and bioaugmented digesters.

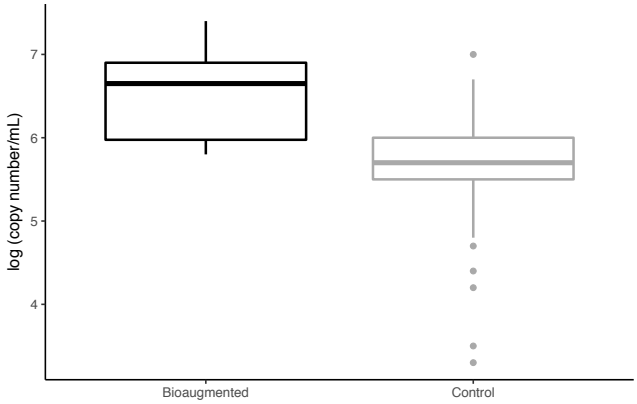


Figure 6.10. Expression level of target methyl coenzyme M reductase gene (*mcrA*) in control and bioaugmented digesters.

Overall, the results showed that there was a slight difference in the expression levels of selected lignocellulolytic enzymes in the pretreated digesters, which marked a comparatively more efficient breakdown of lignocellulose (Biswas et al., 2014). While there was not a significant difference in *Acs1*copy gene numbers, higher expression levels of *mcrA* gene highlighted that the increase in methane production upon fungal bioaugmentation was mostly due to higher activity of hydrogenotrophic methanogens.

6.3. Microbial Community Dynamics

Based on the results obtained from the sequencing analysis, an average number of 51159 raw reads per sample was produced with an average length of 250 bp. Alpha diversity indices are given in Table 6.1. Overall, highest indices values were achieved in the control digesters and bioaugmentation caused the microbial diversity to decrease. The microbial evenness of the bioaugmented digesters were also lower since the highest estimated richness (Chao1 indice) was calculated in RW_C and the lowest in EB_B. Only in the RB trials, the richness was higher after bioaugmentation (RB_B) application compared to its control (RB_C).

The Shannon diversity indices were calculated between 3.99-4.40 and 3.44-3.95 in the control and bioaugmented digesters, respectively. There was quite a remarkable difference in the Simpson indices in the EB, RT and RW digesters and EB_C had the most even microbial community among other digesters.

Table 6.1. Summary of the estimated richness and evenness of the microbial communities in control and bioaugmented digesters.

Sample	Chao1	Shannon	Simpson	Pielou's evenness
EB_C	231	4.34	0.98	0.80
ET_C	235	4.21	0.97	0.77
LB_C	221	4.12	0.96	0.76
LT_C	291	4.30	0.97	0.76
RB_C	204	3.99	0.96	0.75
RT_C	308	4.30	0.97	0.75
RW_C	412	4.40	0.97	0.73
RR_C	290	4.23	0.97	0.75
EB_B	133	3.44	0.94	0.70
ET_B	201	3.68	0.95	0.69
LB_B	171	3.69	0.95	0.72
LT_B	234	3.70	0.94	0.68
RB_B	344	3.95	0.95	0.68
RT_B	278	3.81	0.93	0.68
RW_B	212	3.67	0.93	0.69
RR_B	240	3.87	0.95	0.70

Microbial diversity differences between the samples are shown PCA plot in Figure 6.11 as the beta diversity. There was a significant difference between the bioaugmentation and control set-ups. In each set-up, the samples were divided into 2 groups based on using the main crop or straw parts. More specifically, main crop-fed digesters (i.e. EB, ET, LB, LT) AD were clustered together in groups in the bioaugmented and control digesters. Whereas, straw-added digesters (i.e. RB, RT, RW, RR) showed higher similarity within and were clustered together.

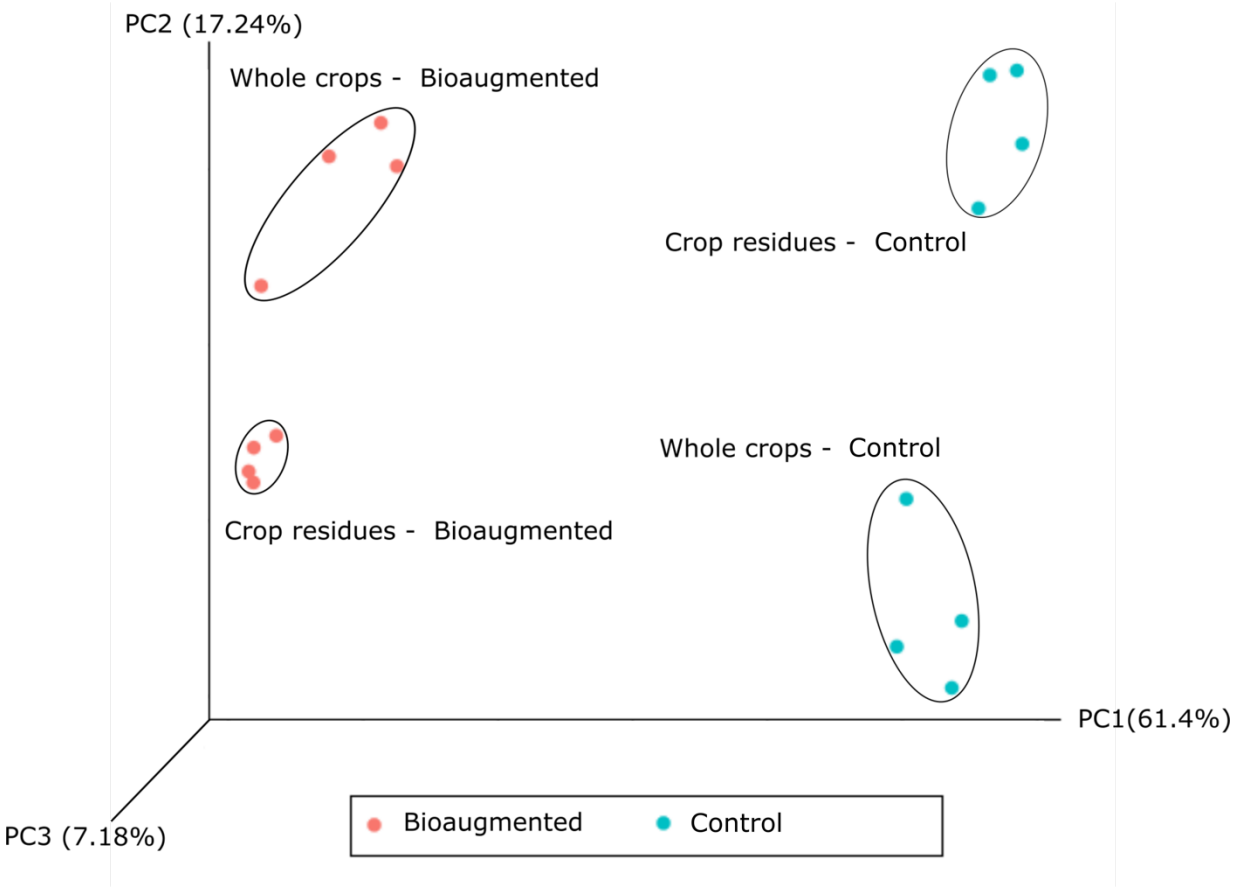


Figure 6.11. Two dimensional plot of a 3D principal component analysis based on the beta diversity of microbial communities in control and bioaugmented digesters.

Figures 6.12 and 6.13 represents the relative abundances of the bacterial phyla and families in AD systems, respectively. *Firmicutes* (52%-75%) and *Bacteroidetes* (19-40) were the most abundant phyla in all digesters, followed by *Synergistetes* (8-9%), *Proteobacteria* (1%-9%), OP9 (2%-4%) and *Thermotogae* (1%-4%) in the bioaugmented digesters. In the control digesters, *Proteobacteria* and *Tenericutes* were also detected in low abundances.

Similar bacterial families were detected in all digesters with varying proportions. Accordingly, most dominant bacterial families in the bioaugmented digesters were as follows: an unclassified family of the order MBA08, *Porphyromonadaceae*, *Anaerobaculaceae*, *Caldicoprobacteraceae* (, and unclassified family of the order *Bacteroidales*, *Clostridiaceae*, *Ruminococcaceae*, TIBD11, *Thermotogaceae*, *Tissierellaceae*, *Bacteroidaceae* and *Syntrophomonadaceae*. Meanwhile, *Porphyromonadaceae*, an unclassified family of the order MBA08 and *Clostridiaceae* were the most abundant bacterial families in the control digesters, followed by *Caldicoprobacteraceae*, *Tissierellaceae*, *Bacteroidaceae*, *Erysipelotrichaceae*, *Ruminococcaceae*, an unclassified family of the order *Bacteroidales*, *Lachnospiraceae* and *Syntrophomonadaceae*.

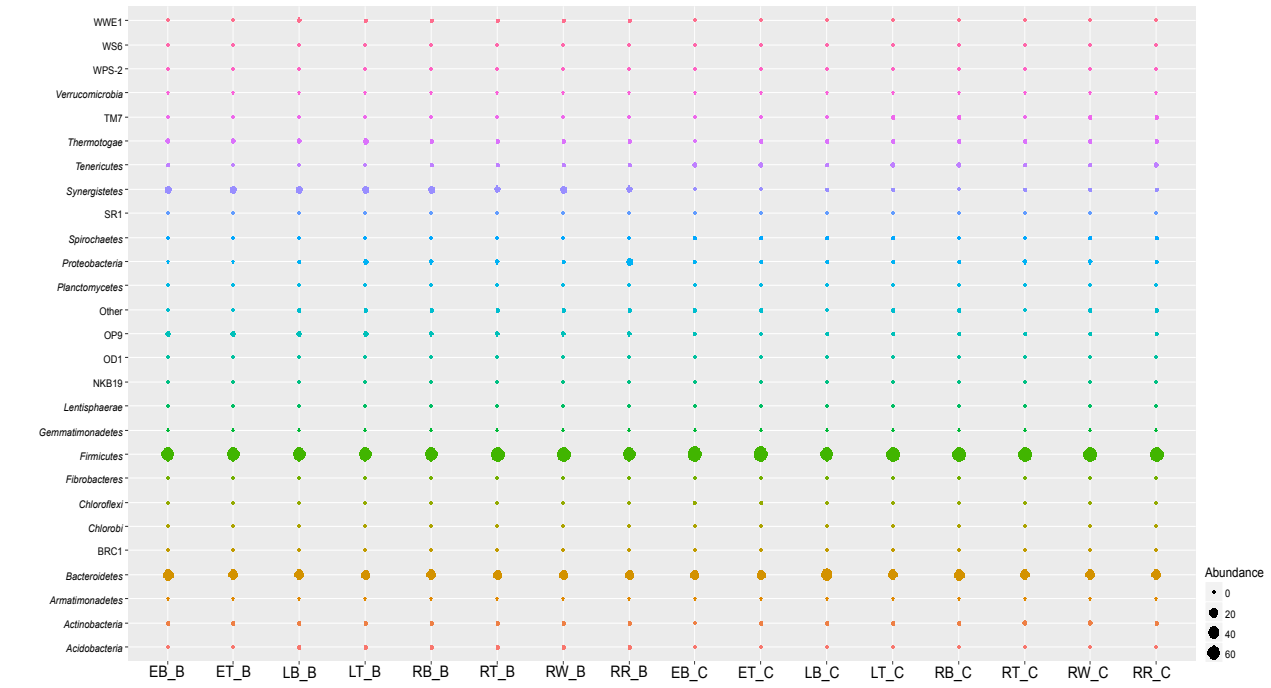


Figure 6.12. The relative abundances of bacterial phyla in control and bioaugmented digesters with a proportion of at least 0.1% in one sample.

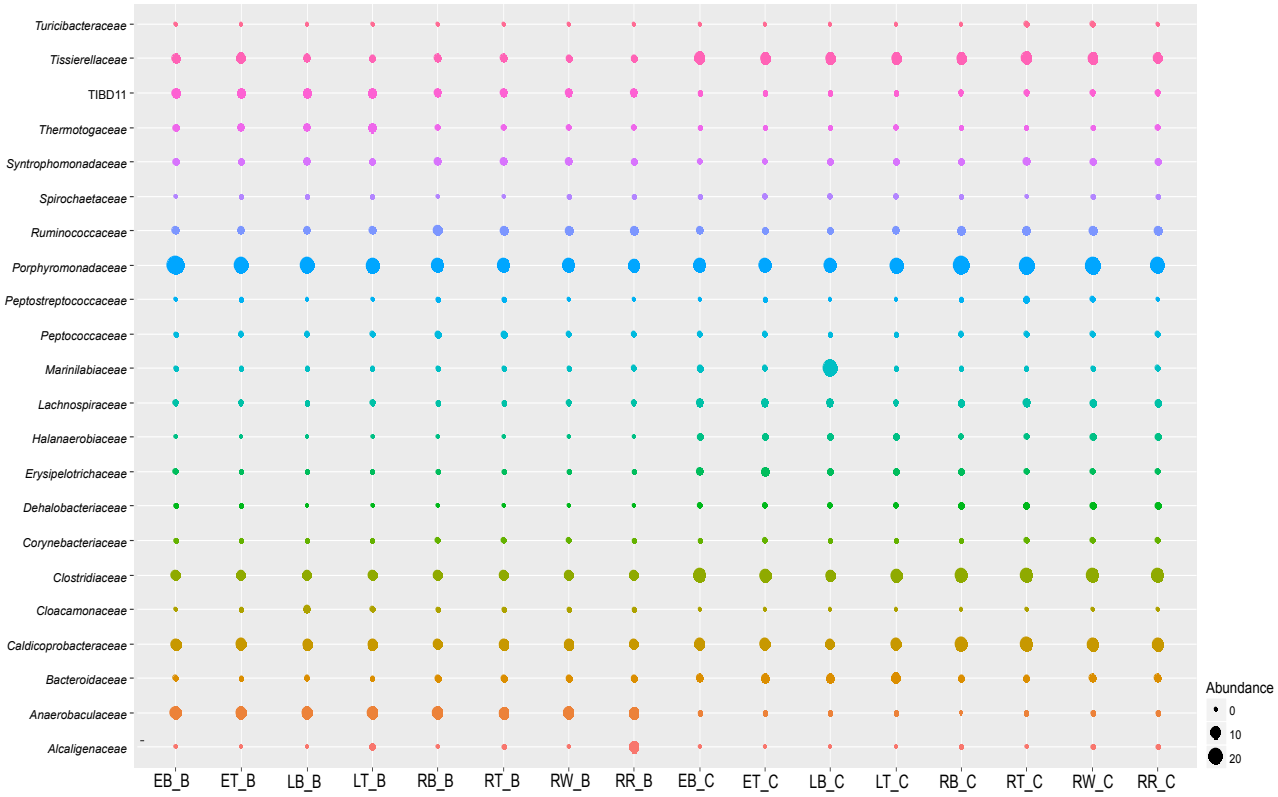


Figure 6.13. The relative abundances of bacterial families in control and bioaugmented digesters with a proportion of at least 0.1% in one sample.

At the genus level (Figure 6.14), *Clostridium* and *Caldicoprobacter* were the most abundant bacteria in all digesters, followed by *Syntrophomonas* and *Sedimentibacter*. *Anaerobaculum* (phylum *Synergistetes*) got only enriched in the bioaugmented digesters.

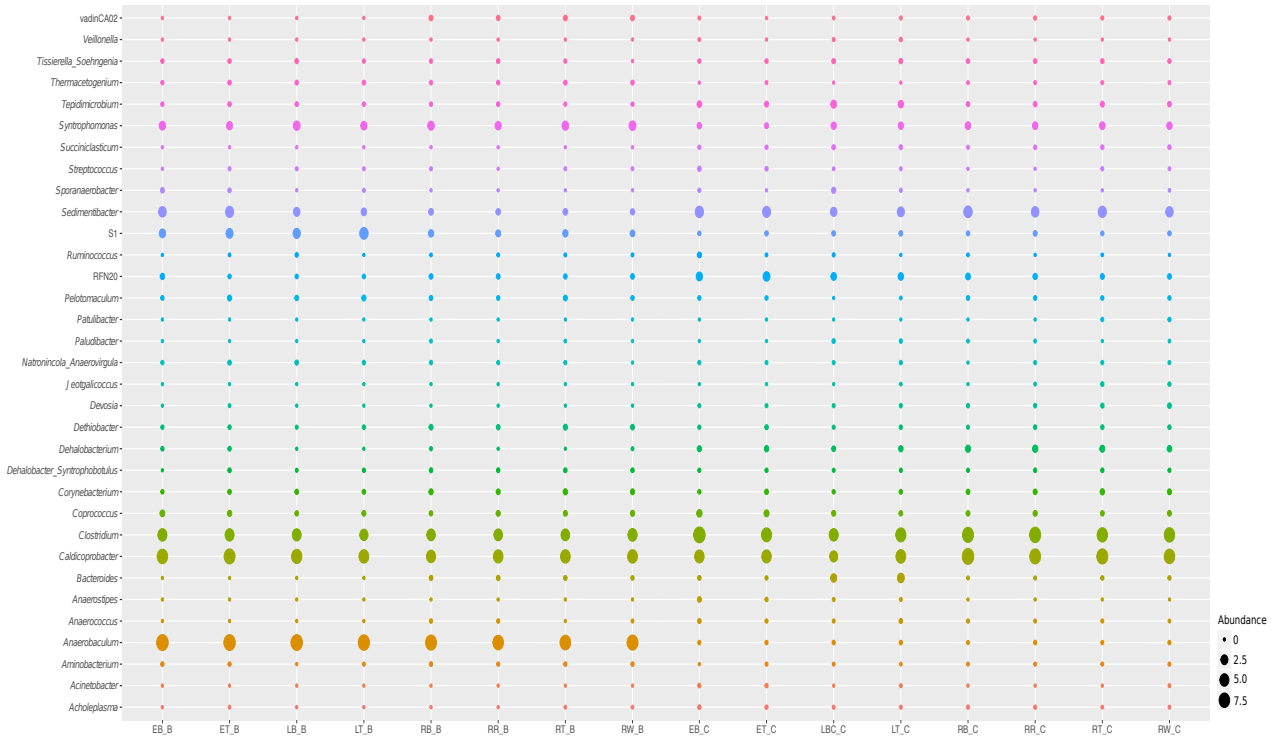


Figure 6.14. The relative abundances of bacterial genera in control and bioaugmented digesters with a proportion of at least 0.1% in one sample.

Methanogenic archaeal diversity in the AD systems are given in Figures 6.15 and 6.16 at the family and genus level, respectively. Methanogenic archaea accounted on average for 1.3% of the whole AD microbiome. As can be seen clearly from the figures, *Methanosarcinaceae* dominated the digesters with and without fungal bioaugmentation. Other detected methanogens were *Methanomicrobiaceae*, *Methanomassiliicoccaceae* and *Methanobacteriaceae*. At the genus level, *Methanosarcinaceae* was represented by *Methanosarcina*. Furthermore, *Methanosphera* (family *Methanobacteriaceae*), *Methanomassiliicoccus* (family *Methanomassiliicoccaceae*), *Methanoculleus* (family *Methanomicrobiaceae*) and *Methanobrevibacter* (family *Methanobacteriaceae*) were also detected in the digesters.

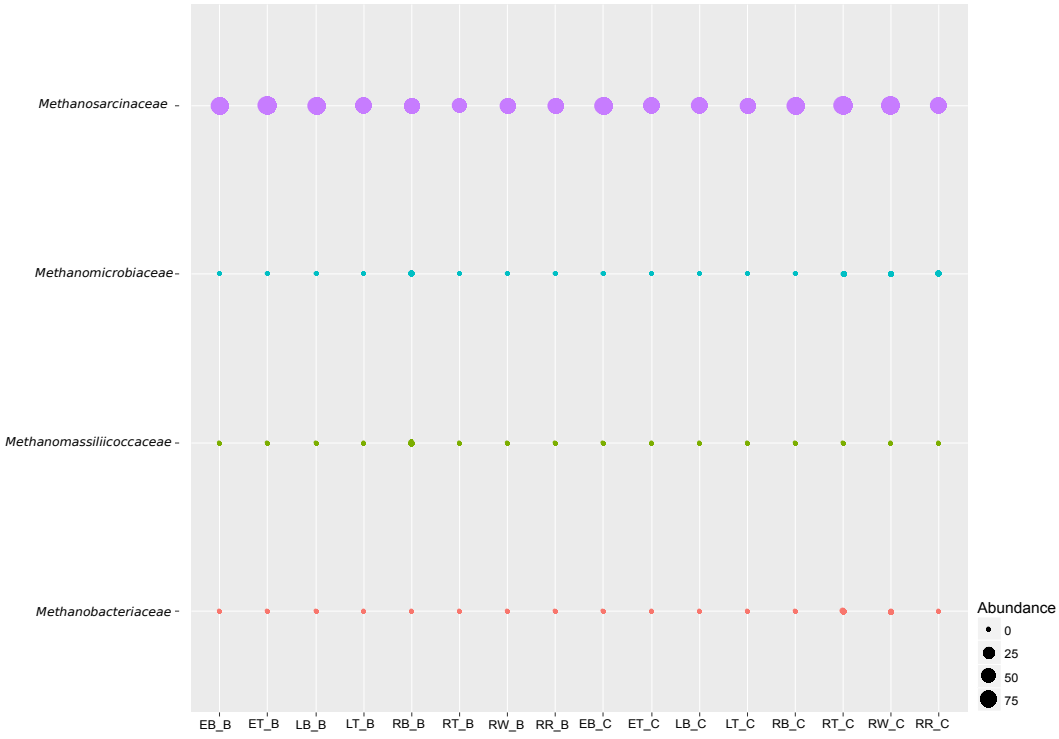


Figure 6.15. The relative abundances of methanogenic families in control and bioaugmented digesters with a proportion of at least 0.1% in one sample.

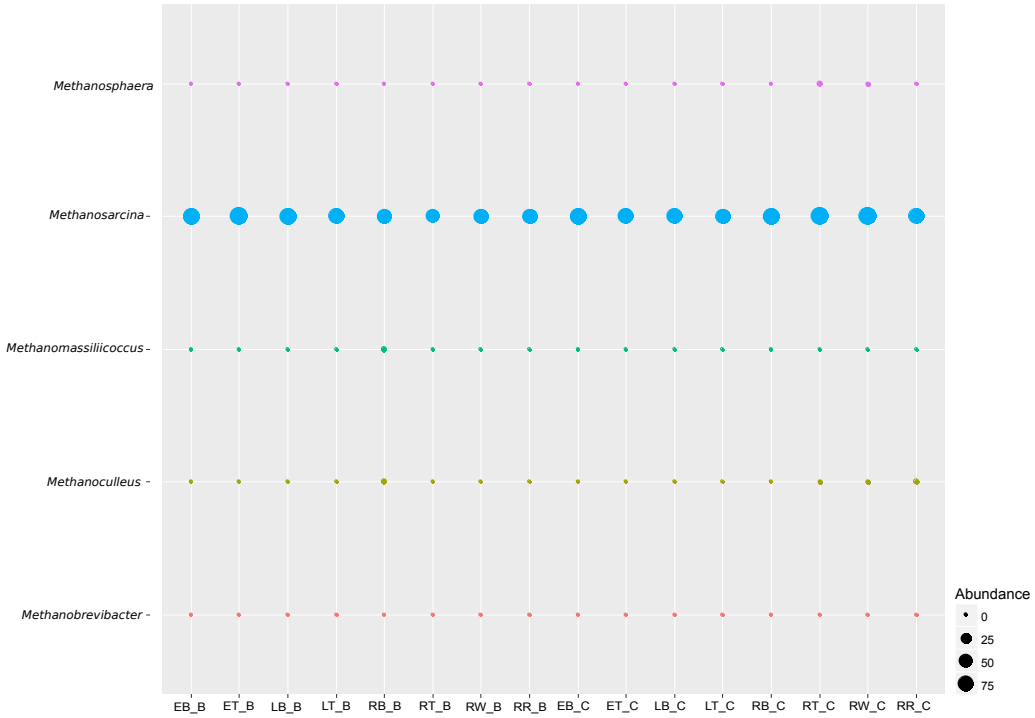


Figure 6.16. The relative abundances of methanogenic genera in control and bioaugmented digesters with a proportion of at least 0.1% in one sample.

According to the clustering of OTUs as given in Figure 6.17, two main groups and four subgroups were formed. Bacterial community structures varied in the samples due to the implementation of fungal bioaugmentation and further formed two main groups. Whereas the first subgroup was composed of EB_B, ET_B and LB_B digesters, the second subgroup was formed by the digesters fed with straws namely RB_B, RT_B, RR_B and RW_B and as well as LT_B. Meanwhile, the third subgroup comprised of only the control digester with late-harvested barley (LB_C) and differed from the other control digesters which together formed the forth subgroup. The most dominant OTU belonged to MBA08 and *Porphyromonadaceae* in the bioaugmented and control digesters, respectively.

Figure 6.17. Heatmap displaying the unique sequence abundance of microbial communities in control and bioaugmented digesters.

Major lesson learned from the microbial ecology of this particular AD systems is that fungal bioaugmentation was the driving factor to cluster microbial groups, followed by the use of whole crop or straw parts. Despite the core microbiome of AD were represented by the typically-detected bacteria (i.e. *Firmicutes* and *Bacteroidetes*); *Synergistetes*, which was mainly assigned to the genus

Anaerobaculum, only enriched in the bioaugmented digesters. Higher abundance of *Synergistetes* marked that efficient syntrophic oxidations were achieved since syntrophic oxidizers relieves the accumulation of propionate and butyrate (Deng et al., 2018). Relatively higher abundance of these species can be attributed to a more efficient fermentation in the anaerobic digesters upon fungal bioaugmentation. Similarly, *Thermotogae*, which was only represented by the family *Thermotogaceae*, were only abundant in the bioaugmented digesters fed with the whole crops (i.e. EB_B, ET_B, LB_B, LT_B). Although bacterial diversity differed with respect to the application of fungal bioaugmentation, methanogenic archaeal diversity was similar and dominated by *Methanosarcina*. *Methanosarcina* sp. are able to use both acetoclastic and hydrogenotrophic methanogenesis pathways and reported to be more tolerant to stress conditions than other methanogens (De Vrieze et al., 2012). It is known that the addition of pure and/or mix culture of microorganisms into AD via bioaugmentation can stress out the existing microbiome (Nzila, 2017). Hence, higher tolerance of *Methanosarcina* sp. can be one of the reasons for the absence of distinction between bioaugmented and non-bioaugmented digesters.

These findings are in agreement with other studies focusing on the microbial communities of biogas reactors fed with lignocellulosic substrates (Grohmann et al., 2018; Li et al., 2018; Tsapekos et al., 2017b). Samples taken from lab-scale biogas reactors fed with sugarcane filter cake alone or together with bagasse showed the predominance of the bacterial families *Bacteroidaceae*, *Prevotellaceae* and *Porphyromonadaceae* (phylum *Bacteroidetes*) and *Synergistaceae* (phylum *Synergistetes*), and the methanogenic genera *Methanosarcina* and *Methanobacterium* (Leite et al., 2016). *Firmicutes* and *Bacteroidetes* are stated as the most commonly found bacterial phyla in biogas plants treating lignocellulosic biomass (Bozan et al., 2017). In another study, *Clostridium* (phylum *Firmicutes*) dominated the samples together with *Methanobrevibacter* and *Methanosarcina* as the most abundant methanogens in agricultural AD systems (Liu et al., 2017). A few recent study also focused on the microbial characterization of fungal-bioaugmented AD few with different substrates (Aydin et al., 2017; Yıldırım et al., 2017). Similar bacterial communities were reported as the most abundant phyla, namely *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* as well as *Synergistetes*. On the other hand, *Methanosaeta* and *Methanolinea* were reported as the most abundant methanogens when the substrates were microalgae and animal manure, respectively. The variations can be easily attributed to the initial microbial composition of the anaerobic seed sludge, different bioaugmentation cultures and other environmental and operating conditions.

7. COMPOSTING OF ANAEROBIC DIGESTATES

During the digestate composting, while the conditions change from anaerobic to aerobic, a drastic change in microbial community occurs. Since the microorganisms are the key players in a composting process, knowledge on the dynamics of microbial community is crucial for any kind of process optimization (Sundberg et al., 2013). In the last decade, the microbiology of composting processes has received considerable attention from researchers (Franke-Whittle et al., 2014; Green et al., 2004; Karadag et al., 2013; Tian et al., 2013; Wang et al., 2015), and with the development of high-throughput sequencing technology, the composition and dynamics of microbial communities in various composts have been investigated (de Gannes et al., 2013; Wang et al., 2016, 2018; Zhang et al., 2016). However, knowledge regarding the microbial communities involved in the anaerobic digestate composting is comparatively limited. Accordingly, an extensive investigation into microbial dynamics can help to optimize the composting process of the digestates and enhance the compost quality. Hence, the aim of this present study was to compare organic matter degradation and microbial community dynamics during the composting of lignocellulosic anaerobic digestates obtained from the AD trials. The specific investigation and comparison of bacterial and fungal diversity in anaerobic digestates may provide valuable insights into the compost microbiome by using 16S and 18S rRNA gene amplicon sequencing, respectively.

7.1. Abiotic Parameters

Temperature variations in compost piles through the process is given in Figure 7.1. In all piles, a rapid temperature increase was observed within first 5 days due to initiated microbial activities favoured by the decomposition of easily-biodegradable organic matter. Highest temperature was measured on Day 6 between 52-54 ^{o}C. After this peak, the temperature began to decline gradually and remained in mesophilic temperature ranges for the following days and reached to ambient temperature after Day 25. Temperature is one of the key parameters in composting to regulate microbial activities and hence organic matter degradation. In most cases, thermophilic phase of a composting process lasts for 7-10 days above 55 ^{o}C. However, shorter periods and lower temperatures were also reported (Bustamante et al., 2013). The characteristics of the raw feedstock is important here since the presence of some antimicrobial compounds, especially in sludge samples, may hinder the temperature increase.

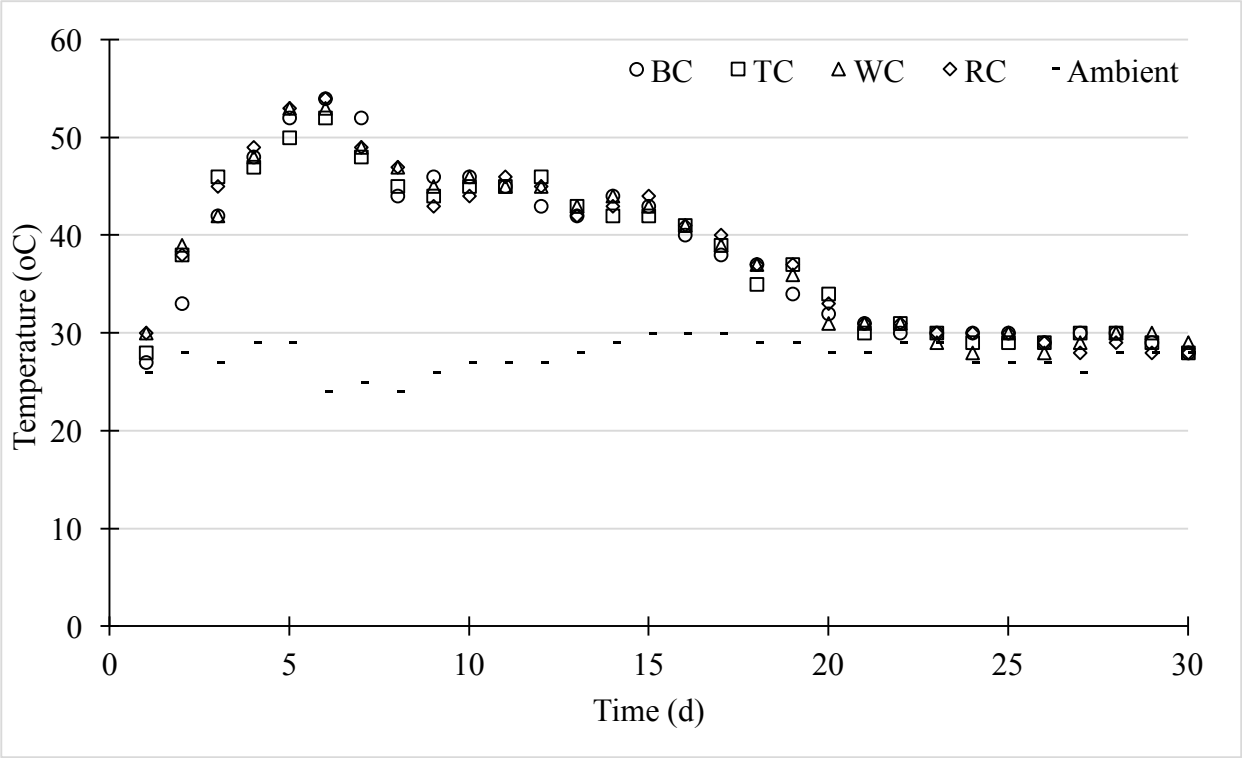


Figure 7.1. Temperature variation in compost piles.

Initial pH values were measured between 8.65-8.90 in the piles and after a slight increase on the early days, it remained quite constant with little fluctuations between 7.50 and 8.50 (Figure 7.2). There was not a remarkable difference in pH between compost piles and maintained in alkali conditions. Although most of the composting processes exhibit acidic conditions especially during the first days, some studies also reported alkaline pH (Chroni et al., 2009; Franke-Whittle et al., 2014) This is most of the time because of the feedstocks such as manure and/or digestate that cause buffering in the system and maintain alkaline pH (Ince et al., 2018).

Figure 7.3 illustrates the variations in MC during the composting process. Prior to composting, each digestate was mixed with wheat straw as bulking agent to set the moisture content to 45-50%. Following the operation, MC decreased gradually and fixed around 35% at the end of the process. TC marked relatively higher MC reduction among other compost piles.

C:N ratio is also another important parameter to evaluate the efficiency of a composting process (Arab and McCartney, 2017). As can be seen from Figure 7.4, initial C:N ratio values ranged between 32:1-35:1 and followed a diminishing trend during the process as expected due to microbial activities. C:N ratio of the compost piles remained constant around 16:1-17.1 at the end.

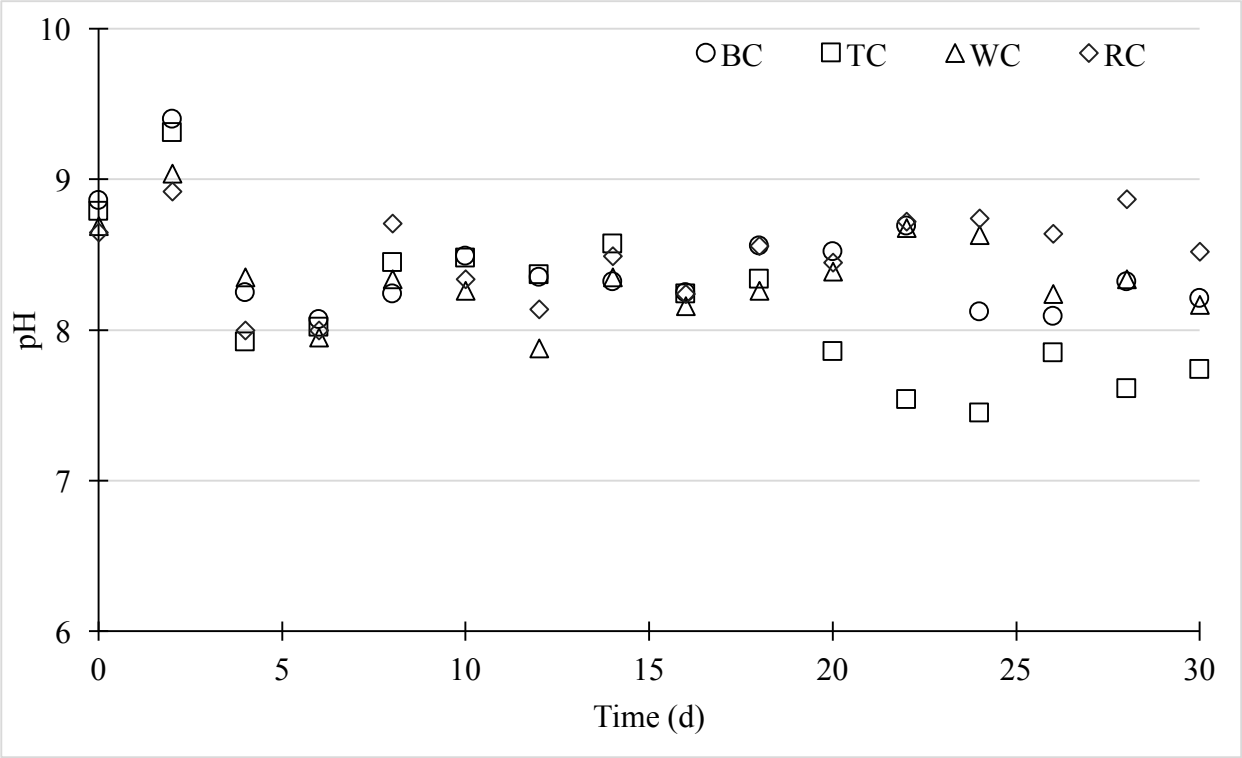


Figure 7.2. pH variation in compost piles.

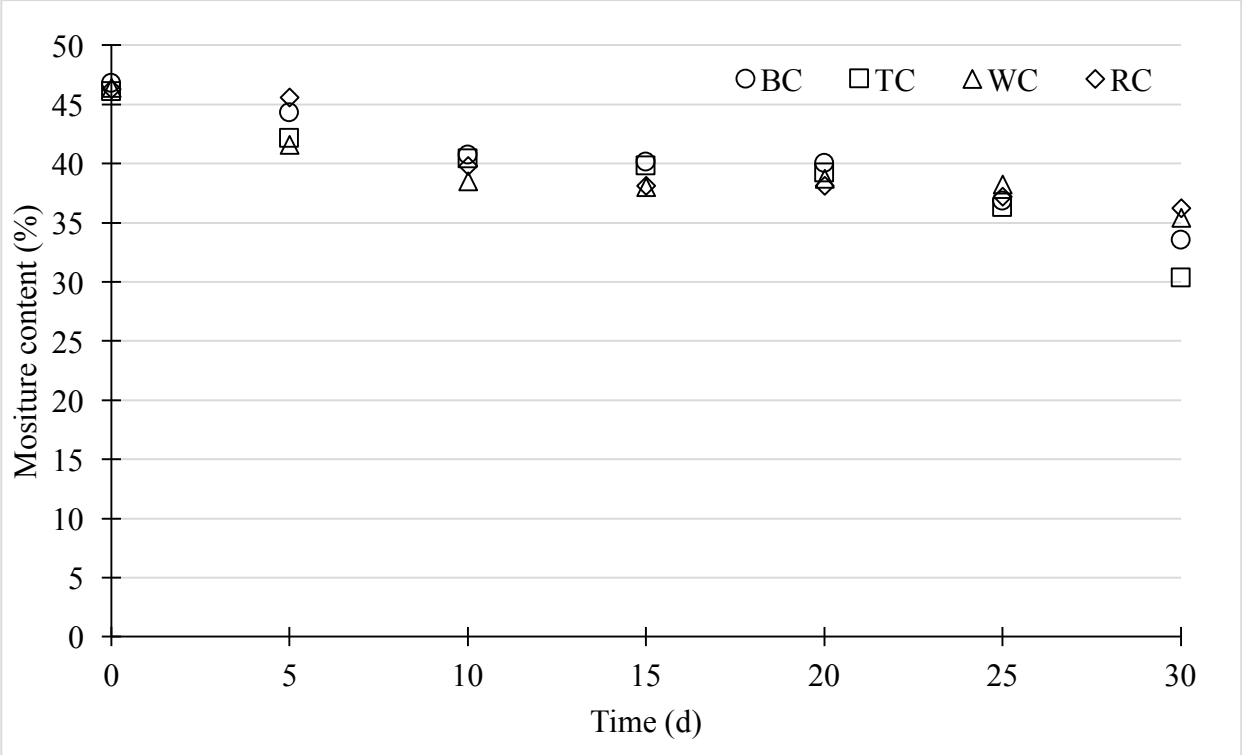


Figure 7.3. Moisture content variation in compost piles.

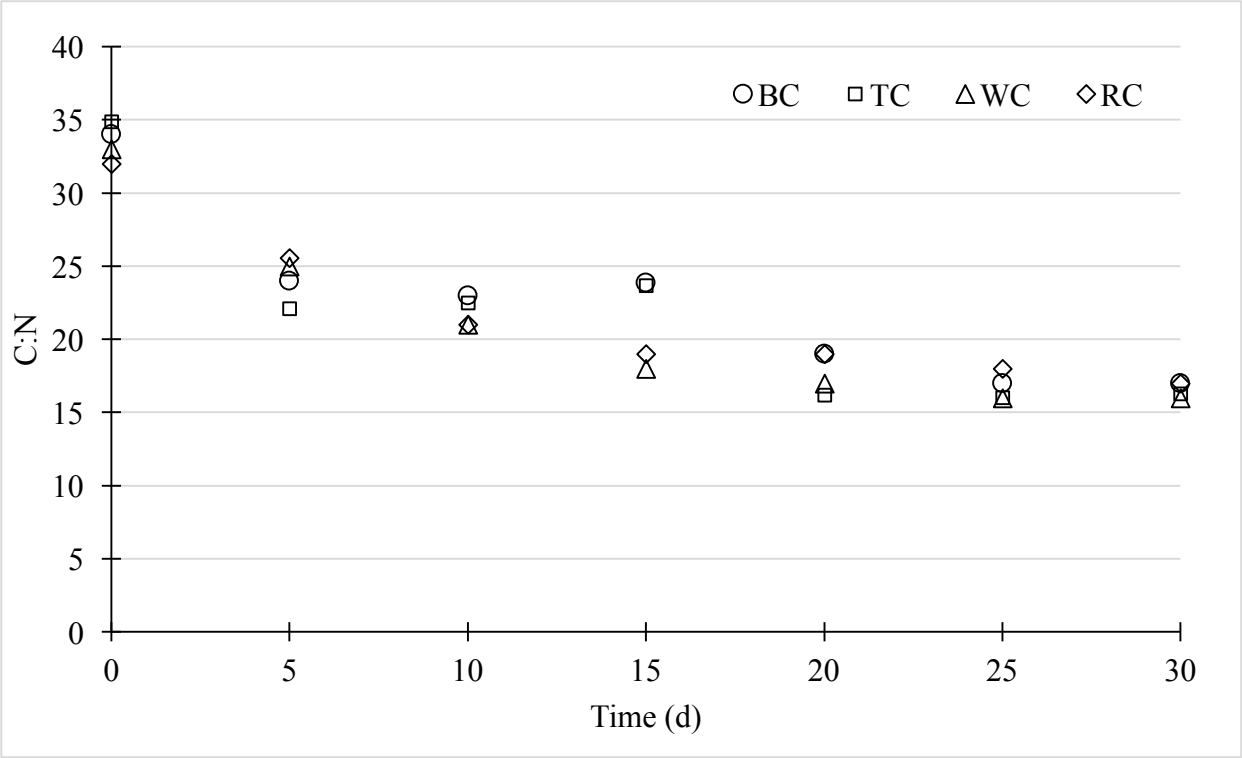


Figure 7.4. C:N ratio variation in compost piles.

Overall, the composting process of the anaerobic digestates exhibited similar variations in terms of temperature, pH, MC and C:N ratio. According to ‘‘Regulations Regarding the Production, Import, Marketing and Inspection of Organic, Organomineral Fertilizers and Soil Amendment Products and Other Products, Microbial and Enzyme Based Products’’ being published in the Official Gazette dated 29.03.2014 and numbered 28956 and, ‘‘Compost Regulation’’ being published in the Official Gazette dated 05.03.2015 and numbered 29286, C:N ratio should be between 10:1 and 30:1, MC should be lower than 30% and pH should be in the range of 5.5 to 8.5. According to this information obtained from the regulations, the final compost products of this particular study is suitable for soil amendment, except that MC is slightly higher than 30%. Hence, the final compost products were air-dried for another 30 days before being amended as soil conditioner.

7.2. Microbial Community Dynamics

An average number of 179,902 raw reads per sample were obtained from the sequencing analysis with an average length of 230 bp for bacterial communities. The relative abundances of bacterial genera in compost piles are presented in Figure 7.5. A very diverse bacterial community was detected in samples (BC, RC, TC and WC), and the highest number of OTUs belonged to mainly 4 phyla as *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. Clearly, *Luteimonas* (phylum *Actinobacteria*) was by far the most abundant bacterial genera in all samples, as the relative

abundance of *Luteimonas* was 15%, 20%, 12% and 13% in BC, RC, TC and WC, respectively. It was followed by *Bacillus* (7%-12%, phylum *Proteobacteria*), *Ochrobactrum* (5%-13%, phylum *Proteobacteria*) and *Thermobifida* (2%-7%, phylum *Actinobacteria*). Despite some minor variations with respect to abundances, all 4 samples were represented by same groups of bacteria and there was not a significant difference between the samples. Other predominant bacterial genera were as follows: *Cellvibrio* (phylum *Proteobacteria*), *Taibaiella* (phylum *Bacteroidetes*), *Bordetella* (phylum *Proteobacteria*), *Pseudomonas* (phylum *Proteobacteria*), *Olivibacter* (phylum *Bacteroidetes*), *Gelidibacter* (phylum *Bacteroidetes*) and *Acidovorax* (phylum *Proteobacteria*).

Figure 7.5. The relative abundances of bacterial genera in compost piles with a proportion of at least 0.1% in one sample.

The results are in accordance with the data presented previously since *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* are commonly reported as the most dominant bacterial phyla in composting processes (Galitskaya et al., 2017; Wei et al., 2018). Phylum *Firmicutes* are known to grow at high temperatures and widely distributed especially in the thermophilic phase of composting of agricultural biomass (Zhang et al., 2016). Meanwhile, *Actinobacteria* is also

considered thermophilic/thermotolerant and also plays important role in terms of break down of organic materials (Jurado et al., 2014).

At the genus level, *Luteimonas* is typically found in food waste and/or manure composting processes together with soil environments (Maeda et al., 2010; Wu et al., 2016) *Bacillus*, is often detected in lignocellulosic composting systems as thermotolerant bacteria (de Gannes et al., 2013), where it contributes to waste degradation during the composting process (Wei et al., 2018). Species belong to *Cellvibrio*, on the other hand, is mesophilic bacteria and similarly grown on cellulose fibres. Hence, it is an important adaptation in such environments due to being capable of degrading polysaccharides (Zhang et al., 2017). *Pseudomonas* has been famously reported as plant disease-suppressive bacteria, that can improve the composting quality during the maturation phase (Wei et al., 2018)**.** *Thermobifida* has been stated to be effective on cellulose and hemicellulose degradation while secreting hemicellulases and cellulases (Zhang et al., 2015)**.** Different from the above-mentioned typical composting bacteria, *Luteolibacter*, *Olivibacter*, *Taibaiella* and *Ochrobactrum* were also detected in the samples together with *Luteimonas*, which are commonly found in soil environments and most probably introduced to composting piles with the addition of wheat straw as the bulking agent.

An average number of 273,414 raw reads per sample were obtained from the sequencing analysis with an average length of 174 bp for fungal communities. Identified fungal genera during the composting process are given in Figure 7.6. Similar to bacterial community, there was not a significant difference within the samples and fungal community was mainly represented by the phylum *Ascomycota*. The members of this phylum are widely distributed in compost processes, and their dominance has been reported (Liu et al. 2015; Zhang et al. 2015)**.** Remarkably, most of the reads belonged to the genus *Thermomyces* (phylum *Ascomycota*). The relative abundance of *Thermomyces* was 25%, 22%, 25% and 24% in BC, RC, TC and WC samples, respectively. *Aspergillus* (12%-14%, phylum *Ascomycota*), *Ascobolus* (2%-8%, phylum *Ascomycota*), *Galactomyces* (5%-7%, phylum *Ascomycota*), *Neurospora* (5%-7%, phylum *Ascomycota*), *Mucor* (2%-5%, phylum *Zygomycota*), *Penicillium* (1%-5%, phylum *Ascomycota*), *Saccharomyces* (1%-4%, phylum *Ascomycota*) and *Fusarium* (1%-3%, phylum *Ascomycota*) were detected as the following predominant fungal genera in compost piles. At a relatively-low abundance, other detected fungal genera included *Curvularia* (phylum *Ascomycota*), *Hygrocybe* (phylum *Basidiomycota*), *Microsporum* (phylum *Ascomycota*) and *Pseudallescheria* (phylum *Ascomycota*).

The genus *Thermomyces* are thermophilic moderate growth rate fungi while *Galactomyces* produce cellulolytic enzymes, which enhance the organic matter degradation in a composting process (Arab et al., 2017). In the study of (Zhang et al., 2015), *Thermomyces* and *Aspergillus* were reported as the dominant functional fungal genera in lignocellulose degradation in maize straw composts. In another study, *Thermomyces, Mucor* and *Penicillum* were also found to be the dominant fungal genera during composting of manure silage and hardwood/softwood shavings, followed by *Galactomyces* and *Pseudallescheria* at minor abundances. Similarly *Thermomyces* and *Penicillium* have been also isolated from compost samples containing hardwood bark and manure (Neher et al., 2013).

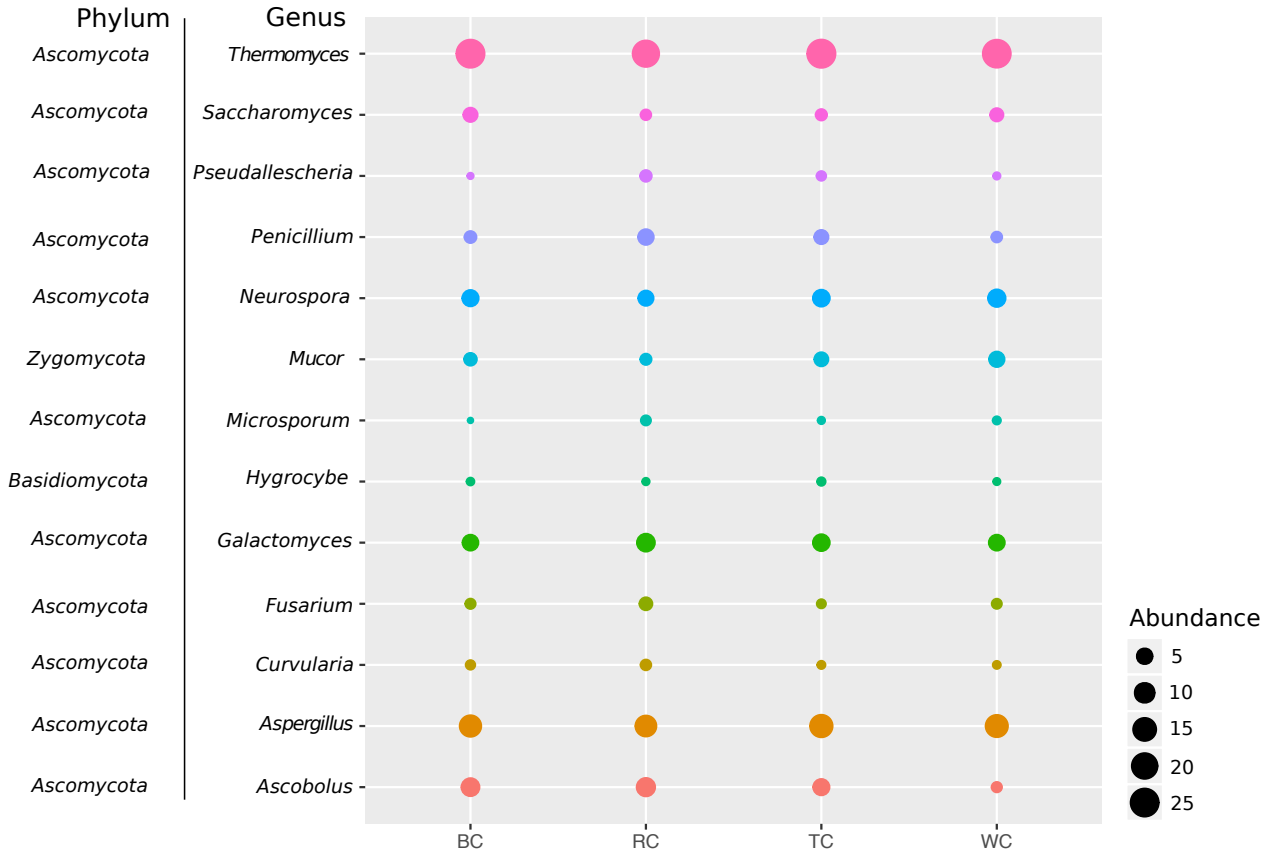


Figure 7.6. The relative abundances of fungal genera in compost piles with a proportion of at least 0.1% in one sample.

Some species of the genera *Aspergillus*, *Fusarium* and *Mucor* are known to be pathogenic to humans and cause diseases, therefore needs special investigation (Dehghani et al., 2012). Other than these commonly-found compost fungi, the genus *Hygrocybe* (phylum *Basidiomycota*) was also found in the samples, which prefers grasslands as the habitat, and most probably brought to compost systems onto wheat straws.

8. SOIL AMENDMENT OF FINAL COMPOST PRODUCTS

Compost amendment to agricultural soils has great influence on plant growth and soil quality by affecting the microbial community composition. In this particular study, the aim was to monitor changes in soil bacterial communities when final composts were amended as soil conditioner. Initial bacterial composition in the soil of the crops (i.e. barley, triticale, wheat, rye) was determined by 16S rRNA gene amplicon sequencing prior to compost amendment. Following the compost application, samples were collected after 3 months. Specific importance was given to triticale as a hybrid crop and its possible effects on soil microbiome.

8.1. Initial Bacterial Diversity in Soil

Rarefaction curves were depicted to evaluate the alpha diversity representing the initial bacterial diversity of each soil sample and given in Figure 8.1. As can be seen clearly, highest number of observed species was detected in the soil belonged to barley (BS), followed by rye (RS) and triticale (TS). The soil belonged to wheat (WS) was found to have the least diverse bacterial community.

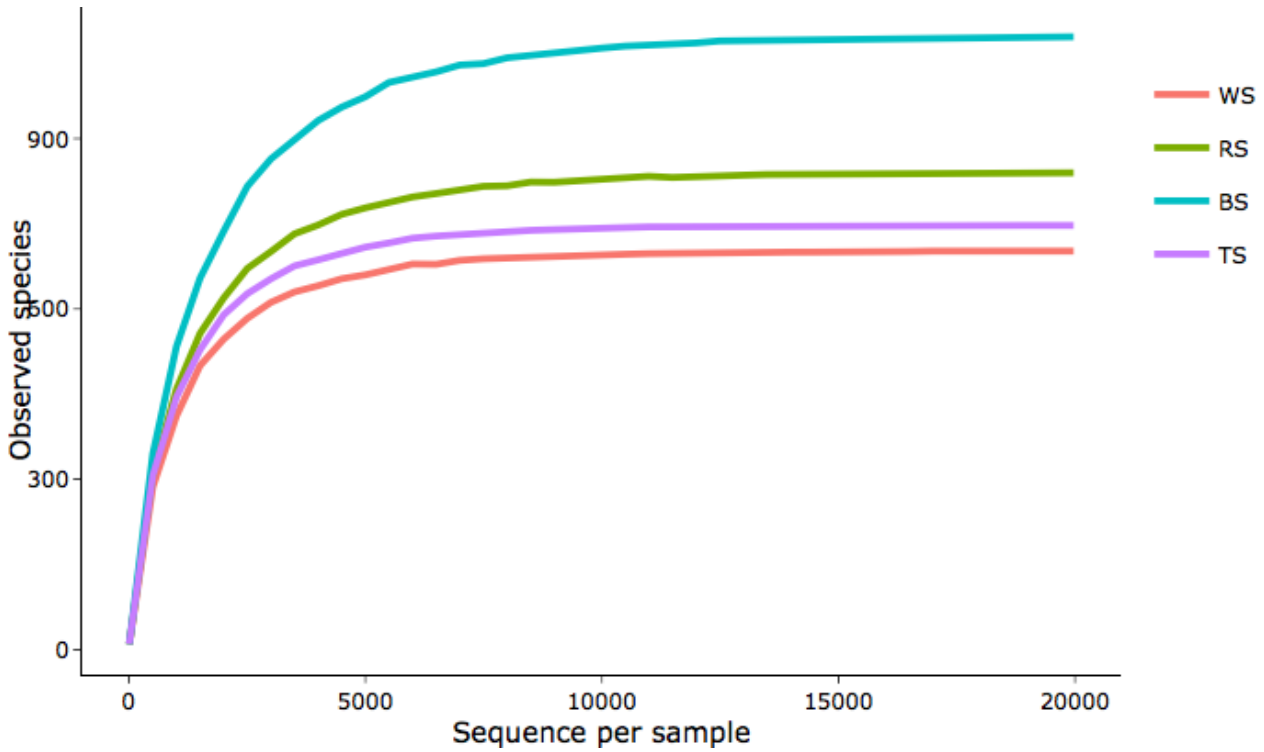


Figure 8.1. Alpha diversity rarefaction curves of initial bacterial communities in soil samples.

The initial bacterial community composition of soil samples is depicted in Figure 8.2. At the phylum level, *Actinobacteria* and *Proteobacteria* were the most dominant bacterial phyla in all samples. The relative abundance of *Actinobacteria* was determined as 41% in RS, 31% in BS, 31% in TS and 30% in WS. Meanwhile, the relative abundance of *Proteobacteria* was 29%, 31%, 36% and 36% in RS, BS, TS and WS samples, respectively. Other abundant bacterial phyla included *Bacteroidetes* (10-15%), *Acidobacteria* (6-10%) and *Gemmatimonadetes* (4-6%). Bacterial diversity of TS and WS were comparatively more similar to each other and clustered together, followed by BS and RS.

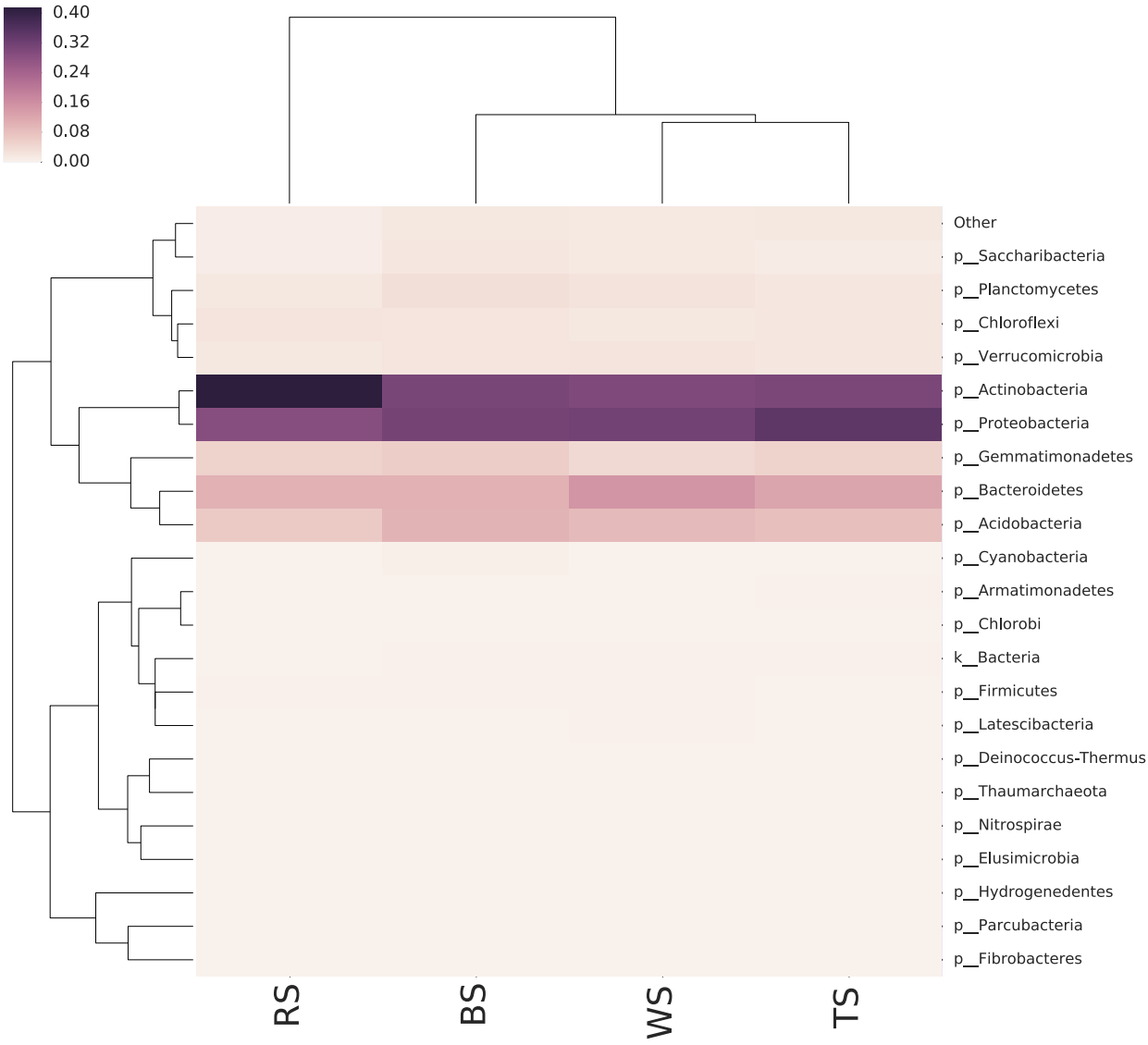


Figure 8.2. Heatmap displaying the unique sequence abundance of initial bacterial phyla in soil samples.

Figure 8.3 displays the initial bacterial families in soil samples. Accordingly, *Oxalobacteraceae* (phylum *Proteobacteria*) and an unclassified family of the class *Acidobacteria* (phylum

Acidobacteria) dominated the bacterial community at the family level, followed by *Sphingomonadaceae* (phylum *Bacteroidetes*), *Chitinophagaceae* (phylum *Bacteroidetes*), an unclassified family of the order Acidimicrobiales (phylum *Actinobacteria*), *Sphingobacteriaceae* (phylum *Bacteroidetes*)**,** *Gemmatimonacadeae* (phylum *Gemmatimonadetes*) and *Streptomycetaceae* (phylum *Actinobacteria*).

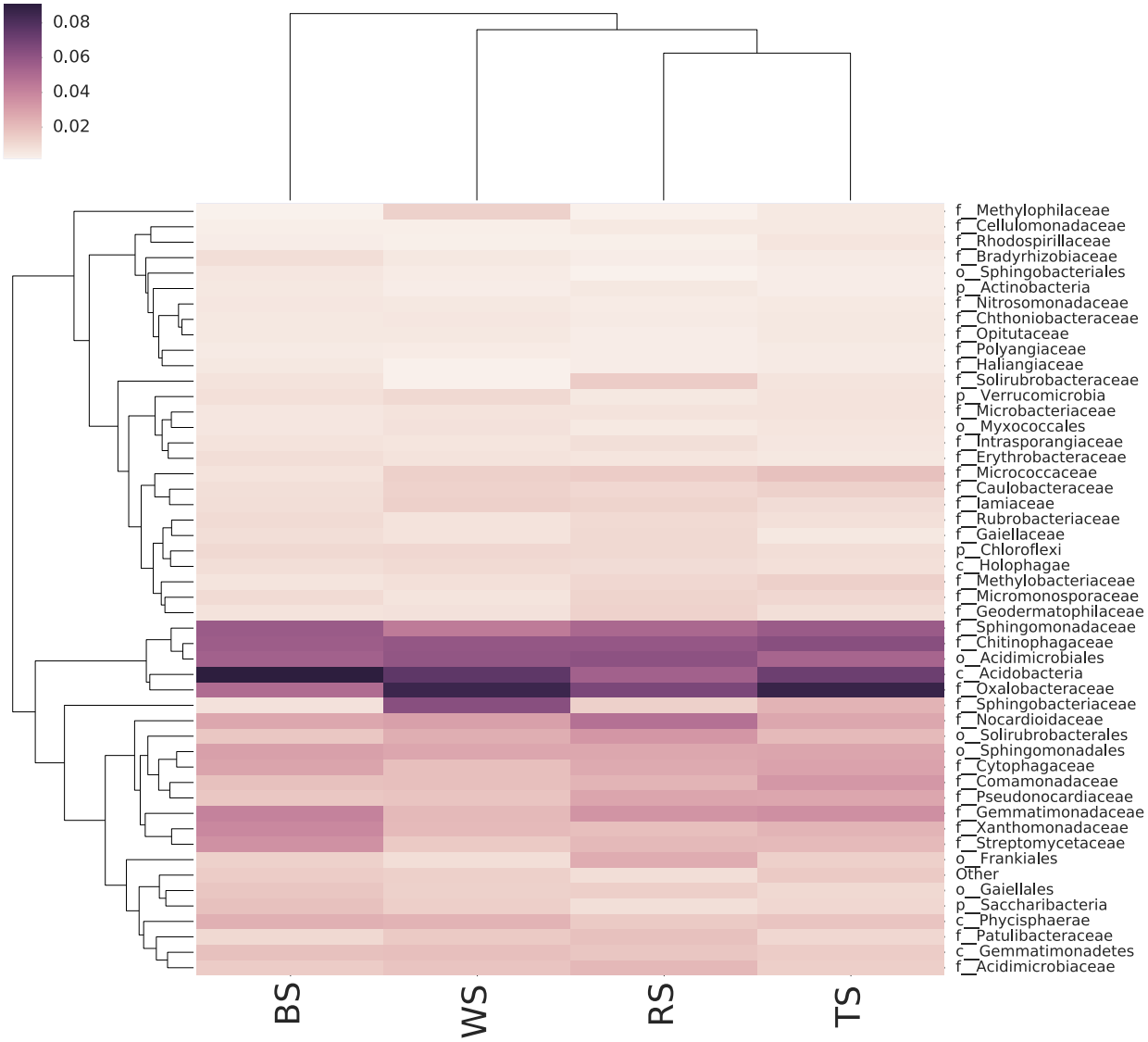


Figure 8.3. Heatmap displaying the unique sequence abundance of initial bacterial families in soil samples.

At the genus level, although same taxonomic genera of bacteria were detected, their relative abundance varied among the samples (Figure 8.4). In WS samples, most abundant bacterial genera were *Massilia*, an unclassified genus of the order *Acidimicrobiales*, an unclassified genus of the class *Acidobacteria*, *Arcticibacter* and *Sphingomonas*. The relative abundance of *Arcticibacter* in WS was significantly higher than the other soils. In BS samples, an unclassified genus of the class

Acidobacteria dominated the bacterial community, followed by an unclassified genus of the order *Acidimicrobiales*, *Sphingomonas*, *Massilia* and *Streptomyces*. In RS samples, most abundant bacterial genera were an unclassified genus of the order *Acidimicrobiales*, *Massilia*, *Sphingomonas,* an unclassified genus of the class *Acidobacteria*, *Flavisolibacter* and *Nocardioides*. Differently, *Missilia* was the most dominant bacterial genus in TS samples, followed by an unclassified genus of the class *Acidobacteria*, *Sphingomonas*, an unclassified genus of the order *Acidimicrobiales* and *Flavisolibacter*.

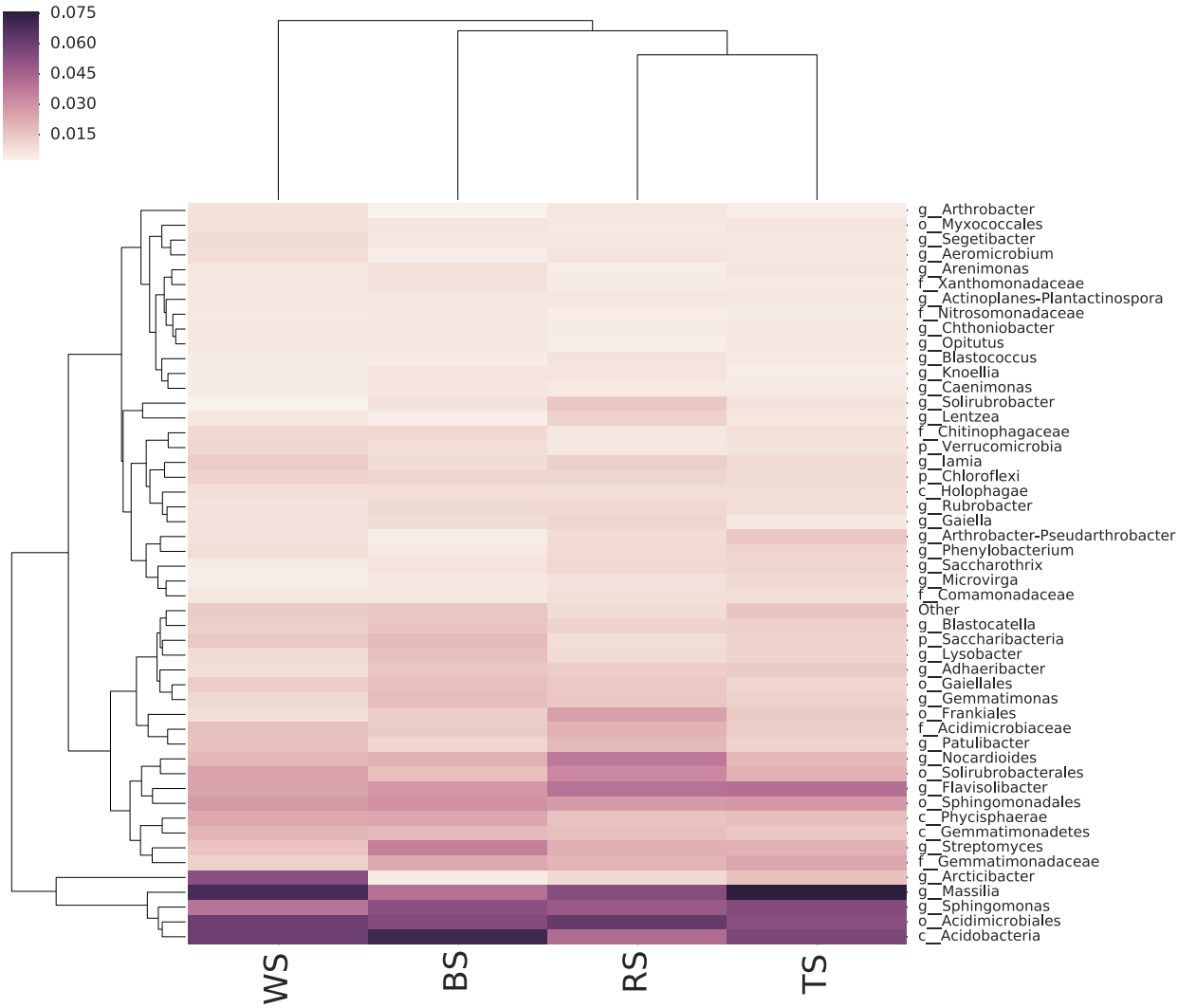


Figure 8.4. Heatmap displaying the unique sequence abundance of initial bacterial genera in soil samples.

Potentially ligninolytic bacteria were found common in soils, such as *Sphingomonas* (phylum *Proteobacteria*), *Streptomyces* (phylum *Actinobacteria*). These bacteria, are capable of contributing to the degradation of phenolic compounds such as lignin, although their efficiency is often much lower than that of fungi (Brown and Chang, 2014).

The results are also in accordance with other studies on crop soils. *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* were reported to be most dominant phyla in wheat crop soil samples (Donne et al., 2014). At the family level, *Streptomycetaceae* (phylum *Actinobacteria*), *Rhizobiaceae* (phylum *Proteobacteria*), *Oxalobacteraceae* (phylum *Proteobacteria*), *Flavobacteraceae* (*Bacteroidetes*) and *Sphingobacteriaceae* (*Bacteroidetes*) were the most abundant bacterial families. Similarly, in the study of (Kaiser et al., 2016), most dominant bacterial phyla and proteobacterial classes in grassland soil samples were reported as *Actinobacteria* (23.75%), *Alphaproteobacteria* (20.43%), *Acidobacteria* (18.39%), *Deltaproteobacteria* (7.22%). *Bacteroidetes* (5.15%), *Chloroflexi* (5.09%), *Betaproteobacteria* (4.64%) and *Gammaproteobacteria* (4.32%).

8.2. Bacterial Diversity Changes in Soil upon Compost Amendment

Soil samples were collected after 3 months following the compost amendment and compared to initial soil bacterial diversity. Similarity of bacterial communities in soil samples before and after compost amendment is depicted in Figure 8.5. Accordingly, initial bacterial communities (i.e. BS, RS, TS, WS) were clustered together in one group and bacterial communities upon compost amendment (i.e. BS.3, RS.3, TS.3) were clustered in one group. Surprisingly, soil samples belonged to wheat were significantly different from other samples and clustered another group.

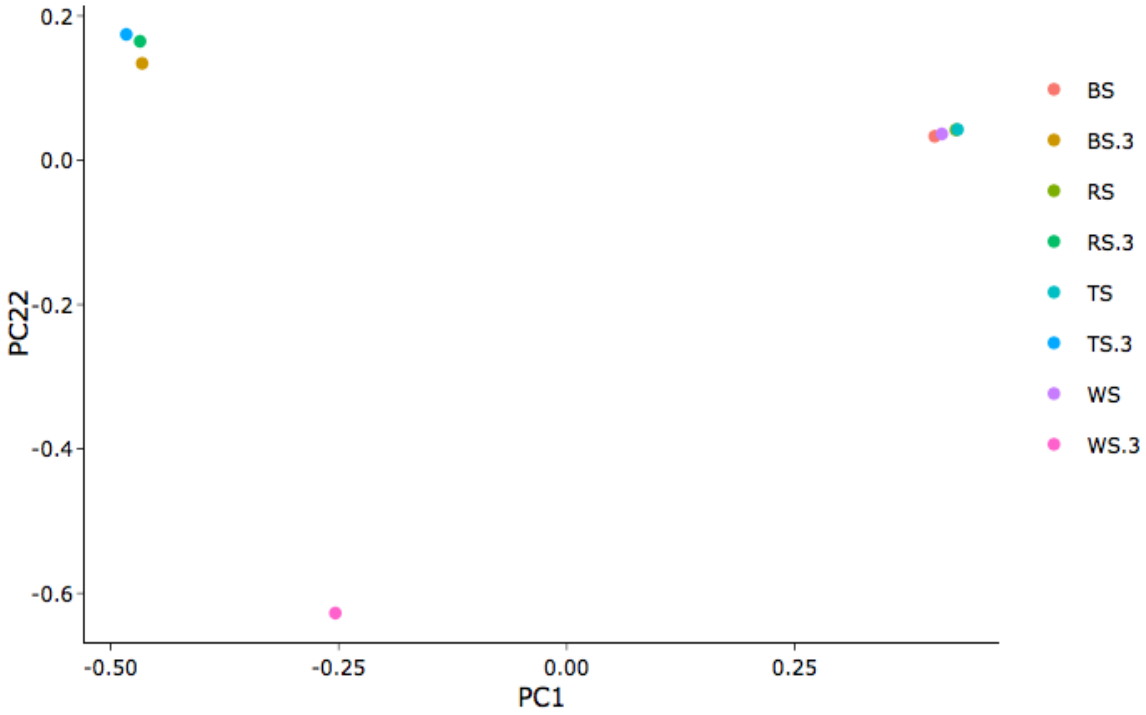


Figure 8.5. Principal component analysis based on the beta diversity of bacterial communities in soil samples before and after compost amendment.

The bacterial community composition of compost-amended soil samples is depicted in Figure 8.6. The relative abundance of *Proteobacteria* was 41%, 42%, 41% and 37% in RS.3, BS.3, TS.3 and WS.3 samples, respectively. Meanwhile, the relative abundance of *Acidobacteria* was found as 22%, 17%, 22% and 19% in RS.3, BS.3, TS.3 and WS.3 samples, respectively. The relative abundance of *Bacteroidetes* increased in compost-amended soil samples, 11%, 19%, 12% and 18% in RS.3, BS.3, TS.3 and WS.3 samples, respectively. Although *Proteobacteria* and *Actinobacteria* clearly dominated the initial bacterial communities, a shift in the predominance from *Actinobacteria* to *Acidobacteria* was observed in all samples upon compost amendment, while *Proteobacteria* was still the most dominant bacterial phylum. There was also a remarkable difference in the relative abundance of *Bacteroidetes* between WS.3, BS.3 and RS.3, TS.3 samples. The phyla *Verrucomicrobia* and *Gemmatimonadetes* were also detected at relatively high abundances in compost-amended soil samples except WS.3. Differently, *Firmicutes* (10%) were also comparatively abundant in WS.3.

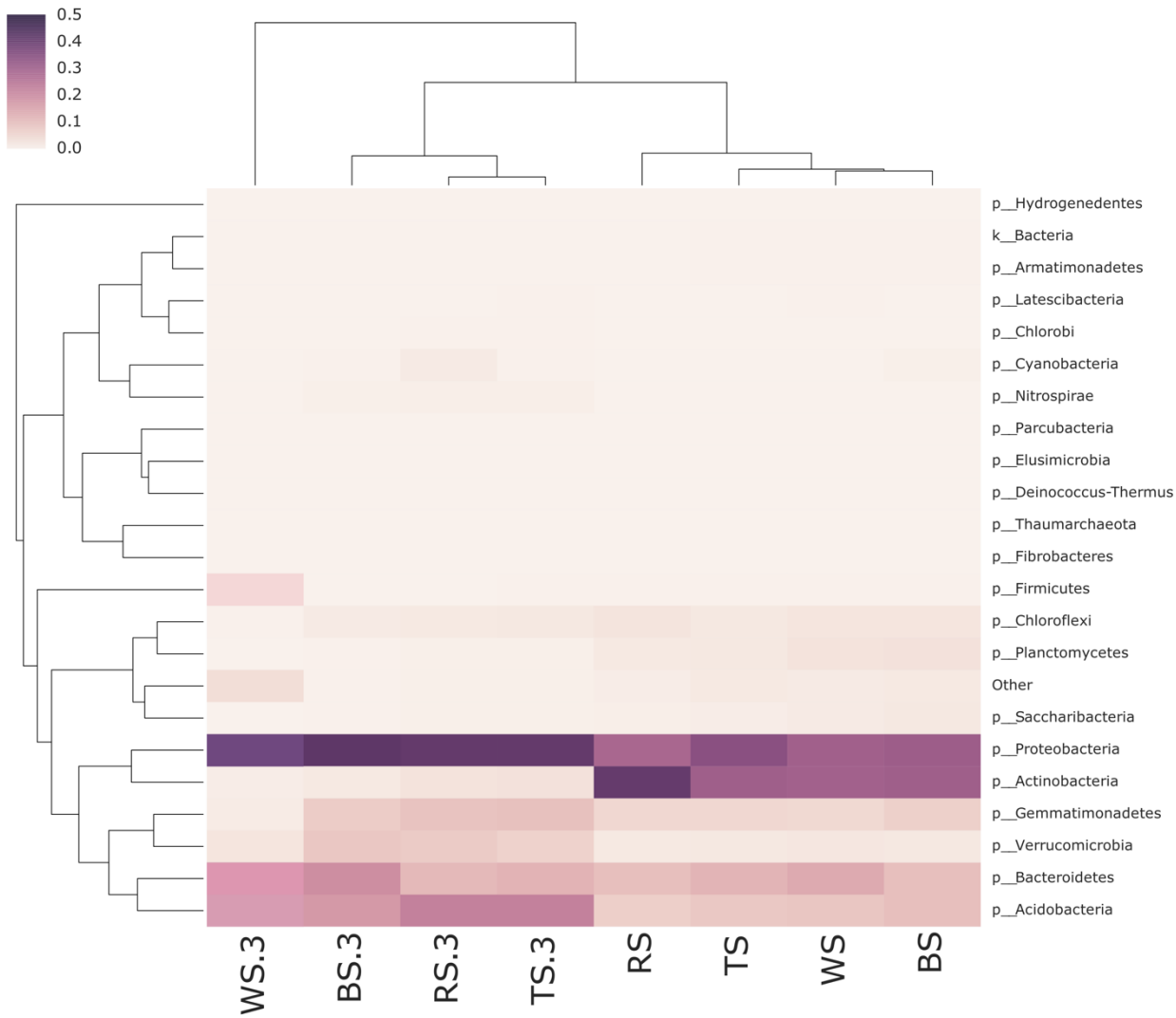


Figure 8.6. Heatmap displaying the unique sequence abundance of bacterial phyla in soil samples before and after compost amendment.

The bacterial communities in compost-amended soil samples are given in Figure 8.7. at the family level. The predominance of an unclassified family belonging to the class *Acidobacteria* (phylum *Acidobacteria*) can be clearly seen in all compost-amended samples. The dominance of *Oxalobacteraceae* (phylum *Proteobacteria*) BS, RS and TS shifted to an unclassified family belonging to the class *Acidobacteria* in soils upon compost amendment. Despite some minor abundance differences, similar bacterial families were detected before and after compost amendment, such as *Sphingomonadaceae* (phylum *Bacteroidetes*), *Oxalobacteraceae* (phylum *Proteobacteria*), *Chitinophagaceae* (phylum *Bacteroidetes*) and *Gemmatimonacadeae* (phylum *Gemmatimonadetes*). Different from the initial bacterial diversity before compost amendment, *Flavobacteriaceae* (phylum *Bacteroidetes*) was also abundant in BS.3, RS.3 and TS.3 samples; whereas, *Ruminococcaceae* (phylum *Firmicutes*), Prevotellacea (phylum *Bacteroidetes*), *Bacteroidaceae* (phylum *Bacteroidetes*) and *Lachnospiraceae* (phylum *Firmicutes*) were detected in WS.3 samples.

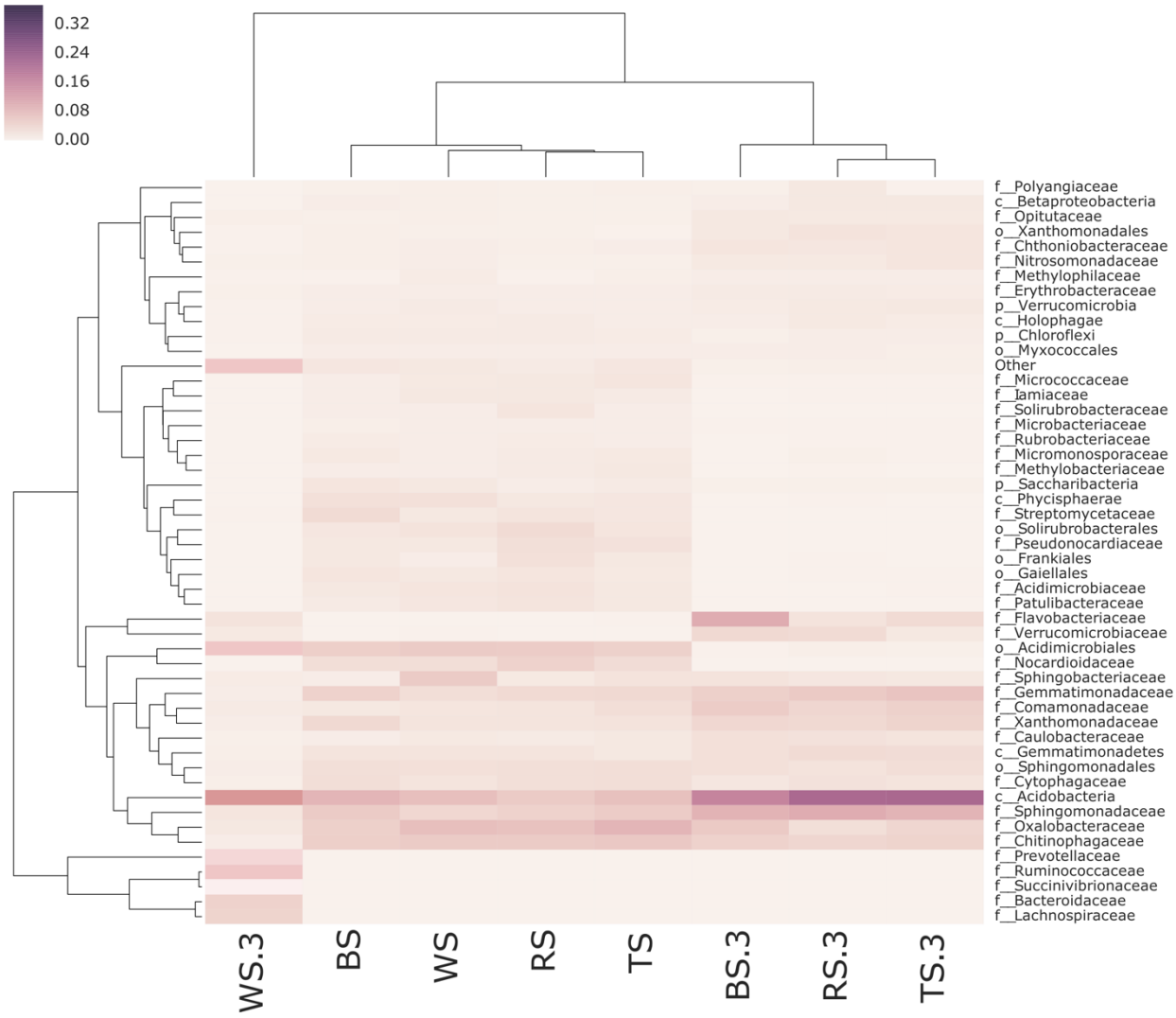


Figure 8.7. Heatmap displaying the unique sequence abundance of bacterial families in soil samples before and after compost amendment.

At the genus level, the most abundant bacterial genera included an unclassified genus of the class *Acidobacteria*, followed by *Sphingomonas*, *Flavobacterium* and *Massilia* (Figure 8.8.). The dominance of *Massilia* before compost amendment shifted to an unclassified genus of the class *Acidobacteria*. The relative abundance of *Flavobacterium* was significantly higher in BS.3 than that of RS.3 and TS.3. Although *Arcticibacter* was among the predominant genera in WS before compost amendment, *Bacteroides* was clearly abundant in WS.3. Furthermore, *Blastocatella* was enriched in BS., RS.3 and TS.3 soils upon compost amendment.

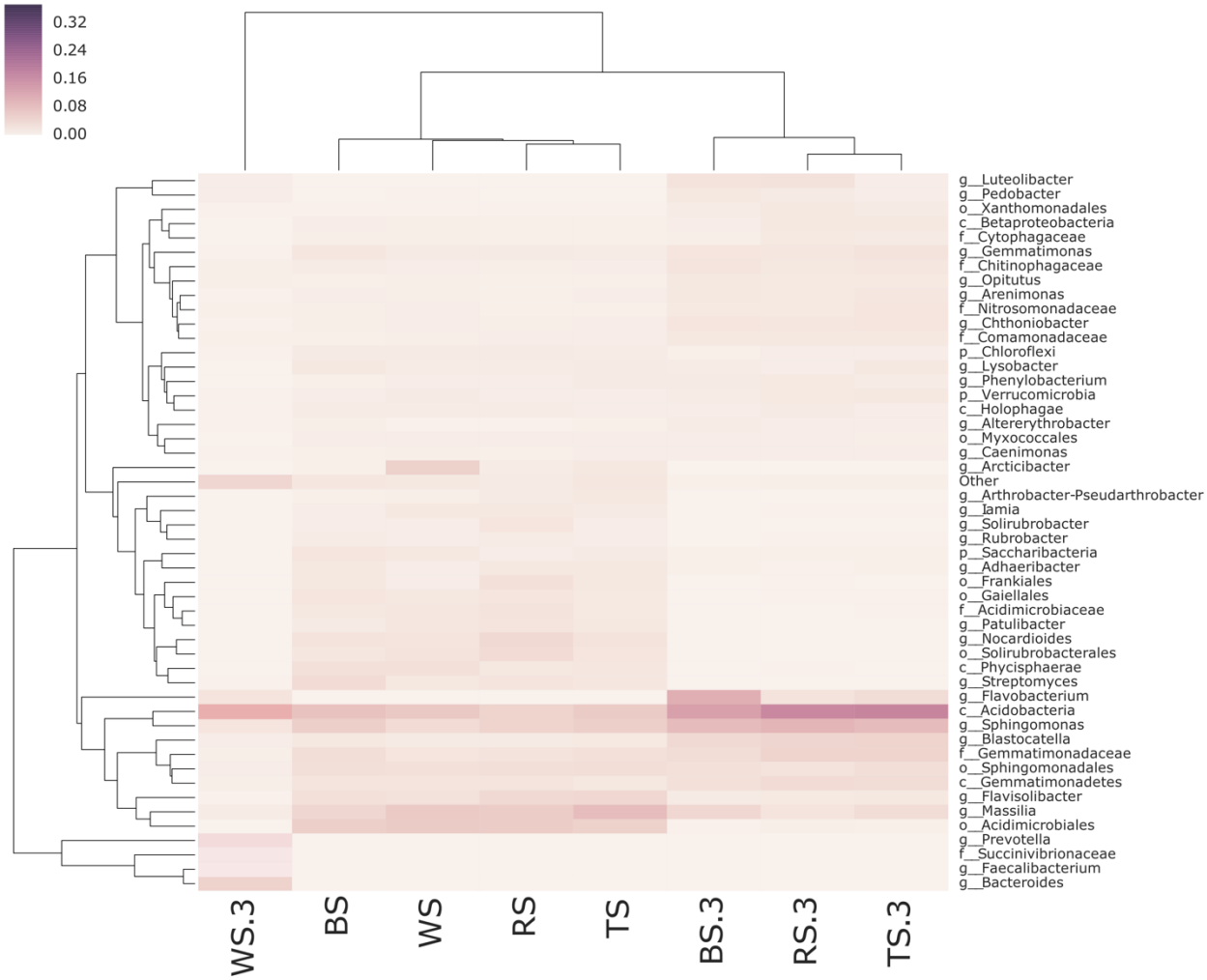


Figure 8.8. Heatmap displaying the unique sequence abundance of bacterial genera in soil samples before and after compost amendment.

Most abundant bacterial genera found in compost samples (i.e. *Luteimonas*, *Olivibacter*, *Ochrobactrum*) were also detected in compost-amended soil samples but in very low abundances. For instance, the relative abundance of *Luteimonas* was between 0.2%-0.3% in compost-amended soils; whereas it was not detected in the soil samples before compost amendment. The results indicated that introduction of new species to soils via compost amendment caused a shift in the

predominant soil bacterial communities and showed a minor contribution of compost bacteria in the soils. Furthermore, the soil that belonged to wheat were comparatively more affected via compost amendment, that could be because wheat-soil samples had the least diverse initial bacterial community. Furthermore, the difference in bacterial diversity in compost-amended soils of wheat and other cereal crops might be the results of the re-introduction of wheat straw via composts since it was used as the bulking agent during composting. Amendment of wheat straw-containing compost products might have caused some synergistic effects on the wheat-soil environment.

Similar results were reported in the literature. For instance, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were reported as the most abundant phyla in the wheat crop rhizosphere**,** followed by *Acidobacteria* and *Firmicutes* (Donn et al., 2015). Other studies also reported significant responses of soil bacteria to compost amendments. In the study of Wu and colleagues (2016), the most abundant phyla were found similar as *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Chloroflexi* and *Verrucomicrobia*. The authors also reported that the relative abundance of *Proteobacteria* decreased as *Bacteroidetes* increased via compost-amended soils.

9. CONCLUSIONS

The main focus of this thesis was the optimization of lignocellulose-based AD, assessing a variety of fungal treatment techniques, including pretreatment and bioaugmentation. Changes on the bacterial and archaeal communities were evaluated together with selected key enzyme expressions in AD. Anaerobic digestates were further composted and amended to agricultural fields as soil conditioner. The major contributions of this thesis are summarized below.

Harvesting stage of the cereal crops (i.e. barley, triticale, wheat, rye) was an important parameter for the chemical composition of the crop materials and further for methane production. Milk in the grave (early harvest) stage provided higher methane yield in all conditions. Furthermore, early-harvested barley was by far the highest methane-yielding co-substrate in all AD trials. This should be considered when barley is selected as the co-substrate.

Fungal pretreatment with *T. versicolor* positively influenced biomethanation of lignocellulosic biomass by 10-18% in terms of methane yields, resulting in remarkable cellulose degradation and more diverse biogas microbiome. The effect of harvesting stage on lignocellulosic structure as well as further methane yield was most apparent on barley but not significant on triticale. Early-harvested barley achieved the highest methane yield, 378 mL CH_4/g VS and 386 mL CH_4/g VS in control and pretreated digesters, respectively, where fungal pretreatment did not have a significant effect on methane yield in this trial. Fungal pretreatment showed the highest impact on triticale-fed digesters in terms of methane yield, contributing 18% increase between control and pretreated digesters. Harvesting residues of triticale also performed better than those of barley, rye and wheat in all trials. VFA profiles also differed among the pretreated digesters based on the harvesting stage and higher VFA speciation was achieved via fungal pretreatment. There was not a significant difference in the selected key enzyme expression levels between the control and pretreated digesters, and were slightly higher in the pretreated digesters. Microbial communities clustered into groups in terms of similarity based on the fungal pretreatment and selection of whole crops or crop residues. Generally, typically-detected bacterial species (mainly belonged to *Firmicutes* and *Bacteroidetes*) dominated the digesters; except that *Synergistetes* was only enriched in the fungal-pretreated digesters and represented by *Anaerobaculum*. Although *Methanosarcina* was the predominant methanogenic archaea, a more diverse methanogenic population was identified in the fungal-pretreated digesters in which *Methanoculleus* and *Methanobacterium* also took role during biomethanation.

Fungal bioaugmentation with anaerobic fungus *Orpinomyces* sp. increased the methane yield by 15-33%, in which early-harvested barley was found to be the highest-yielding co-substrate to cow manure with 430 mL CH_4/g VS upon fungal bioaugmentation. There was no significant effect of harvesting stage in triticale-fed digesters; however, fungal bioaugmentation further boosted the methane yield by 33%. The increment in methane yield in straw-fed digesters was between 15-23%, and triticale straw performed slightly higher than that of barley, wheat and rye. Furthermore, fungal bioaugmentation shortened the hydrolysis and acidogenesis stages; where peak VFA production could not be tracked in this particular set-up that encountered some time between Day 0 and Day 10. Remarkable cellulose degradation (up to 85%) was also achieved in the bioaugmented digesters. The application of fungal bioaugmentation and the use of whole crop or crop residues were the most critical parameters to cluster microbial groups with respect to similarity. In terms of key enzyme expression levels, *xynD* and *xynF* genes together with *mcrA* were expressed significantly higher in the bioaugmented digesters, meanwhile other examined genes were expressed slightly higher than that of the control digesters. Similar to pretreatment trials, microbial communities in the anaerobic digesters clustered into groups in terms of similarity based on the fungal bioaugmentation and selection of whole crops or crop residues. Most abundant bacterial communities belonged to *Firmicutes* and *Bacteroidetes*, while *Synergistetes* was only enriched in the bioaugmented digesters and mainly represented by *Anaerobaculum*. Methanogenic archaeal composition; on the other hand, did not change by fungal bioaugmentation, and the majority of methanogens was represented by *Methanosarcina*. This can be easily attributed to stress conditions to AD microbiome caused by the augmentation of *Orpinomyces* sp.

The composting process showed similar trends in terms of temperature, pH, moisture content and C:N ratio, while having highly-similar initial feedstock materials. Thermophilic phase lasted comparatively shorter than usual compost systems and the temperature did not rise above 55 ^{o}C. On the other hand, most of the characteristics of the final compost products were in accordance with the legislations, expect MC, which was therefore air-dried prior to soil amendment. In terms of microbial diversity, there was not a remarkable difference between the compost samples. Most abundant bacterial genera were represented by *Luteimonas* (phylum *Actinobacteria*), *Bacillus* (phylum *Proteobacteria*), *Ochrobactrum* (phylum *Proteobacteria*) and *Thermobifida* (*Actinobacteria*). Meanwhile, *Thermomyces* (phylum *Ascomycota*). *Aspergillus* (phylum *Ascomycota*), *Galactomyces* (phylum *Ascomycota*), *Neurospora* (phylum *Ascomycota*) and *Mucor* (phylum *Zygomycota*) were detected as the predominant fungal genera in all samples.

Initial bacterial communities in all soil samples were dominated by *Proteobacteria* and *Actinobacteria*. A shift in the predominance from *Actinobacteria* to *Acidobacteria* was observed upon compost amendment in soils belonged to rye, triticale and barley, while *Proteobacteria* was still the most abundant bacterial phylum in all samples. In addition, the relative abundance of *Bacteroidetes* also increased in all soil environments following the compost amendment, and the most affected soil belonged to wheat with its comparatively distinct bacterial community.

10. RECOMMENDATIONS

Fungal pretreatment and fungal bioaugmentation enhanced methane yields by 10-18% and 15-33% in anaerobic digesters, respectively; however, the choice between each process should be done following a cost-analysis in scaled-up implementations.

Phase separation (two-phase AD) can be a good approach in bioaugmentation implementation. Fungal bioaugmentation can be directly applied in acid digester to enhance hydrolysis/acidification. Hence, possible stress conditions to methanogens can be omitted.

Laboratory-scale and/or pilot-scale continuous operation is recommended for a better understanding of the effect of selected fungal treatment, in which different operating conditions can be applied and assessed.

The energy output can be further improved by subjecting different feedstock compositions as well as implementing sequential pretreatment application approaches (i.e. mechanical + fungal). In addition to that, mix culture of fungi can be used in treatment processes to synergistically improve the anaerobic biodegradability of lignocellulosic biomass.

Co-composting is recommended for anaerobic digestates to ensure a more efficient composting process, such as yard or kitchen wastes as co-substrates.

Long-term monitoring (more than 6 months) is highly recommended to observe the changes in soil microbiome upon compost amendment.

Implementation of other omic techniques, such as metatranscriptomics or proteomics, can also enable to fully understand the microbial interactions in each step.

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APPENDIX A: OVERALL PERFORMANCE OF ANAEROBIC DIGESTERS

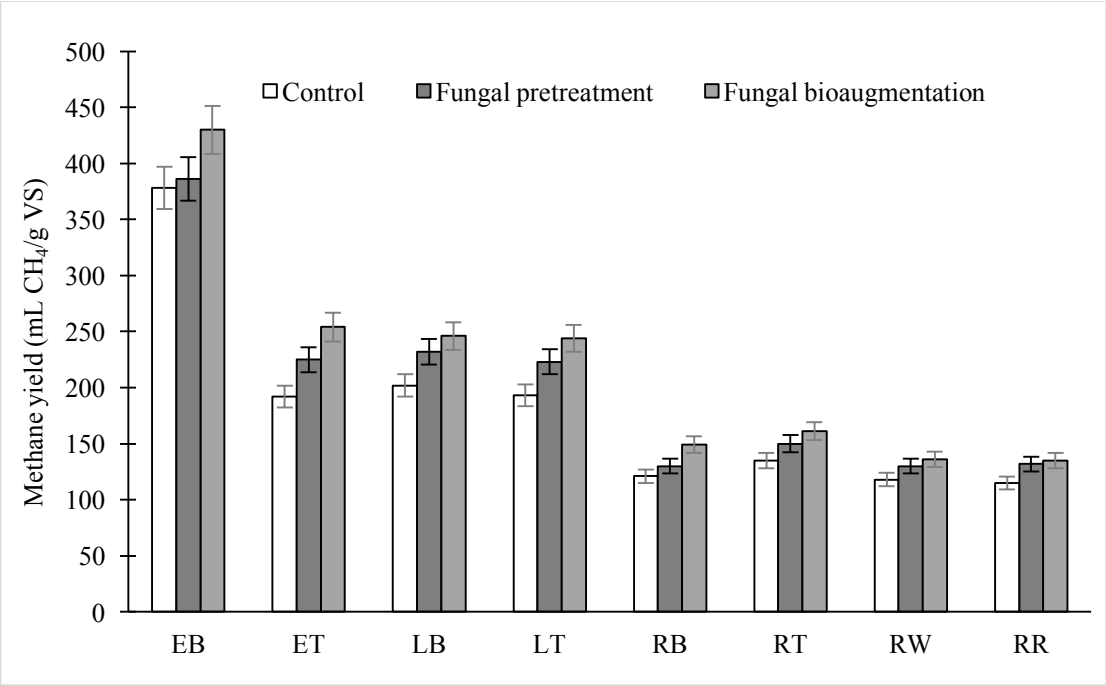
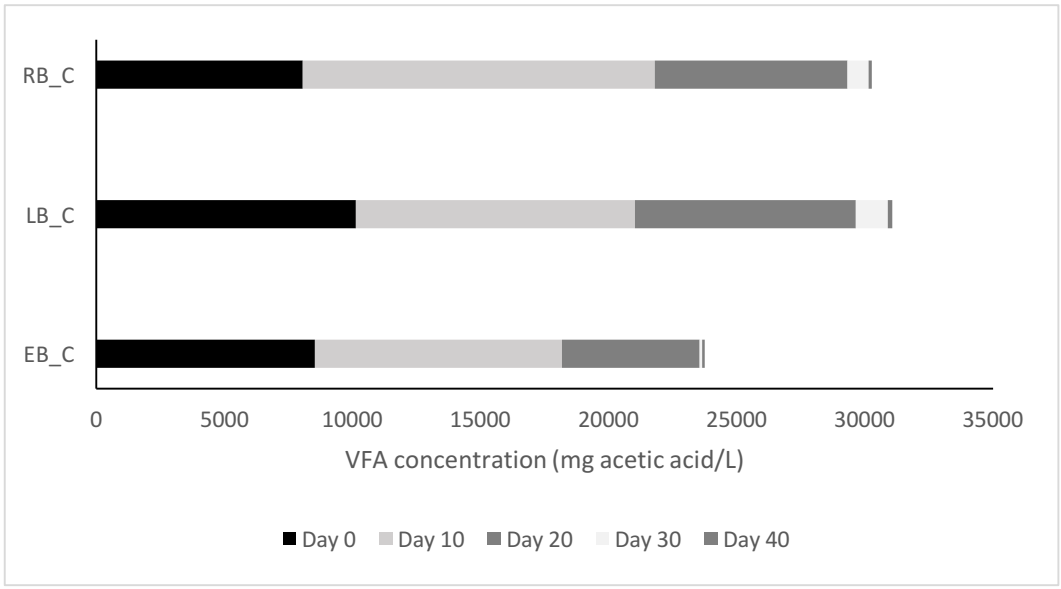
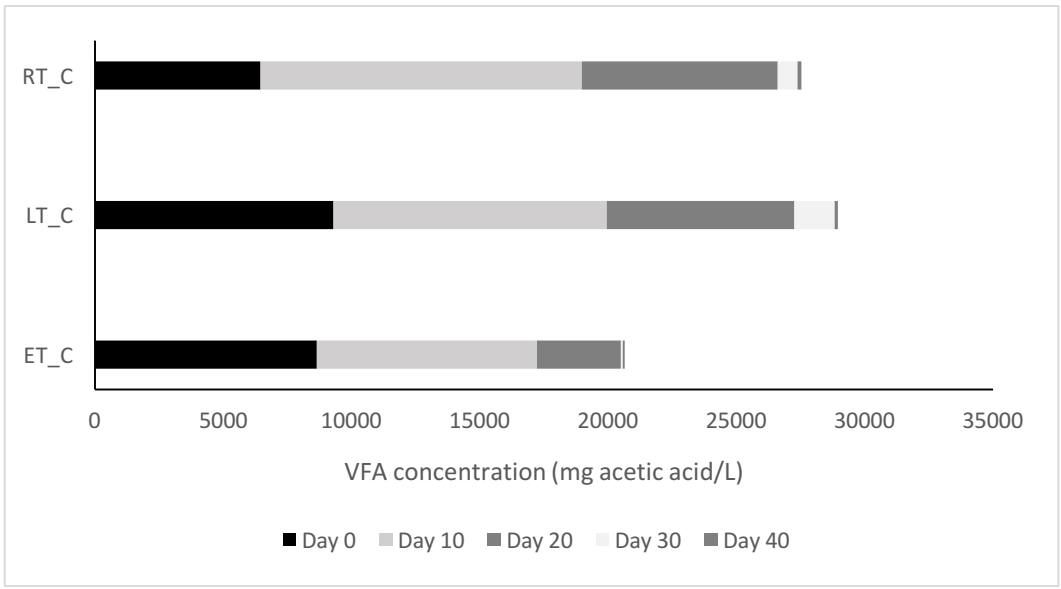


Figure A.1. Overall methane yields of anaerobic digesters.





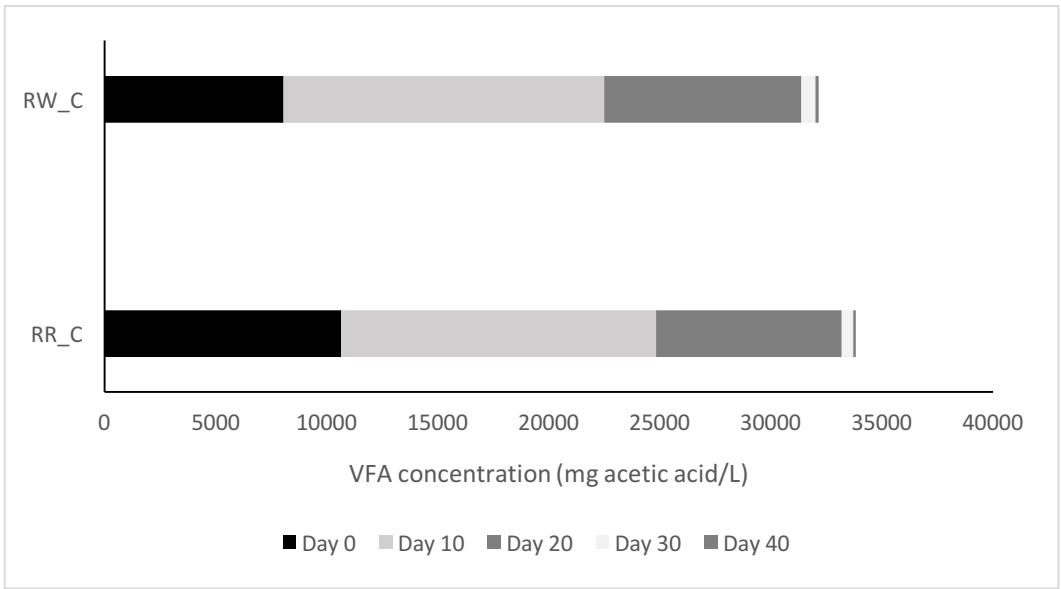
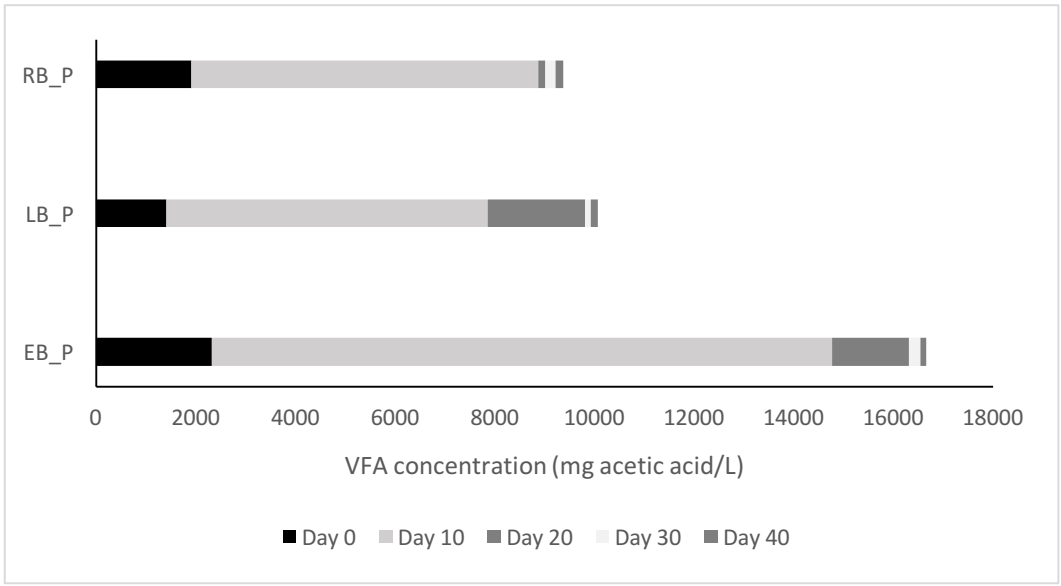
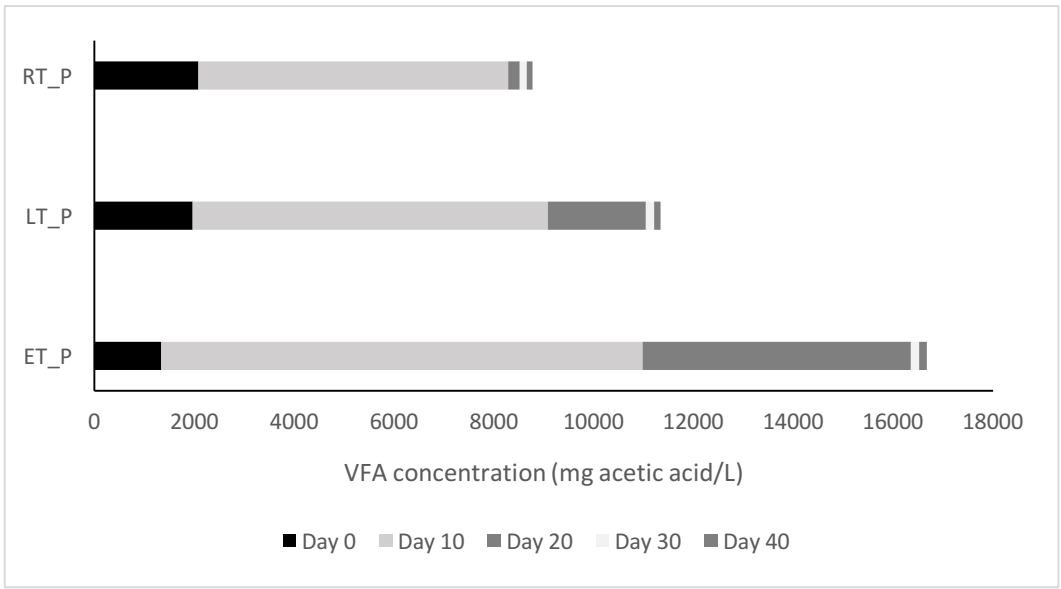


Figure A.2. Total VFA production in control digesters.





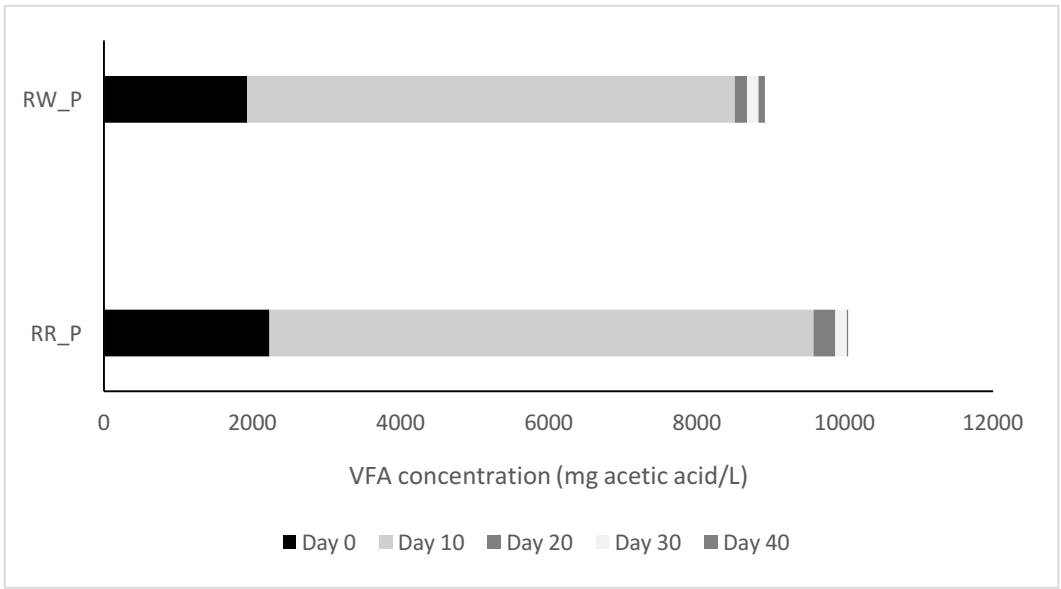
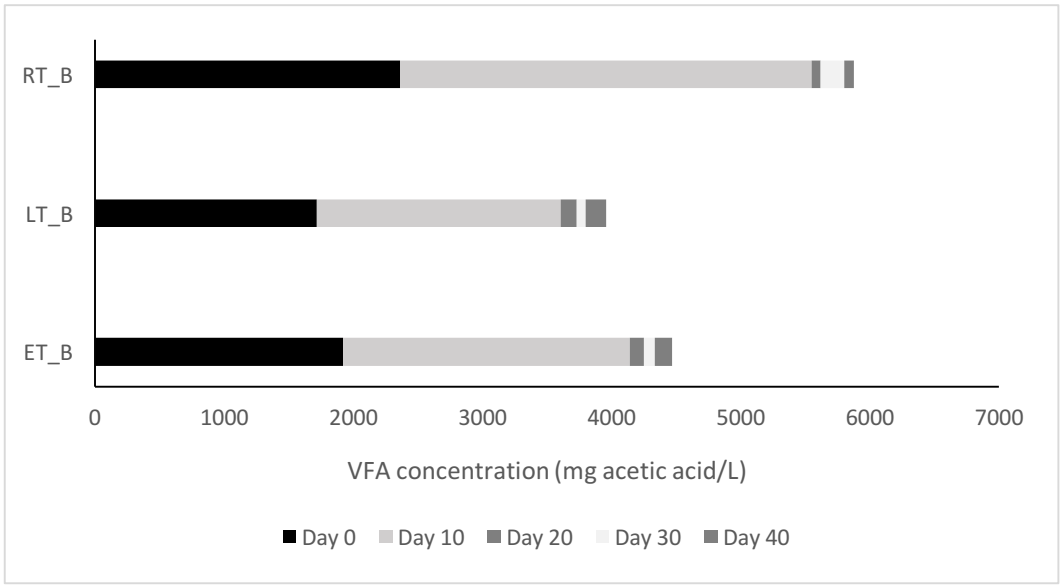
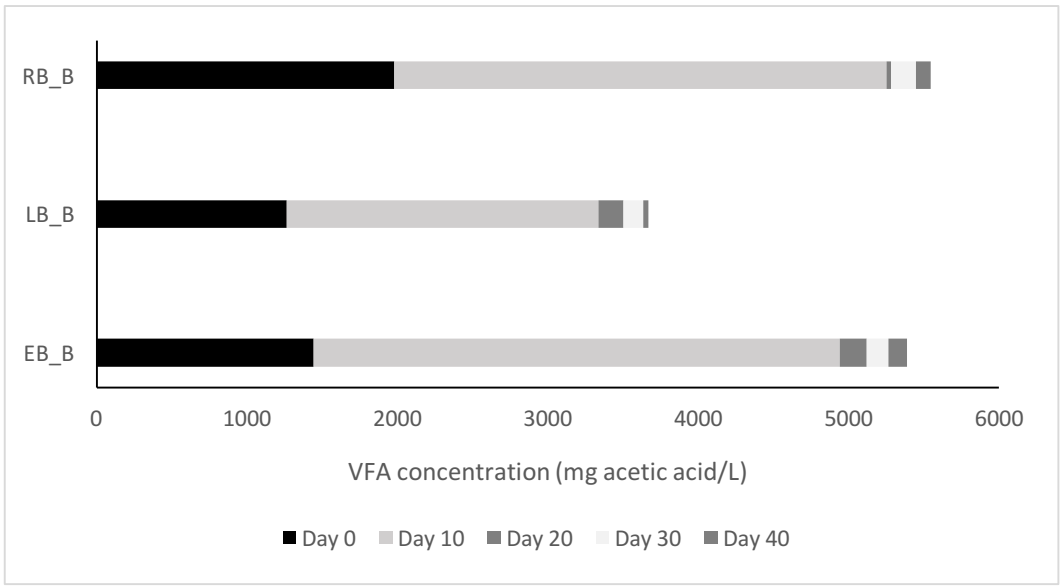


Figure A.3. Total VFA production in pretreated digesters.





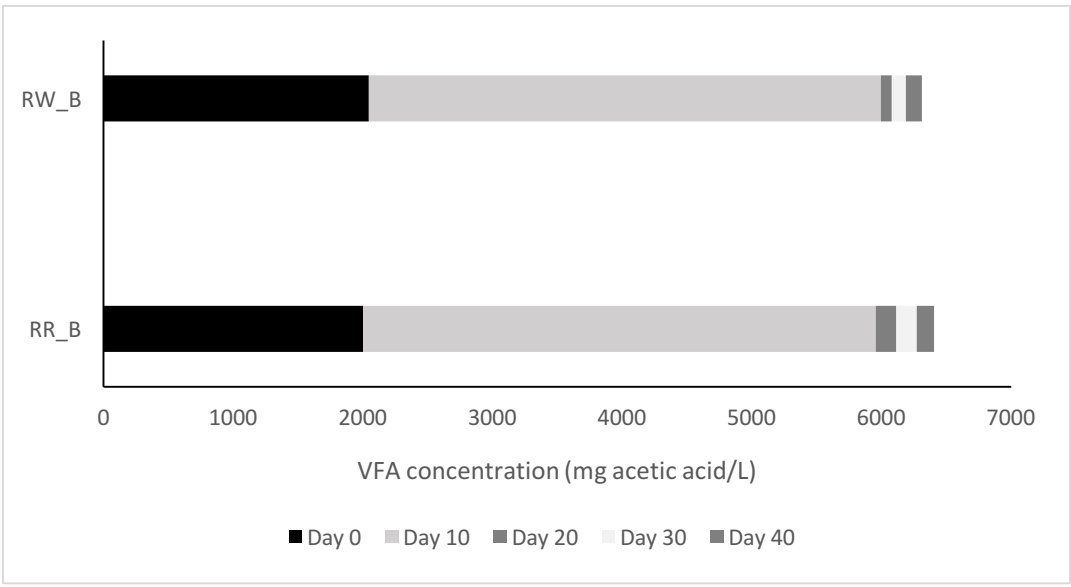


Figure A.4. Total VFA production in bioaugmented digesters.

APPENDIX B: METAGENOMIC ANALYSIS

Table B.1. Read processing summary for control and pretreated AD samples.

Samples	Raw seqs (R1+R2)	Trimmed seqs (R1+R2)	Dada2 infered	Chimera seqs	Chimera free seqs	Unique seqs	Seqs (after size iltration)	Final unique seqs
EB_C	138604	124780	59123	2566	56557	250	55879	196
ET_C	120142	111352	53411	2068	51343	260	50462	191
LB_C	156484	143424	68520	4048	64472	236	63830	187
LT_C	184464	171708	83022	4424	78598	333	77473	238
RB_C	126128	115746	55929	2926	53003	226	52367	174
RT_C	173746	161970	77496	3914	73582	354	72441	254
RW_C	250278	234588	112583	6952	105631	475	104048	338
RR_C	198120	183978	89093	4674	84419	323	83402	240
EB_P	141482	126032	60032	2474	57558	191	57025	150
ET_P	146472	135332	65423	2362	63061	284	61721	178
LB_P	138588	123414	58829	2725	56104	199	55443	145
LT_P	152372	140496	67678	3252	64426	268	63548	190
RB_P	149928	137718	66171	2178	63993	282	63323	228
RT_P	187390	174004	84352	3233	81119	334	79984	241
RW_P	275154	256724	124947	4417	120530	420	119260	313
RR_P	210710	193558	93787	3120	90667	348	89623	265
Seed sludge	132778	118146	56746	1391	55355	155	55062	131

Table B.2. Read processing summary for bioaugmented AD samples.

Samples	Raw seqs (R1+R2)	Trimmed seqs (R1+R2)	Dada2 infered	Chimera seqs	Chimera free seqs	Unique seqs	Seqs (after size filtration)	Final unique seqs
EB_B	148272	126900	60604	6194	54410	145	54336	133
ET_B	177482	162012	78189	4591	73598	226	73424	201
LB_B	144038	125534	59324	5040	54284	179	54231	171
LT_B	183160	168034	80817	5363	75454	257	75303	234
RB_B	253062	234338	111959	8359	103600	379	103372	343
RT_B	190680	173916	83164	5274	77890	299	77764	278
RW_B	213762	182408	86046	3518	82528	222	82445	212
RR_B	170704	153030	72713	4403	68310	258	68164	239

Table B.3. Read processing summary for compost bacteria samples.

Samples	Raw seqs (R1+R2)	Trimmed seqs (R1+R2)	Dada2 infered	Chimera seqs	Chimera free seqs	Unique seqs	Seqs (after size filtration)	Final unique seqs
WC	205772	188762	88541	952	87589	210	87526	199
RC	173840	162708	78071	1078	76993	265	76911	253
BC	126564	116426	54421	748	53673	185	53587	170
TC	213432	202132	97793	1261	96532	317	96392	296

Table B.4. Read processing summary for compost fungi samples.

Samples	Raw seqs (R1+R2)	Trimmed seqs (R1+R2)	Dada2 infered	Chimera seqs	Chimera free seqs	Unique seqs	Seqs (after size filtration)	Final unique seqs
WC	252198	251570	124430	1318	123112	183	122335	142
RC	258276	257660	127949	1505	126444	241	125186	183
BC	320870	320090	159146	1551	157595	247	155889	195
TC	262312	261736	130186	1091	129095	238	127613	177

Table B.5. Read processing summary for initial soil samples.

Samples	Raw seqs (R1+R2)	Trimmed seqs (R1+R2)	Dada2 infered	Chimera seqs	Chimera free seqs	Unique seqs	Seqs (after size filtration)	Final unique seqs
WS	183018	167766	61976	4323	57653	779	57149	702
RS	175002	163442	66721	5206	61515	909	61091	840
BS	175186	164118	68305	5270	63035	1235	61997	1080
TS	130626	122260	50264	3795	46469	842	45907	747

Table B.6. Read processing summary for soil samples after compost amendment.

Samples	Raw seqs (R1+R2)	Trimmed seqs (R1+R2)	Dada2 infered	Chimera seqs	Chimera free seqs	Unique seqs	Seqs (after size filtration)	Final unique seqs
WS.3	51298	50876	23152	803	22349	394	21623	290
RS.3	102516	101758	44905	4991	39914	1322	37674	981
BS.3	65646	65016	28200	2634	25566	857	24263	656
TS.3	105818	104928	45300	4722	40578	1305	38540	989

APPENDIX C: IMAGES

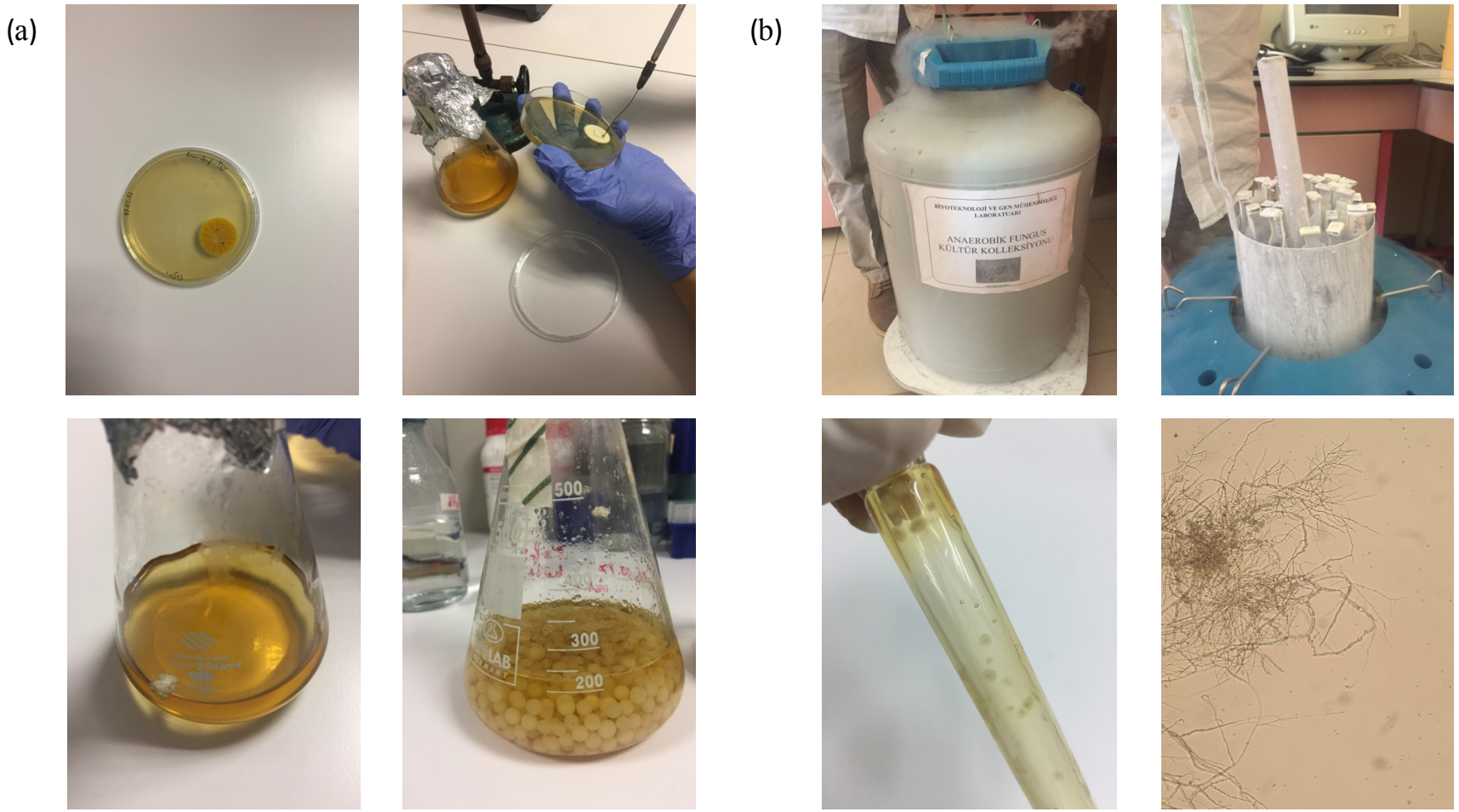


Figure C.1. Images from the cultivation of (a) *Trametes versicolor* (b) *Orpinomyces* sp.