A FARM-BASED BIOREFINERY FOR CHEMICAL PRODUCTION FROM AGRICULTURAL RESIDUES

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Biosystems Engineering – Master of Science

2021

ABSTRACT

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The purpose of this study is to investigate a new process of chemical production from agricultural residues. The studied system reduces the environmental impacts and power requirements of current chemical production methods. The feedstocks, corn stover and swine manure, are utilized by a three-step biological and physical conversion process to produce value-added chemicals lactic acid and chitin. Anerobic co-digestion was applied on the feedstocks to produce biogas and generate carbohydrate-rich solid digestate. The solid digestate was then processed by a mechano-biocatalytic one-pot hydrolysis to release mono-sugars.*Rhizopus Oryzae*, a fungus, was used to convert mono-sugars into lactic acid and chitin. Under the steady state, anaerobic co-digestion produced 249±71 mL biogas/g volatile solids loading/day with a methane content of 62% (v/v). The mechano-biocatalytic process produced hydrolysate with high titers of glucose, xylose and acetic acid (32.90, 21.35, 4.06 g/L, respectively). The liquid hydrolysate was then fermented by *R. oryzae* to produce lactic acid (14.23 g/L) and mixed biomass (119 g dry matter) with a chitin content of 18% (w/w). A mass and energy balance on a farm-based biorefinery concluded that 199 m^3 of biogas, 22 kg of lactic acid and 34 kg chitin per day can be produced by processing 1,000 kg dry feedstock per day. The energy balance showed that a positive net energy output of 2,200 MJ/1,000 kg dry feedstock was achieved by proposed process. Therefore, the studied system not only addressed the environmental challenges of agricultural residues handling and disposal but also produces value-added chemicals to generate revenues from the residues.

ACKNOWLEDGEMENTS

I would like to thank everyone who have helped me with this thesis and throughout the last 5 and a half years.

My advisor Dr. Wei Liao for his guidance, knowledge and patient. Not only for this thesis but for all he has done to help me both inside the lab and out of it.

Dr. Susie Liu for being on my committee and guiding me through the fermentation methods.

Dr. Ilsoon Lee for taking time to serve on my committee and giving me help on my thesis.

Dr. Sibel Uludag-Demirer for always being willing to help and talk. Also, for always knowing where I screwed up.

All my fellow co-workers including Carly Daiek, Ashley Cutshaw, Yurui Zheng, Doug Clements, Adam Smerigan, Annaliese Marks, Yuan Zhong, Meicai Xu and Jack Blackhurst for lending their help and giving constant encouragement.

Finally, I'd like to thank my family and friends who have gotten me to this point at Michigan State University.

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KEY TO ABBREVIATIONS

SM	Swine Manure
CS	Corn Stover
HRT	Hydraulic Retention Times
ADREC	Anaerobic Digestion Research and Education Center
TS	Total Solids
VS	Volatile Solids
TKN	Total Kjeldahl Nitrogen
TP	Total Phosphorus
COD	Chemical Oxygen Demand
TOC	Total Organic Carbon
NREL	National Renewable Energy Laboratory
PDB	Potato Dextrin Broth

CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

The chemical industry is one of the most important industries to our way of life. Without it many of the materials we use and the nutrients we need would have insufficient supplies. In 2018 world chemical sales totaled almost $3.4 trillion, the top chemical producing nations are China, European Union, and the United States [1]. Commodity chemicals include polymers, bulk petrochemicals and intermediates, and inorganic chemicals etc. [2] Currently a majority of them are produced from fossil sources. In fact, most petroleum refineries convert 5-20% of their crude oil into petrochemicals and some existing refineries have increased the output of value added chemicals to 45% [3]. The reason refineries are heavily invested in the chemical industry is that petroleum and natural gas fit perfectly into the traditional chemical manufacturing value chain. That is, they take lower value feedstocks and produce high value low volume products. The problem with this method however is as chemical demand increases fossil fuel usage and thus greenhouse gas production increases [2]. In 2013 the energy demand for the chemical industry was 15 EJ per year this accounted for 28% of the total global industrial energy demand [4]. Associated with this power usage the chemical industry produced 1.5 Gt of CO_2 emissions which accounts for 5.5% of global CO_2 emissions [4]. Because of this the importance of biomass-derived carbon-neutral biorefineries has been amplified.

Production of Chemicals from Fossil Resources

Despite the drawbacks of using fossil resources, there are many benefits to their use, including consistent composition and high density of the feedstock [5]. Once the fossil feedstock enters a refinery, it is pretreated and processed into a “platform chemical”. Platform chemicals

are building blocks used to produce value-added products. Platform chemicals are classified by the number of carbons in the molecule. In petroleum refining the most used platform chemicals are C2-C4 olefins, also known as lower olefins. Olefins are produced using fluid-catalytic cracking, steam cracking and dehydrogenation. It is estimated that 400 million tons of lower olefins are produced annually [6]. These products are very valuable and can greatly increase the profit margin of refineries.

Steam cracking is used to produce 50% of olefins. The feedstocks (naphtha and other saturated hydrocarbons) are first vaporized in the absence of oxygen at 650 ˚C. Fractionation and compression are then performed to separate and recover olefins. The process requires vessels that can withstand high heat and pressure. The main crux of cracking is the immense amount of power it requires. It accounts for approximately 8% of energy consumption in the chemical sectors and contributes to 180-200 million tons of CO_2 emissions every year [7]. Fluid-catalysts cracking, another new cracking technology, improves steam cracking by using catalysts in lower temperatures and pressures. However, the energy consumption and greenhouse gas emissions are still high [8].

Another method of producing chemicals from fossil fuels focuses on natural gas. The natural gas is processed and converted into syngas (carbon monoxide and hydrogen). Syngas can be produced using three different processes, partial oxidation, steam methane reforming, and autothermal reforming [9]. Syngas is then further processed to produce lower olefins for chemical production. Syngas can also be used to directly produce chemicals such as waxes, alcohols and aldehydes [10]. However, the problem with syngas derived from fossil fuels is the same as steam cracking. It requires either high temperatures or high pressure that causes process complexity and high energy demand [11].

Biomass-Derived chemicals

Because of the downsides to deriving chemicals from fossil fuels, it has been a goal of scientific community since the early 1990's to find an economically feasible pathway to produce chemicals in a more environmentally friendly way [12]. This spawned a new type of chemistry, green chemistry, which is defined by Anastas and Warner as "to promote chemical technologies that reduce or eliminate the use or generation of hazardous substances in the design, manufacture and use of chemical products" [13]. Based on the definition of green chemistry, there are numerous new feedstocks that can be used for chemical production including, industrial (manure), agricultural (corn stover, rice husks, logging) and domestic (food wastes) residues [14]. Biomass as one of renewable resources is the most suitable feedstock to be used for chemical production.

When using biomass to produce a chemical, it involves multiple steps. The biomass first needs to be pretreated and converted into a platform chemical using physical, chemical, thermochemical, biological pretreatment or their combinations [15], [16]. The biomass-derived platform chemicals include sugars, ethanol, lactic acid, and succinic acid. The platform chemicals are then catalyzed using chemical or biological means to produce value-added chemicals [12].

Physical pretreatment focuses on reducing the particle size or separating of components without changing the state or composition of the compound. Physical pretreatment methods include milling, irradiation, and extrusion [17], [18]. It is apparent that physical pretreatment reduces particle size and crystallinity of biomass and makes the following processes more

efficient. However, the high energy demand of physical treatment processes is the main drawback.

Chemical treatment refers to process that directly converts biomass to sugars by changing the chemical structure of the substrate. These processes are performed at relatively mild temperatures and pressures [19]–[21]. Chemical treatment methods use an array of chemicals to release the lignin and break down cellulose and hemicellulose into simple sugar monomers. The downsides of these chemical treatment methods are the use of strong chemicals and generation of toxic waste effluents.

Biological pretreatment methods rely on microbes and enzymes to hydrolyze the cellulose and hemicellulose into mono-sugars. Fungal strains have been used to degrade lignin and increase cellulose availability [20]. Enzymatic hydrolysis is another common treatment method. It is often combined with other pretreatment methods. It has been shown that higher glucose content and production rate are achieved when enzymatic hydrolysis is combined with a pretreatment method (microwave, acid and alkali) [16]. Enzymatic hydrolysis also was shown to have a possible efficiency of 91% [17].

STUDIED PROCESS

This study focuses on developing an integrated farm-based biorefining concept that synergistically combines anaerobic digestion, one-pot mechano-biocatalytic treatment, and fungal fermentation to convert corn stover and swine manure into value-added lactic acid and glucosamine precursor (chitin) (Figure 1). The mixture of corn stover and swine manure is first treated by anaerobic digestion to generate methane for power generation and solid digestate for carbohydrate production. The solid digestate is consequently treated by a low-energy and simple mechano-biocatalytic process (simultaneous ball milling and enzymatic hydrolysis) to generate a

hydrolysate rich in C5 and C6 sugars according to a previous study [22]. The hydrolysate is then fermented using a fungal strain to produce lactic acid. The biomass of the fungal strain contains chitin which can be further processed to produce glucosamine. The power generated from methane is used to power the farm-based biorefining. The technologies of anaerobic digestion, one-pot mechano-biocatalytic hydrolysis, and fungal fermentation used in this study are discussed below.

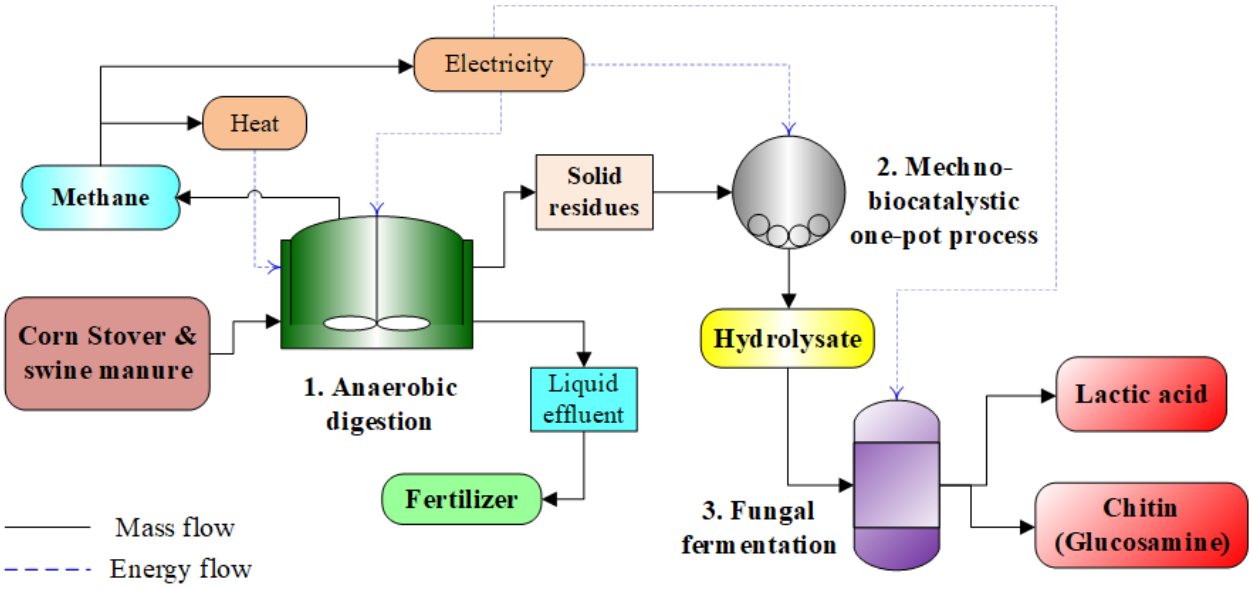


Figure 1. The studied farm-based biorefinery

Anaerobic digestion as a biological process has been widely used in waste management. The biogas produced from the digestion can be used as an energy source to farm-based biorefining. Corn stover and swine manure both have previously been used for anaerobic digestion. Swine manure has been shown to produce 430 L CH_4/kg VS [23]. However, swine manure has a carbon/nitrogen ratio of 6-8 which is too low for the digestion to efficiently utilize the nutrients [24]. Low carbon/nitrogen ratio also leads to ammonia accumulation that negatively influences digestion performance. Anaerobic digestion of corn stover has been shown to produce 403.7 L CH4/kg VS [25]. Pretreating the corn stover with an alkali solution can increase methane

production by 37% [26]. However, corn stover has a carbon/nitrogen ratio of between 61 and 84, which leads to accumulation of volatile organic acids that eventually reduce the pH of digestion and cause digestion failure [27]. It is apparent that anaerobic digestion of either corn stover or swine manure has drawbacks caused by an imbalanced carbon/nitrogen ratio. These drawbacks can be negated by adding a co-substrate. Such digestion is referred to as anaerobic co-digestion. The co-digestion of corn stover and swine manure has been studied multiple times [28]. Fujita et al. showed that the addition of corn stover to digestion increased biogas production by 65% compared to the case of swine manure as the only substrate. He concluded the increase is probably due to the easily biodegradable carbohydrates in corn stover [28]. Another study concluded that the preferred SM/CS ratio of 2/1 can enhance the digestion performance of biogas production, and an increase of corn stover concentration led to reduction of the biogas productivity [24]. A microbiology study that focused on the dynamic changes of microbial communities during co-digestion of corn stover and swine manure elucidated that along with showing co-digestion increased methane content, the highest methane production was achieved with the highest relative abundance of *Methanosaeta* [29].

Mechano-catalysis has been applied to release reducing sugars from biomass. Many studies have focused on mechano-chemical pretreatment and enzymatic hydrolysis (Table 1). Loustau-Cazalet et al. developed a vibro-ball-milling (VBM) process with and without chemical (H_2O_2 Urea, NaOH, H_3PO_4, and Betaine Cl) supplement to pretreat corn stover for enzymatic hydrolysis of monosaccharide production [30]. Enzymatic hydrolysis of the biomass pretreated by the VBM process with chemical supplement converted 98% of cellulose into glucose, which is much higher than the process without chemical supplement (56% glucose conversion). Mais et al. applied a combined ball milling and enzymatic hydrolysis on steam explosion treated

Douglas-fir wood chips to generate monosaccharides [31]. A glucose conversion of 85% was achieved at an enzyme loading of 25 filter paper unit (FPU)/g cellulose during a 48-hour hydrolysis. Lee et al. used a planetary ball mill with alumina balls to prepare corn stover for the following enzymatic hydrolysis [32]. High monosaccharide conversion of 92% and relatively high sugar concentration of 35 g/L were achieved. Falls et al. applied NaOH augmented ball milling followed by enzymatic hydrolysis and obtained 91% of monosaccharide conversion and 33 g/L monosaccharide in the hydrolysate from switchgrass [33]. Balch et al. recently developed a bacterial fermentation process in a ball-milling reactor to realize a one-step conversion of switchgrass into biofuel [34]. Total fractional carbohydrate solubilization was 88% after 5 days cultivation with an initial carbohydrate concentration of 8.5 g/L (including glucan, xylan and arabinan) in the culture broth. However, these approaches either use corrosive chemicals, high-temperature thermal process, complicated treatment steps, low sugar titer and yield, or still require multiple steps to generate mono-saccharides form biomass. A consolidated pretreatment and hydrolysis process has been recently developed to incorporate both ball milling and enzymatic hydrolysis in one single step [22]. The new process simplifies the process and improves the process efficiency. Under the preferred reaction, glucose concentration reached 27 and 55 g/L for solid digestate and corn stover, respectively with cellulose conversions of 59 and 89%.

Table 1. Comparison of the studied mechano-biocatalytic process and selected mechanical pretreatment and hydrolysis processes

Process	Substrate	Process description	Sugar conversion (%)	Sugar concentration (g/L) [a]	Reference
Ball milling and hydrolysis	Corn stover	Dry ball milling followed by enzymatic hydrolysis	92 [b]	35 [b]	[32]

Table 1 (continued)

Ball milling and hydrolysis	Corn stover	Wet ball milling followed by enzymatic hydrolysis	54	4	[35]
Ball milling and hydrolysis	Miscanthus	Dry ball milling followed by enzymatic hydrolysis	28 [c]	4	[36]
Ball milling and hydrolysis	Solid digestate	Dry ball milling followed by enzymatic hydrolysis	76 [d]	17 [d]	[37]
Chemical augmented ball milling and hydrolysis	Corn stover	NaOH augmented ball milling followed by enzymatic hydrolysis	85 [e]	33	[38]
Chemical augmented ball milling and hydrolysis	Corn stover	Vibration ball milling with NaOH followed by enzymatic hydrolysis	98 [f]	20[f]	[30]
Lime and ball milling and hydrolysis	Switchgrass	Lime treated biomass followed by ball milling and then enzymatic hydrolysis	91[g]	34	[33]
Mechano-chemical treatment and hydrolysis	Miscanthus	Solvent ball milling followed by enzymatic hydrolysis	82	12	[39]
NaOH-extrusion pretreatment	Miscanthus	Extrusion pretreatment with NaOH followed by enzymatic hydrolysis	55 [h]	32 [h]	[40]
Simultaneous ball milling and hydrolysis	Douglas-fir wood	Steam explosion and alkaline hydrogen peroxide treatment before simultaneous ball milling and hydrolysis	67 [i]	60	[31]

Table 1 (continued)

Thermo-mechano-chemical-biological extrusion	Sugar cane bagasse	Two steps of thermos-chemical pretreatment and enzymatic hydrolysis happened in a twin-screw extruder	63 [j]	10 [j]	[41]
Mechano-biocatalytic one-pot process	Corn stover	Direct balling mill and enzymatic hydrolysis in a single reactor without corrosive chemicals and thermal treatment	83	75	[22]
Mechano-biocatalytic one-pot process	Solid digestate	Direct balling mill and enzymatic hydrolysis in a single reactor without corrosive chemicals and thermal treatment	65	41	[22]
Mechano-biocatalytic one-pot process	Miscanthus	Direct balling mill and enzymatic hydrolysis in a single reactor without corrosive chemicals and thermal treatment	40	36	[22]
Mechano-biocatalytic one-pot process	Switchgrass	Direct balling mill and enzymatic hydrolysis in a single reactor without corrosive chemicals and thermal treatment	46	39	[22]

a. The sugar concentration includes all monosaccharides.
b. The enzyme loading was 42 FPU/g cellulose in the biomass. The hydrolysis time was 24 hours. The sugar conversion and concentration are glucose only.
c. The enzyme loading was 15 FPU/g cellulose in the treated biomass. The hydrolysis time was 24 hours.
d. The enzyme loading was 30 U/g dry biomass. The hydrolysis time was 72 hours.
e. The enzyme loading was 15 FPU/g cellulose in the treated biomass. The hydrolysis time was 24 hours.
f. The enzyme loading was 25 FPU/g cellulose in the treated biomass. The hydrolysis time was 72 hours.

g. The enzyme loading was 20 FPU/g biomass. The hydrolysis time was 72 hours. The sugar conversion and concentration are glucose only.
h. The hydrolysis time was 72 hours.
i. The enzyme loading was 25 FPU/g cellulose in the treated biomass. The hydrolysis time was 24 hours.
j. The hydrolysis time was 48 hours. The sugar conversion and concentration are glucose only.

Fungal fermentation utilizing *Rhizopus oryzae* has also been previously studied. *R. Oryzae* immobilized in situ within sponge-like cubic particles was able to produce 145 g/L of lactic acid with a glucose concentration of 150 g/L in the feed [42]. Another paper studied the consumption of xylose by *R. Oryzae*. The study concluded that xylose consumption only occurs under growing conditions, and xylose consumption ceases once deprivation of nutrients occurs. In addition, complete xylose consumption requires a carbon to nitrogen ratio of 61/1 which is much lower than the 201/1 ratio needed for glucose consumption [43]. Studies have also been conducted on utilizing wastes such as food derived sub-products. One such study focused on enhancing the value of animal feed. *R. Oryzae* was shown to produce a protein and fat rich mycelia on a feed mixture of fruit wastes, coffee grounds and potato peels [44]. The biomass of *R. Oryzae* also has potential to produce value-added chemicals. The cell wall contains 9% phosphate, 10% glucosamine and 21% N-acetyl glucosamine. Sulfuric acid can recover 89% of the cell wall and a pure fungal chitosan concentration of 0.12 g/g cell wall [45]. These studies elucidate the *R. Oryzae* fermentation is a method that can produce high concentrations of lactic acid.

CURRENT PRODUCTION METHODS OF TARGET CHEMICALS

Lactic Acid

Lactic acid currently is in high demand from a variety of industries. This is due to its wide array of potential applications. Currently it is used on a large scale in the food, chemical

and pharmaceutical industries. There are also emerging technologies that utilize lactic acid such as controlled drug delivery, tissue engineering and biodegradable polymers [46]. The market in 2020 was valued at $2.7 billion and according to Grandview Research is expected to grow at a rate of 8% annually from 2021 to 2028 [47]. The chemical can be produced using chemical or biological methods. Chemical processes produce a racemic form of D/L lactic acid whereas optically pure lactic acid is produced by biological routes [48]. Currently, most industrial-scale lactic acid is produced via the chemical synthesis route however this route relies on fossil fuels which is neither renewable nor sustainable [49]. Chemical synthesis of lactic acid can be divided into two stages. The first stage is a catalytic reaction of acetaldehyde with hydrogen cyanide to produce lactonitrile. Lactonitrile is then hydrolyzed using sulphuric or hydrochloric acid to produce crude lactic acid and ammonium salts. The downside of the chemical synthesis is that a racemic mixture of lactic acid does not guarantee good conditions for downstream processes [50]. Fermentation producing lactic acid has been shown to have an economic advantage when compared to chemical synthesis [51]. The current commercial method to produce lactic acid via fermentation is to provide a culture medium with excessive carbohydrates and sufficient nutrients as well as a pH regulatory agent. After fermentation, crude calcium lactate is purified using filtration and hydrolyzed by concentrated sulfuric acid to obtain lactic acid [46]. The main difficulty of fermentation is the reduction of pH caused by the production of acid. If pH becomes too low, it negatively affects the fermentation performance.

Glucosamine

Glucosamine is an amino sugar that is derived by substituting of a hydroxyl group on a glucose molecule with an amino group. It is used in a wide array of fields including food, cosmetics, and pharmaceutical industries. It has been shown to help treat osteoarthritis in humans

due to glucosamines presence in our joints [52]. Glucosamine is used by more than five million people as a dietary supplement making it the fourth most used dietary supplement [53]. The market was valued at $249.1 million in 2019 and expect to grow annually at 1.5% [54]. It has also been identified as a promising antimicrobial agent due its antimicrobial properties [55]. Most glucosamine is produced using acid hydrolysis of chitin from shellfish shells. Concentrated hydrochloric acid breaks down the polymer and deacetylates N-acetylglucosamine to form glucosamine [56]. The main drawback of this technology is that it relies on a limited raw material. As the demand for glucosamine increases, this drawback becomes more pertinent. Another method to produce glucosamine is using enzymatic hydrolysis. However the enzymes used for this method currently cannot efficiently hydrolyze chitin derived from shellfish [57].

KNOWLEDGE GAPS

This literature review elucidated the need for more processes to produce value added chemicals from renewable resources. Currently the chemical production industry relies on fossil fuels and energy intensive processes. Because of this, novel biomass conversion processes need to be developed. The literature review revealed knowledge gaps that the further research needs to resolve. First, anerobic co-digestion of corn stover and swine manure has been studied, however the effects of high corn stover content has not been investigated. Second, *R. Oryzae* fermentation has been studied to produce lactic acid, the ability of *R. Oryzae* to simultaneously consume glucose and xylose in a hydrolysate high in nitrogen content has not been sufficiently studied. Finally, and most importantly, the feasibility of integrating three processes into a system to co-produce lactic acid and chitin from biomass residues has not been investigated.

OBJECTIVES AND HYPOTHESIS

The overall hypothesis for this study is that by designing an integrated farm-based biorefining system including anaerobic digestion, mechano-biocatalytic hydrolysis and fermentation would help minimalize the environmental impacts of agricultural residues (wastes) and maximize the revenue of farm operations. The objectives of this study are to:

1) study the effects of feedstocks on individual unit operations in the system, and

2) elucidate the relationship between individual units and conclude a technically sound farm-based biorefining system.

CHAPTER 2. A FARM-BASED BIOREFINERY FOR CHEMICAL PRODUCTION FROM AGRICULTURAL RESIDUES

ABSTRACT

The purpose of this study is to investigate a new process of chemical production from agricultural residues. The studied system reduces the environmental impacts and power requirements of current chemical production methods. The feedstocks of corn stover and swine manure are utilized by a three-step biological and physical conversion process to produce value-added chemicals of lactic acid and chitin (a precursor of glucosamine). Anerobic co-digestion was first applied on the feedstocks to produce biogas and generate carbohydrate-rich solid digestate. The solid digestate was then processed by a mechano-biocatalytic one-pot hydrolysis to release mono-sugars. A fungal strain - *Rhizopus Oryzae* was used to convert mono-sugars into lactic acid and chitin. Anaerobic co-digestion of swine manure and corn stover took two HRTs (40 days) of continuous operation to reach steady-state digestion (considering both digestion performance and microbial community). Under the steady state, anaerobic co-digestion produced 249±71 mL biogas/g volatile solids loading/day with a methane content of 62% (v/v). The modified one-pot process on the solid digestate generated a liquid hydrolysate with high titers of glucose, xylose, and acetic acid (32.90, 21.35, 4.06 g/L, respectively). The liquid hydrolysate was then fermented by *R. Oryzae* to produce lactic acid (14.23 g/L) and mixed biomass (119 g dry matter) with a chitin content of 18% (w/w). A mass and energy balance on a farm-based biorefinery concluded that 199 m^3 of biogas, 22 kg of lactic acid and 34 kg chitin per day can be produced by processing 1,000 kg dry feedstock per day. The energy balance showed that a positive net energy output of 2,200 MJ/1,000 kg dry feedstock was achieved by the farm-based biorefinery integrating anaerobic digestion, mechano-biocatalytic one-port hydrolysis, and fungal

fermentation. Therefore, this study concluded that the studied system not only addressed the environmental challenge of agricultural residues handling and disposal but also produce value-added chemicals to generate revenues from the residues. The farm-based refining concept creates a win-win-win scenario for the environment, rural community, and bioeconomy.

INTRODUCTION

The energy demand for the global chemical industry was 15 EJ per year in 2013, which accounted for 28% of the total global industrial energy demand. This energy usage is compounded by the fact that the industry produced 1.5 Gt of CO_2 emissions which accounts for 5.5% of global CO_2 emission [4]. Chemical production is heavily connected to fossil fuel usage. Most petroleum refineries convert 5-20% of their crude oil into petrochemicals [3]. These chemicals are highly valuable chemicals, total chemical sales total $3.4 trillion in 2018 [1].

Because of environmental impacts of the current chemical production methods, there has been an increasing interest in the use of renewable resources to produce chemicals. Agricultural residues are a very promising renewable resource [58]. 140 billion metric tons of biomass is generated annually, including energy crops, forestry, and agricultural residues. It is the equivalent of 50 billion tons of oil [59]. It is the reason that research on the conversion of biomass into value added chemicals has been an emphasis since the early 1990s [12] . Chemical, physical, thermal, chemical, and biological approaches have been intensively studied to carry out the conversion. These approaches have been greatly advanced in the past decades [16]. However, there are still many drawbacks to them. Many of them either demand high energy or rely on strong chemicals, both of which can cause harm to the environment. In addition, the reliance on chemicals also complicates downstream processes. Therefore, new technologies are needed to

replace these harsh processes and further advance chemical production systems to realize a true green chemical industry.

Because of the need for new conversion technologies [22], this study focuses on developing a farm-based biorefinery to efficiently utilize corn stover and swine manure for value-added lactic acid and chitin (glucosamine) production. The mixed feedstocks underwent anaerobic co-digestion with a hydraulic retention time of 20 days. This co-digestion has previously been studied ([28], [29], [60]). The solid digestate produced by the co-digestion was treated by a modified mechano-biocatalytic process to produce mono-sugars. Finally, a fungal fermentation using *R. Oryzae* converted the mono-sugars into value-added chemicals of lactic acid and chitin (glucosamine). Lactic acid and glucosamine are widely utilized in multiple industries including food, chemical and pharmaceuticals.

MATERIALS AND METHODS

Anaerobic Digestion

Anaerobic digestion was performed in a custom made 20 L reactor. The reactor was placed in a hot room at ADREC to keep the process temperature at 50 ˚C. The reactor was continuously mixed with an impeller to ensure proper mixing. Gas lines connected the reactor to a tip meter to record gas data.

The initial feeding of the reactor was 20 kg of mixed feed. The mixed feed consisted of corn stover and swine manure (50.5 g Corn Stover, 949.5 g swine manure). The corn stover was collected from a field in Mid-Michigan and then dried for 24 hours in a 50˚ C hot room. The corn stover was ground in a hammermill (Schutte Model # W-8-LH) supplied by the MSU Crop and Soil Research Center. The swine manure was collected from the Michigan State University

farms. The feed was fixed at a total solids (TS) of 7.5%. After initial feeding the reactor was left to begin digestion, once gas production began daily feeding began. The digestor was targeted to have a hydraulic retention time of 20 days. To accomplish this 1 kg of anaerobic digestate was wasted daily and replaced with 1 kg of mixed feed. This continued until 4 HRT's had been completed at which point the remaining digestate was drained and saved for further analysis.

The wasted digestate collected during feeding underwent further analysis. Every weekday the pH, TS and VS of the sample was measured. The pH was measured using a Fisherbrand™ accumet™ AB15 + Basic (Fisher Scientific Co., Pittsburgh, PA). To measure total solids the sample was mixed and poured into a crucible and placed in an oven at 105 ˚C for 24 hours. To measure volatile solids the crucibles were taken from the oven and placed in a furnace at 550 ˚C for 5 hours. Nutrient characteristics were measured multiple times per week. The characteristics measured were TKN, TP, COD and TOC. These where measured using HACH Nutrient Test Kits and a Hach spectrophotometer. Every HRT the large amount of sample was saved to measure fiber composition (cellulose, hemicellulose, and lignin) according to NREL methods. Another smaller sample was saved to analyze microbial composition.

Microbial Analysis

Once every 10 days during anaerobic digestion samples (1.5 ml) were collected for DNA analysis and stored at -20 ˚C until needed. To remove nutrients from the samples they were centrifuged for 5 mins at 10,000 rpm and the supernatant discarded. The pellet was then washed and resuspended, then centrifuged again. The remaining pellet was used for the DNA extraction using a DNeasy® PowerSoil® DNA Isolation Kit (Qiagen, Germany). DNA extracts were eluted with 100 μL of 10 mM Tris-HCl (pH 8.5) and the concentration and purity were determined

using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA). Extracted DNA samples were stored at -80°C for several weeks before use in real-time PCR quantification and high-throughput sequencing (Illumina MiSeq flow cell).

Illumina sequencing was performed for the 16S rRNA gene region to assess the bacterial community. The PCR conditions were as follows: 1.0 µL DNA template (10x diluted of microbial community DNA), 0.5 µL of 100 µM forward primer (IDT, Pro341F 5'-CCTACGGGNBGCASCAG-3'), 0.5 µL of 100 µM reverse primer IDT, Pro805R 3'-GACTACNVGGGTATCTAATCC-5'), 12.5 µL 2x Supermix (Invitrogen, USA), and 10.5 µL PCR grade water. The PCR program used for all assays were as follows: 96 ˚C for 2 min, followed by 30 cycles of 95 ˚C for 20 s, 52 ˚C for 30 s, and 72 ˚C for 1 min, and a final elongation period of 72 ˚C for 10 min. Amplicons were quality-tested and size-selected using gel electrophoresis (1.0% (w/v) agarose concentration and 1× TAE run buffer). Samples were then diluted to normalize DNA concentrations within 5-10 ng µL-1 by measuring the DNA concentration with the PicoGreen dsDNA quantitation assay (Invitrogen, USA) and Fluostar Optima microplate reader (BMG Labtech, Germany). The normalized PCR products were then sequenced at the Michigan State University (MSU) Research Technology Support Facility (RTSF). Illumina MiSeq (pair-end 250 bp) targeting on V3_V4 hypervariable regions was used to carry out the sequencing. The sequences were analyzed using R statistical software to determine what microbes were present and the relative abundance.

Biomass Conversion

To separate the fiber from the anaerobic digestate a milk bag was used. The milk bag was set above a large beaker and the anaerobic digestate was poured in. Once all digestate was poured into the bag the fiber was squeezed. Once the fiber was sufficiently dry it was removed from the bag and total solids was measured. To avoid contamination the fiber was placed in a medical autoclave and autoclaved at 121 ˚C for thirty minutes.

The one-pot mechano-biocatalysis took place in a 2 L stirred ball mill (JM-2L, Tianchuang Powder Tech Co., Ltd., Changsha, China). The ball to wet-biomass ratio was set at 3:1. The balls were made of agate and the ball mill vessel lined with zirconium. The amount of biomass, enzymes and buffer are calculated. The buffer used is citrate buffer (pH=4.8) containing 0.1 g/L tetracycline. The enzymes used were cellulase (CTEC2 Sigma Aldrich, St. Louis, MO) and xylanase (HTEC 3, enzyme activity: 10000 U mL−1; Novozymes North America, Franklinton, NC). At the beginning of the process the biomass is placed into the mill and half the buffer and enzyme was added. The mixer in the mill was run at 300 rpm for two hours. At this time, the remaining buffer, and enzymes (the other half) are added and another period of two hours of milling occurs. Once the total four hours of the milling at 300 rpm is complete, the speed of the mixer is reduced to 50 rpm. During this time a circulating water bath pumps water (T=50˚ C) into the jacket to warm the reactor. After a total time of 24 hours has elapsed the stirring is stopped, and the grinding media separated from the hydrolysate. A 1 ml sample of the hydrolysate was frozen for sugar analysis. The hydrolysate was autoclaved at 121 deg C for 30 minutes to sterilize the hydrolysate for fermentation.

To prepare the hydrolysate sample for HPLC the sample was centrifuged at 5000 rpm for 5 minutes to separate the liquid hydrolysate. The sample was then diluted 5 times and 1 mL was

filtered through a 0.22 μm polyethersulfone membrane filter (SLGS033SS, EMD Millipore, Billerica, MA). Glucose, xylose, and acetic acid in the hydrolysate were determined by a High-Performance Liquid Chromatography (HPLC) (Shimadzu Prominence, Shimadzu Corp., Kyoto, Japan), which equipped a Bio-Rad Aminex HPX-87H analytical column and a refractive index detector. The mobile phase was 0.005 mol/L sulfuric acid at a flow rate of 0.6 mL/min. The oven temperature was set at 65 °C. HPLC grade standards including glucose (Catalog Number: 49158), xylose (Catalog Number: 95729), and sodium acetate (Catalog Number: S8750) were purchased from Sigma–Aldrich, St. Louis, MO.

Fermentation

Fermentation occurred in a 500 ml Erlenmeyer flask. The flask was placed in a stirrer set at 200 rpm and 27˚ C. The microbe used for fermentation was *Rhizopus oryzae*. The seed was inoculated in PDB with a calcium carbonate concentration of 0.3 g/L. Spores (.5 ml) were introduced into PDB and placed in a heated stirrer at 180 rpm and 27 deg C. 50 g of sterilized hydrolysate was added to the beakers along with 1 g of calcium carbonate. The seed was then introduced to the flask at a 20% seed (10 g). The flasks were then placed in the stirrer and samples were taken every 12 hours for 60 hours. At the end of this period the fungal pellets were separated from the hydrolysate using a centrifuge at 200 rpm. All fermentation samples were saved for analysis in HPLC following the same procedure described earlier. The biomass was dried to measure total dry weight and underwent further testing to determine glucosamine production.

To determine the total amount of glucosamine synthesized the following method was used. This method hydrolyzes and deacetylate the chitin and chitosan molecules. Dried biomass was hydrolyzed using 60 % (v/v) sulfuric acid at room temperature for 24 hours. Sulfuric

concentration was then reduced to 0.5 M using DI water. The samples were autoclaved at 11 ˚C for 1 hour (total time) and neutralized to a pH of 7 using 1 M sodium hydroxide. DI water was again used to dilute the solution to a biomass concentration of 0.1 g/L and a 0.5 mL sample was taken. These samples were mixed with 0.5 mL of 5% (w/v) sodium nitrite (lot # MKBH7064V) and 0.5 mL of 5% (w/v) potassium bisulfate (lot # SZBB3500V). Samples were shaken at 250 rpm for 15 min, and then centrifuged at 1500 x g for 2 min. 0.6 mL of supernatant was mixed with 0.2 mL of 12.5% (w/v) ammonium sulfamate (lot # MKBR8639V) and shaken vigorously for 3 min. To each sample 0.2 mL of 0.5% (w/v) methyl-2-benzothiazolinone hydrazone hydrochloride (lot # BCBG8561V) was added and then samples were boiled for 3 minutes and cooled to room temperature. 0.2 mL of 0.5% (w/v) iron (III) chloride hexahydrate (Mallinckrodt Baker, NJ, USA, lot # 851342) was added to each and samples were allowed to stand for 30 minutes before being measured with a photo spectrometer (Shimadzu UV-1800 UV spectrophotometer) at λ=650nm. Fresh methyl-2-benzothiazolinone hydrazone hydrochloride and iron(III) chloride hexahydrate was prepared each time and analytical grade glucosamine was used to establish a standard curve [61]–[64].

Mass and Energy Balance Analysis

A mass and energy balance were performed on a theoretical biorefinery that utilizes the proposed method. The biorefinery was assumed to consume 1000 kg of dry feedstocks per day (691 kg CS, 309 kg SM). The total solids of this feed is 6.85%. The lower heating value of methane (35.8 kJ/L methane) was used to determine the energy derived from methane. The specific heat of the feed is 4.2 kJ/kg ˚C. A year-round average temperature of the feed is assumed to be 15 ˚C and the operational temperature of anaerobic digestion is 35 ˚C. Ten percent of energy in methane is set to power the equipment for anaerobic digestion. The

electricity consumption of the one-pot process was derived from a previously published study (.56 kWh-e/kg dry solid digestate) [22]. It was also assumed that 1 kg dry mixed feedstock produces 0.233 kg dry solid digestate. Finally, the conditions of fungal fermentation are as follows, 72 hours of the culture time, 200 rpm of the agitation, 0.5 vvm of the aeration, and 30ºC is the culture temperature. The energy demand of fungal fermentation is 0.06 MJ/kg fermentation broth [65].

Statistical Analysis

Statistical analysis was performed using R statistical software. Data was analyzed using one-way analysis of variance (ANOVA) and Tukey pair-wise comparison to determine the significance of the data. A significance value of a=0.05 was used for all tests. Microbial analysis was performed using the R libraries Vegan, ggplot2, phyloseq and MASS on taxonomic and phylogenic data to graph relative abundances of different microbes. All code necessary for statistical analysis can be found in supplementary material.

RESULTS AND DISCUSSION

Anaerobic Digestion

Feedstocks

Characteristics of swine manure and corn stover are listed in Table 2. Swine manure had a TS of 2.67% and a volatile solids of 68.7%. Corn stover had a total solids of 97.52% and a volatile solids of 92.9%. Fiber analysis shows that the swine manure contains 2.64% cellulose and 3.65% xylan, which are much less than corn stover (31.26% cellulose, 23.82% xylan). The data also shows that swine manure has TN concentration of 2,317 mg/L, which is consistent with literature reports [66]. Considering high nitrogen content of swine manure and high

carbohydrate content of corn stover, mixing them can make a feedstock with a better nutrient profile to support a healthy anaerobic digestion [67]

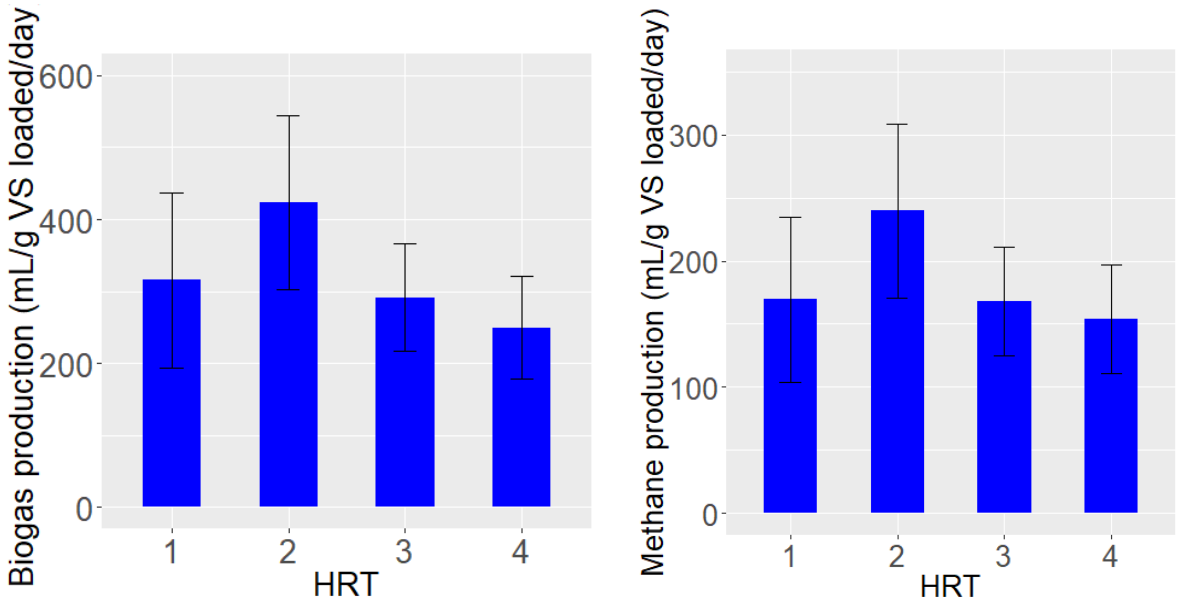
Table 2. Characteristics of swine manure, corn stover, and mixed feed

Parameter	Swine manure	Corn stover	Mixed feed [b]
TS (%)	2.67±0.03	97.52 ± 0.55	6.85
VS (%TS)	68.7±1.26	92.9 ± 0.06	80
Cellulose (%TS)	2.64±0.04	31.26±0.89	21.53
Xylan (%TS)	3.65±0.04	23.82±0.67	16.97
Lignin (%TS)	23.78±0.42	18.32±1.50	20.17
COD (mg/L)	12280	ND[c]	101050
TN (mg/L)	2317±15	ND[c]	21750

a. Values are expressed in mean and standard deviation.
b. The mixed feed for the digestion contains 949.5 g of swine manure and 50.5 g of corn stover.
c. ND (Not determined)

Digestor Performance

Anerobic digestion was run for 80 day. Four hydraulic retention times (HRTs) were run to study the relationship between microbial communities and anaerobic co-digestion performance from the startup to steady state, and further conclude when the steady-state digestion has been achieved. During the first and second HRTs, the daily biogas production was 315±121 and 423±121 ml/g VS loaded/day, respectively, with the daily methane production of 169±65 and 239±69 mL/g VS loaded/day (Figure 2). With significant ($P<0.05$) differences on daily biogas and methane production and large standard deviation (Biogas production were varied) for the first and second HRTs, it is apparent that the co-digestion was not stable in the first 40 days. After the second HRT, daily biogas production was reduced but steady in the third and fourth HRT where the daily biogas production were 291±74 and 248±71 mL/g VS loaded/day, respectively, with corresponding daily methane production of 168±43 and 153±43 mL/g VS loaded/day. Daily biogas and methane production data clearly demonstrate that the 3rd and 4th HRTs are stable, which indicates that the steady-state was achieved after the 2nd HRT.



A B

Figure 2. Biogas production during the semi-continuous AD. A. Daily biogas production; B. Daily methane production

Similar with biogas and methane production, total solids (TS) and volatile solids (VS) and their removal were significantly ($P<0.05$) influenced by digestion time (Figure 2). The first HRT had the highest VS and TS in the effluent (3.5±0.5% and 4.5±0.7%, respectively) along with the lowest VS and TS removal (36.6±8.8% and 34.5±9.7%, respectively). With further increase of HRTs, VS and TS in the effluent were reduced and corresponding removal were increased. They stabilized in the 3rd and 4th HRTs (Figure 3). The average VS and TS in the effluents were 1.5% and 2.1% in the 3rd and 4th HRTs, and corresponding VS and TS removal were averaged at 73.5% and 69.5%. The result of TS and VS removal further verified that the co-digestion stabilized in the 3rd and 4th HRTs.

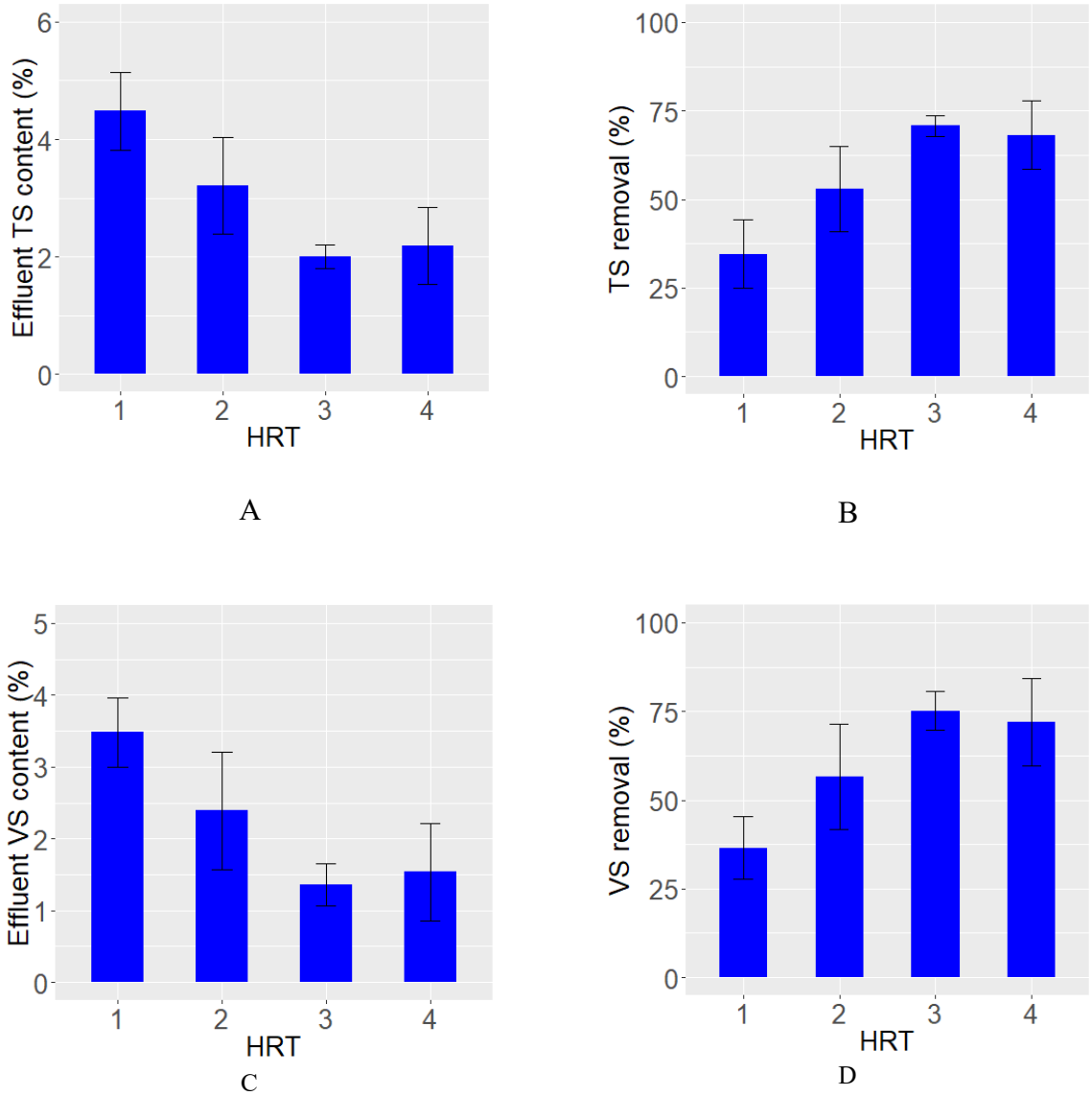


Figure 3. Solids characteristics during AD. A. Effluent TS content; B. TS removal; C. Effluent VS content; D. VS removal

Changes of chemical oxygen demand (COD), total phosphorus (TP), and total nitrogen (TN) during the co-digestion are presented in Figures 4A and 4B. COD and COD removal were significantly ($P<0.05$) affected by HRT. With increase of the digestion time, COD concentration continuously reduced, and COD removal was enhanced. COD concentration and COD removal of the co-digestion in the 4th HRT were 28,910±4,004 mg/L and 71.4±4.0%, respectively, which were significantly ($P<0.05$) lower than (36,163±4,269 mg/L and 64.2±4.2%,) in the 1st HRT. As

for TP and TN, (Figures 4C and 4D). TP and TN concentrations in the 4th HRT were 348±25 and 1,496±357 mg/L, respectively, which were significantly (P<0.05) different from them (719±95 and 1,665±440 mg/L) in the 1st HRT. Changes of TP and TN concentrations between the 3rd and 4th HRTs were much less than them between the 1st and 4th HRTs. The results of COD, TN, and TP also confirmed that the digestion was in the steady state during the 3rd and 4th HRTs.

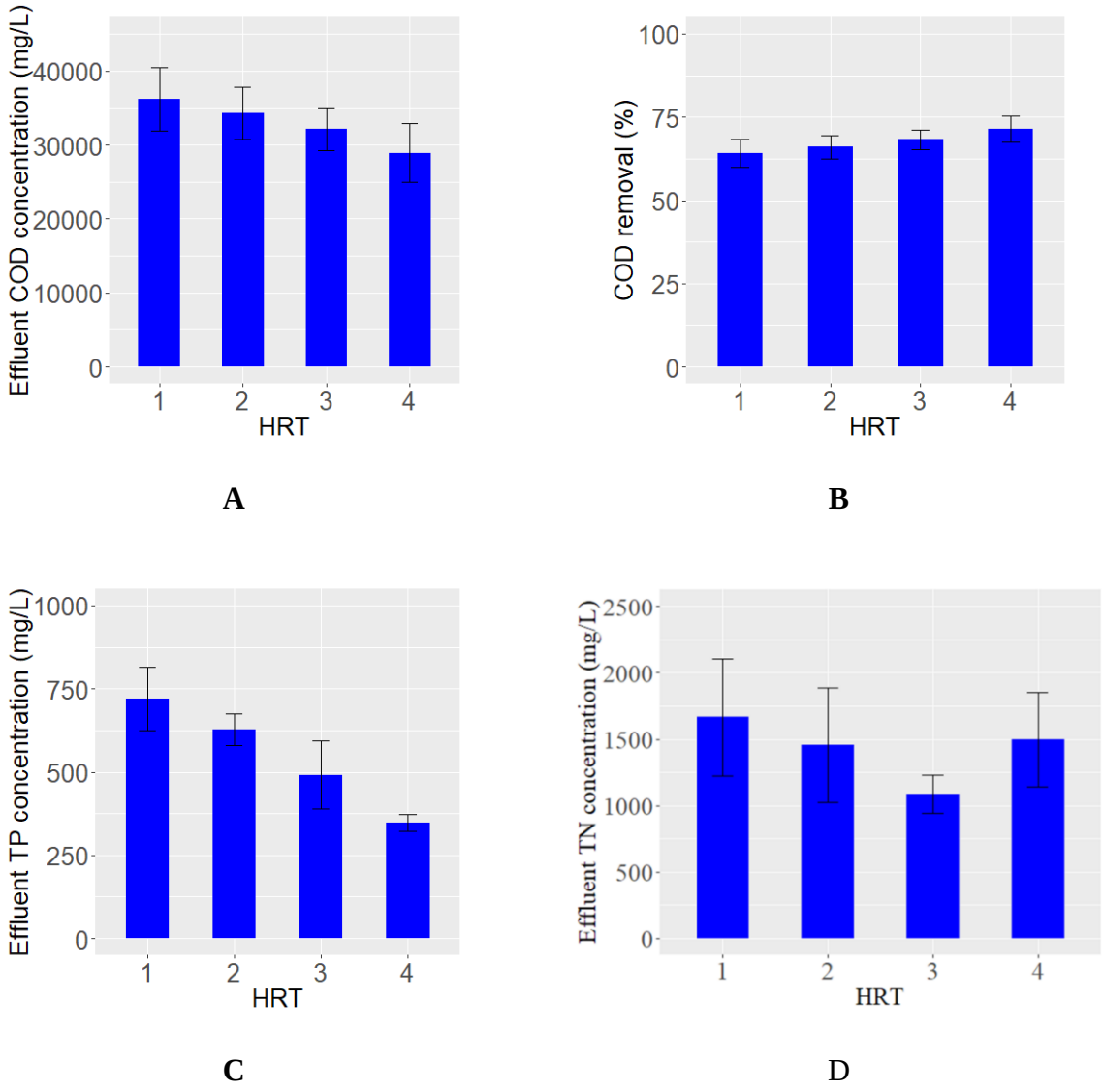


Figure 4. Nutrient concentrations during AD A. Effluent COD concentration; B. COD removal; C. Effluent TP concentration; D. Effluent TN concentration

Fiber Composition

Changes of cellulose and xylan concentrations in the effluent and corresponding removal (Figure 5) were similar with changes of TS and VS (Figure 1). Cellulose and xylan concentrations in the 4th HRT (3.4±0.1% and 3.1±0.1%, respectively) were significantly ($P<0.05$) different from them in the 1st HRT (5.9±0.8 and 5.3±0.5%) (Figures 4a and 4c). Cellulose and xylan removal in the 4th HRT (78.2±0.4 and 74.1±0.8%, respectively) were also significantly ($P<0.05$) higher than them in the 1st HRT (61.6±5.3 and 56.3±4.5%) (Figures 4b and 4d). There were no significant ($P>0.05$) differences on cellulose/xylan concentrations and removal between the 3rd HRT and 4th HRT where the co-digestion reached steady state.

Microbial Analysis

To monitor the changes of microbial community during the digestion, the amplicon sequencing was run on samples from individual HRTs. The sequencing results show that 16S rRNA gene sequences in a sample were between 3,809 and 7,167. The sequences were rarified at 7,000 reads (Figure 6A). The numbers of sequenced microbial species stabilized after sampling 3,000 sequences for all samples (Figure 6A). The rank abundance shows a richness of 250 species (Figure 6B). Statistical analysis on alpha-diversity and evenness of microbial communities shows that digestion time (HRT) had no significant ($P>0.05$) influence on Shannon diversity index (*H*) and Pielou evenness (*J*) (Table 3). This means that the total number of microbial species in microbial community was not significantly ($P>0.05$) varied between different HRTs and feed. A dendrogram was then generated to compare microbial community similarity (beta-diversity) between feed and different HRTs (Figure 7). The first and second separation of clades show a clear sign of community shift between feed and different HRTs. The samples from the 3rd and 4th HRTs are similar each other, and different from the feed and other

HRTs. The result further indicates that the steady state digestion was realized under the 3rd and 4th HRTs.

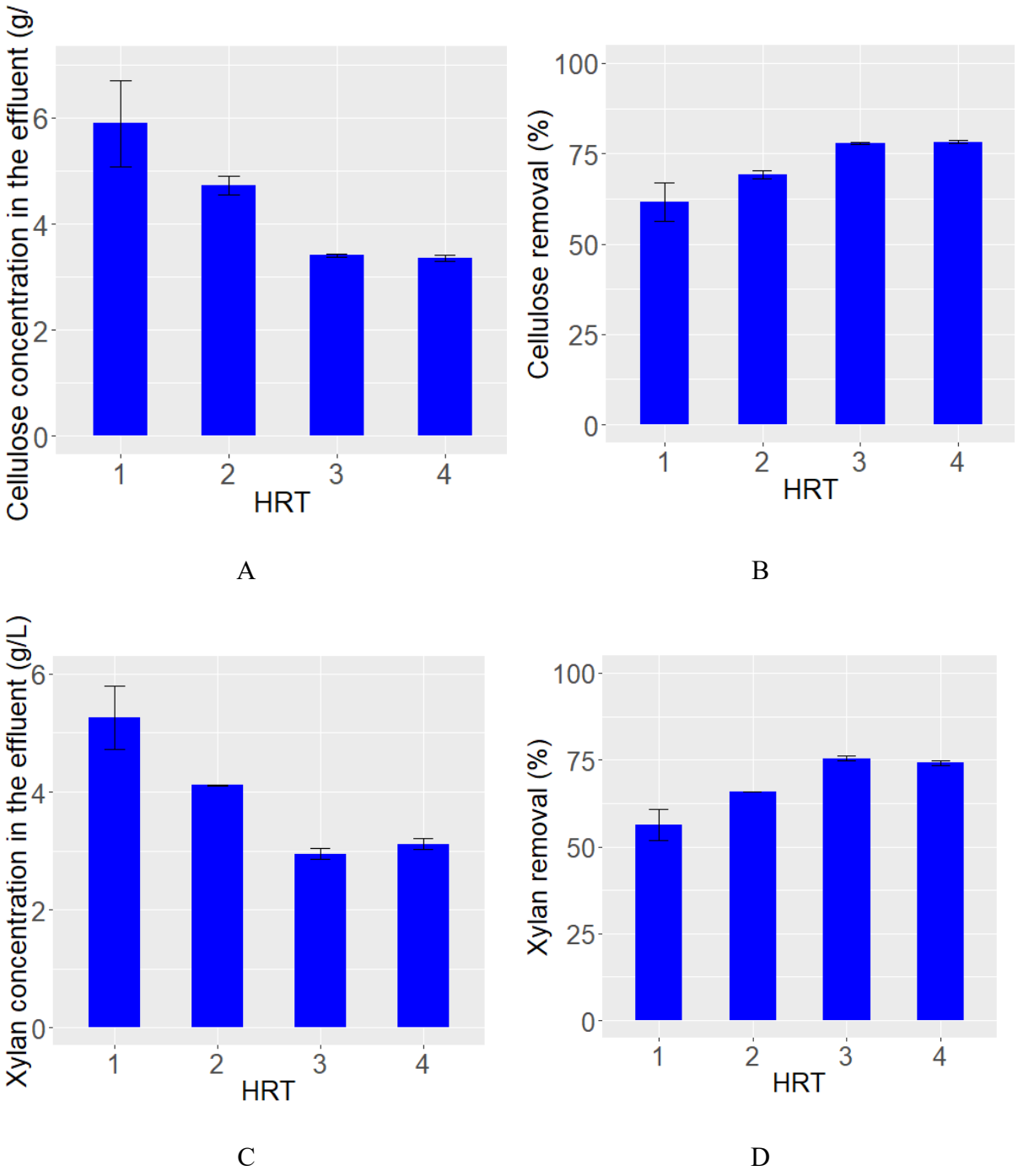
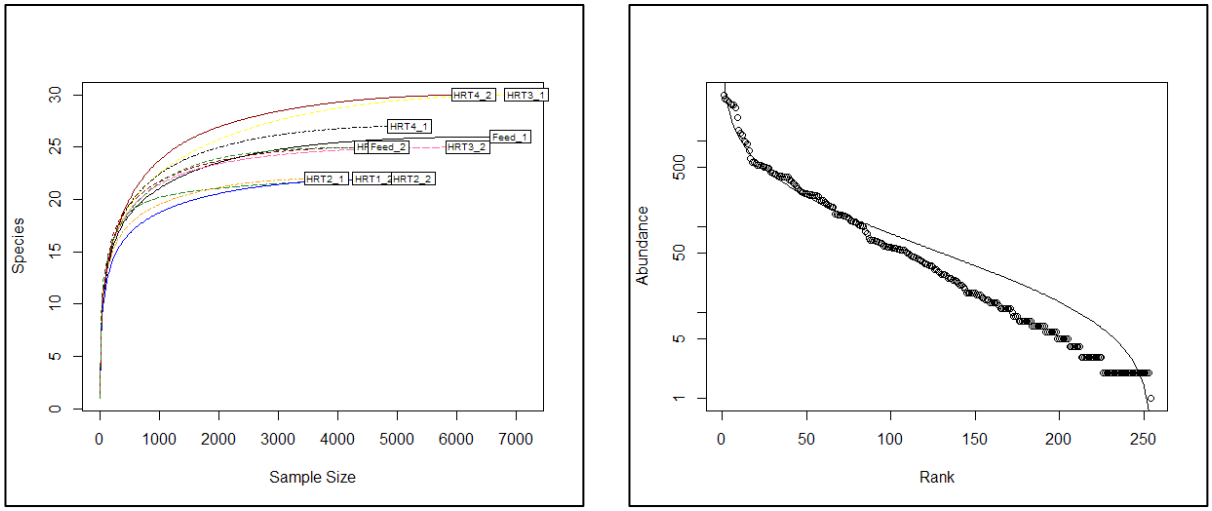


Figure 5. Fiber composition during the semi-continuous AD. A. Cellulose concentration in effluent; B. Cellulose removal; C. Xylan concentration in effluent; D. Xylan removal



A B

Figure 6. Rarefaction and rank abundance of microbial community. A. Rarefaction; B. Rank abundance

Table 3. Diversity of microbial community

HRT	H	J
Feed	2.04±0.11	**0.63±0.04**
1	1.75±0.02	0.55±0.01
2	1.73±0.07	0.56±0.02
3	1.93±0.03	0.58±0.02
4	1.84±0.03	0.55±0.00

[a] H: Shannon's index which indicates the diversity of the microbial community.
[b] J: Pielou's index which indicates the evenness of the microbial community.

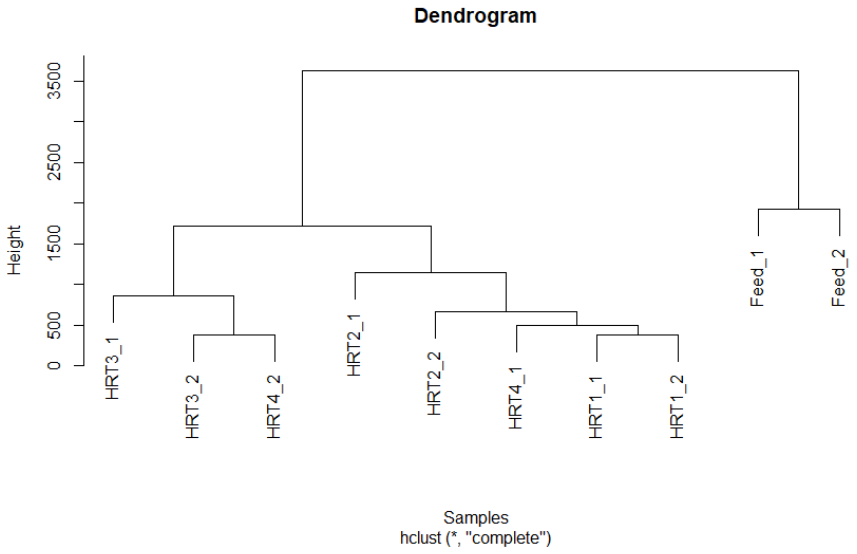


Figure 7. Dendrogram of microbial communities of all samples

A total of 58 microbial species were identified in the digestion samples (Table 8). Bacterial population (85.1-92.8% of the total community) was more abundant than archaeal population (7.2-14.9% of the total community) (Figure 8A). Archaea abundance in the 1st HRT was low at 7.2%, gradually increased to 14.4% in the 3rd HRT, and maintained at 14.9% in the 4th HRT. The microbial community analysis further elucidated that unclassified *Bacteria*, *Bacteriodetes*, *Firmicutes*, and *Euryarcheota* are four dominant phyla in all samples. The most abundant phylum is unclassified *Bacteria* with an average abundance of 50.6% followed by *Bacteriodetes*, *Firmicutes*, and *Euryarcheota* (17.5, 17.1, and 11.9%, respectively) (Figure 8B). The abundance data of microbial community show that HRT has significant ($P<0.05$) influences on distribution of the dominant phyla. Mixing of swine manure with corn stover significantly ($P<0.05$) increased abundance of unclassified *bacteria* in the digestion, which is different from the co-digestion systems using dairy manure as a base feed of co-digestion[21], [68].

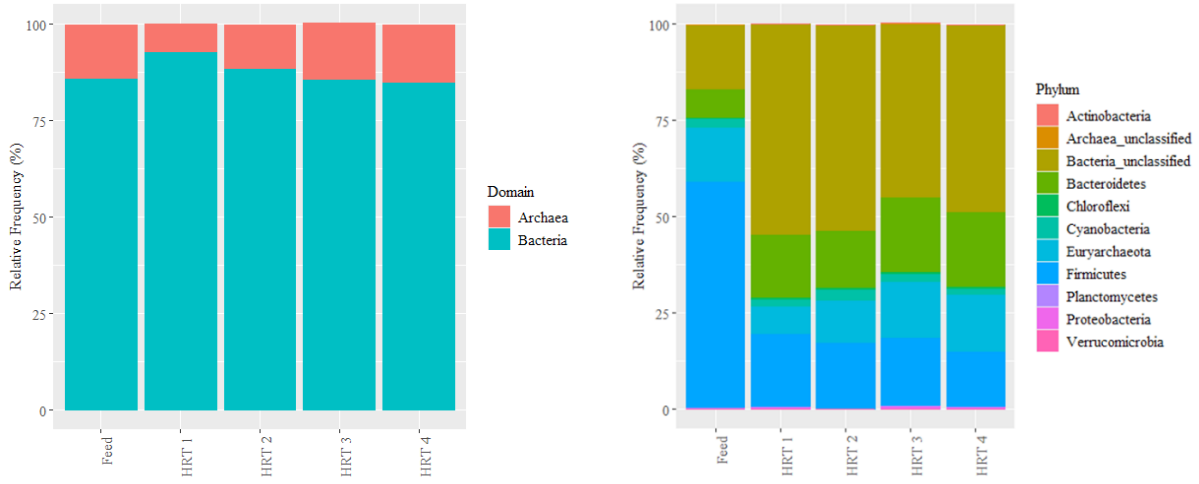


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Figure 8. Changes of microbial community during the semi-continuous AD. A. Abundance at domain level; B. Abundance at phylum level

The phylum *Bacteroidetes* as the second largest microbial group in the digestion are widely existed in many different habitats (i.e., soil and aquatic environment, guts of humans and animals)[69]. Unclassified *Bacteroidales*, Unclassified *Bacteoroidetes* and *Porphyromonadaceae* are three predominant families in the phylum *Bacteroidetes* (Figure 7A). The abundance of Bacteroidetes were gradually increased with the increase of HRTs. It has been reported that *Bacteroidetes* have diverse genes to preferably break down easily hydrolysable carbohydrates (hemicellulose, starch, and sugars) under different environmental conditions[70]–[72]. The result of this study was consistent with the reports. The abundance of *Bacteroidetes* during the digestion were significantly ($P<0.05$) increased compared to the seed (7.3%) (Figure 9A). Supplement of corn stover introduced carbohydrates including an easily degradable xylan into the digestion, which stimulated the accumulation of *Bacteroidetes*. Therefore, the abundances of *Bacteroidetes* peaked in the 3rd HRT (19.2%) and maintained at the high abundance level in the 4th HRT (19.2%) during the steady state digestion.

The phylum *Firmicutes*, the third largest microbial group in the digestion, include species from the families of *Clostridiaceae,* unclassified *Clostridiales,* unclassified *Firmicutes, Lachnospiraceae,* unclassified *Lactobacillales, Peptostreptococcaeae, Ruminococcaceae,* and *Thermoanaerobacteraceae* (Figure 9B). Among them, *Clostridiaceae* and *Ruminococcaceae* are the predominant families. Species in phylum *Firmicutes* can utilize carbohydrates as carbon and energy sources to produce hydrogen and VFAs (acetate and butyrate) [39], [73], [74]. It has been reported that *Clostridiaceae* and *Ruminococcaceae* both contain several key enzymes and sugar transporter proteins to utilize carbohydrates, however, *Ruminococcaceae* are more specialized in the degradation of complex plant materials such as cellulose and hemicellulose[75]. This might be the reason that abundance of *Clostridiaceae* in the digestion (6.8, 7.0, 4.8, and 2.9% for the

1st, 2nd, 3rd, and 4th HRTs) was significantly (P<0.05) less than the seed (35.9%). While, *Ruminococcaceae* were accumulated in the digestion and became the most abundant *Firmicutes* family (6.0, 6.0, 7.7, and 7.0 for the 1st, 2nd, 3rd, and 4th HRTs). Overall, Firmicutes were relatively stable over the entire digestion.

The phylum *Euryarchaeota* was the only archaeal phylum in all AD reactors (Figure 9C). Species from this group are metabolically versatile, meaning that they can produce CH_4 through all three metabolic pathways of aceticlastic pathway using acetate as substrate, methylotrophic pathway using methanol as the substrate, and hydrogenotrophic pathway using carbon dioxide and hydrogen as the substrates [76]. The data indicates that abundance of *Euryarchaeota* was gradually increased with the increase of HRT. It reached 14.4% in the 3rd HRT and kept at 14.7% in the 4th HRT. *Methanosarcinaceae* was the predominant family in the phylum *Euryarchaeota* during the digestion. The abundances of *Methanosarcinaceae* in the 1st, 2nd, 3rd, and 4th HRTs were 7.2, 11.1, 14.4, and 14.7%, respectively. *Methanosarcina* is a group of archaea that uses the aceticlastic pathway to generate methane [77], which means that mixing swine manure with corn stover promoted aceticlastic pathway as the main methane production route during the digestion.

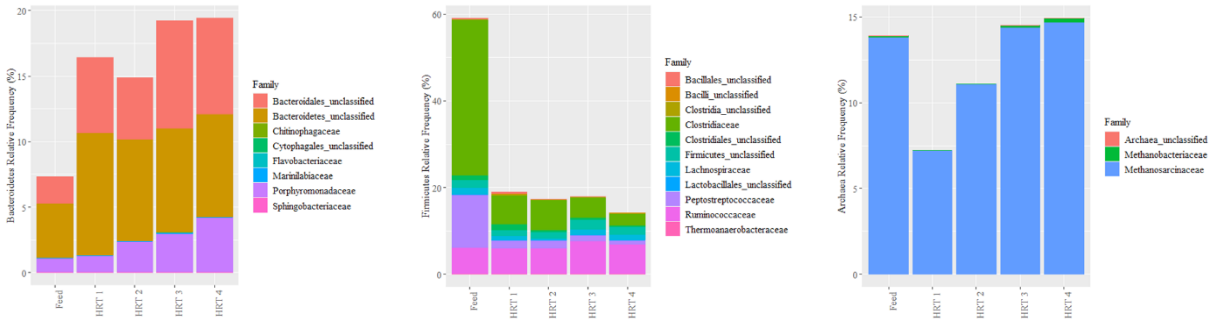

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Figure 9. Changes of microbial community during the semi-continuous AD. A. Bacteroidetes abundance at family level; B. Firmicutes abundance at family level; C. Achaea abundance at family level

The digestion performance and microbial community analyses show that anaerobic microbial communities dynamically adjust their demographics (diversity and abundance of populations) in response to the mixed feed. Mixing carbohydrate-rich residue – corn stover and nitrogen-rich waste – swine manure enables a preferred digestion to simultaneously improve the digestion performance of biogas production and generate solid digestate rich in carbohydrates (cellulose and xylan)[60]. The solid digestate containing relatively high cellulose and xylan (Table 4) can then be used as a feedstock by biorefineries to produce value-added chemicals. Lactic acid and chitin (glucosamine) as the target products of this study are discussed in the following sections.

Ball Mill

The mechano-biocatalytic one pot process developed from a previous paper [22] was modified by this study to carry out mono-sugar release form the solid digestate. Characteristics of solid digestate (washed and unwashed) are listed in Table 4. Fiber composition of the solid digestate remains relatively unchanged between the washed and unwashed solid digestate. It has been reported that washed fiber has much lower total nitrogen content [78], which was verified by the TN content in the hydrolysate (Table 5). Compared to the solid digestate used in the previous paper [22], the digestate had lower contents of cellulose (21.6 and 22.7% for unwashed and washed solid digestate) and lignin (24.7 and 28.1% for unwashed and washed solid digestate) but higher xylan contents (19.6 and 22.5% for unwashed and washed solid digestate). The mechano-biocatalytic process yielded 32.90, 21.35, and 4.06 g/l of glucose, xylose, and acetic acid, respectively, from the unwashed solid digestate. The corresponding cellulose and xylan conversions are 92 and 82%, respectively. As for the washed solid digestate, the mechano-biocatalysis generated the hydrolysate with 36.27, 17.30 and 4.84 g/l of glucose, xylose, and

acetic, respectively. The cellulose and xylan conversions are 97 and 62%, respectively. The results show that both washed and unwashed solid digestates had higher cellulose conversion. Washing the solid digestate increases cellulose conversion but decreases xylan conversion. It may be due to that some amorphous xylan in the solid digestate was removed during the washing process. The remaining xylan in the washed digestate was embedded into cellulose and lignin, and difficult to be hydrolyzed. In addition, it was observed that washed digestate had less chance to be contaminated during the mechano-biocatalysis. The hydrolysates from the mechano-biocatalysis of both original solid digestate and washed solid digestate were prepared for fungal fermentation.

Table 4. Fiber composition of solid digestate

Parameter	Solid digestate	Washed solid digestate
TS (%)	19.1%	18.8%
Cellulose (%TS)	21.57±0.07	22.69±0.06
Xylan (%TS)	19.57±0.07	22.50±0.37
Lignin (%TS)	24.74±0.33	28.08±0.69

a. Data are the average of two replicates with standard deviation.

Table 5.Composition of hydrolysates from one-pot hydrolysis of solid digestates

Parameter	Hydrolysate from the solid digestate	Hydrolysate from the washed solid digestate
Glucose (g/L)	32.90	36.27
Xylose (g/L)	21.35	17.30
Acetic acid (g/L)	4.06	4.84
TN (g/L)	3.07	2.73

a. Data are the average of two replicates with standard deviation.

Fermentation and Chitin Production

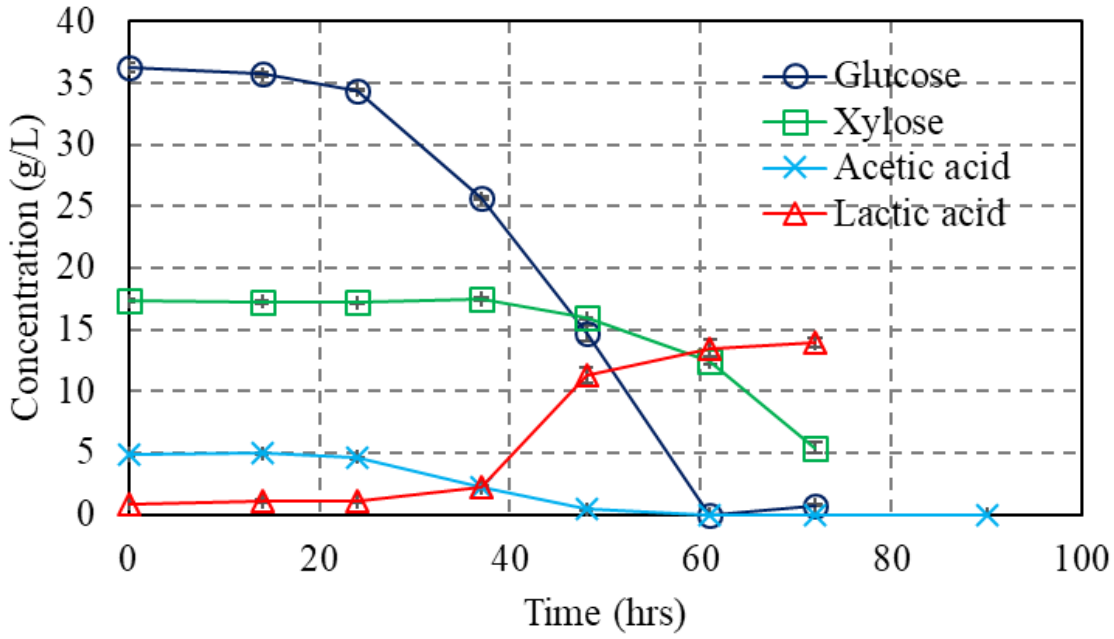
The kinetics of fungal fermentation are presented in Figure 10. Glucose and acetic acid in both hydrolysates were completely consumed by the end of the fermentation. The final concentration of lactic acid and chitin can be seen in Table 6. There was no lactic acid produced from the hydrolysate of original solid digestate during a 72 hours fermentation, however, the

fermentation accumulated fungal biomass with a chitin content of 24% TS. The fermentation on the hydrolysate of washed solid digestate produced 14.2 g/L of lactic acid and 18.4% TS of chitin in the biomass. The chitin content from the fermentation of the hydrolysate from the washed solid digestate was lower than the fermentation of the hydrolysate from the original solid digestate. In both fermentations, glucose and acetic acid are the preferred carbon source by *R. oryzae*, and xylose consumption started after 40 hours. The fungal strain cannot consume all xylose during the fermentation. Approximately 5 g/L of xylose was left in the media for both fermentations. No lactic acid production from the fermentation of the hydrolysate from the original solid digestate may be attributed to a slightly higher nitrogen content of the solid digestate. It has been reported that using the high nitrogen media led *R. oryzae* to accumulation of biomass rather than lactic acid production[79]. The washing process may have lowered the total nitrogen to a level that is favorable for the fungus to simultaneously produce both lactic acid and chitin. In addition, the fungal lactic acid and chitin production from the washed solid digestate had similar conversion of lactic acid and chitin once comparing with a report that the same strain was used to convert potato starch into lactic acid and chitin [80]. The study used a potato hydrolysate medium containing 100 g/L of glucose and produced 30 g/L of lactic acid and 0.25 g chitin/g dry biomass. This comparison further elucidates that the hydrolysate of washed solid digestate provides a suitable medium to enable *Rhizopus oryzae* to simultaneously produce lactic acid and accumulate chitin in its biomass.

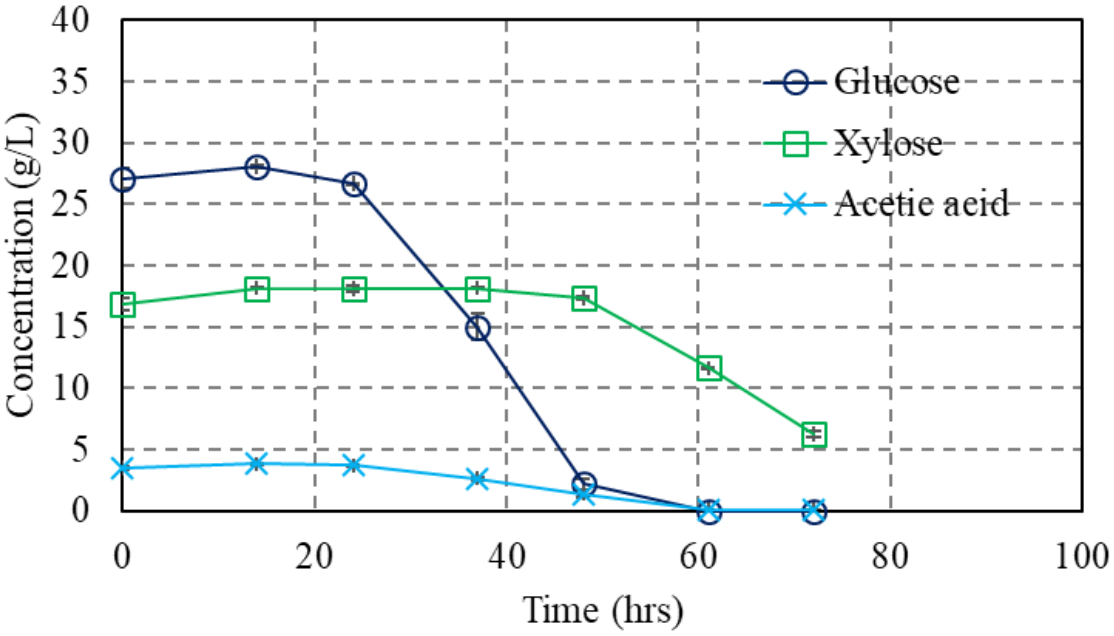
Table 6. Lactic acid and chitin production

Parameter	Hydrolysate from the solid digestate	Hydrolysate from the washed solid digestate
Lactic acid (g/L)	0	14.23 ± 0.52
Chitin (w/w)	24.6± 0.07	20.07 ± 0.04

a. Data are the average of two replicates with standard deviation.



A



B

Figure 10. Rhizopus oryzae fermentation of hydrolysate of solid digestates. A. Hydrolysate of washed solid digestate; B. Hydrolysate of original solid digestate

Mass and Energy Balance

A mass and energy balance for the propose process was conducted based on a biorefinery with a capacity of 1,000 kg dry feedstock per day (Figure 11). 13,602 kg of water in the feed is from the moisture content of swine manure. The anaerobic co-digestion produces 199 m^3 of biogas containing 123 m^3 of methane. The co-digestion also generates 13,201 kg of liquid digestate with a total solid of 0.6% and 1,238 kg of wet solid digestate with a total solids of 18.8%. The liquid digestate could be used as liquid fertilizer. The solid digestate along with 314 kg of water and enzyme were hydrolyzed by a mechano-biocatalytic one-pot process to generate 1,552 kg of liquid hydrolysate. The hydrolysate contains 57 kg of glucose, 27 kg of xylose and 8 kg of acetic acid. Fungal fermentation of the hydrolysate produces 22 kg of lactic acid and 185 kg of dry fermentation residues containing 34 kg of chitin. The energy balance is presented in Table 7. Anaerobic digestion requires an energy input of 1.6 MJ/kg dry feedstock to maintain the digestion temperature and other operational energy demands. Anaerobic co-digestion also generates 4.4 MJ/kg dry feedstock from methane utilization. One-pot hydrolysis and fermentation require 0.5 and 0.09 MJ/kg dry feedstock to convert solid digestate into sugars and ferment them for lactic acid and chitin production. The energy balance shows that integration of three-unit operations can enable an energy positive process with an overall net energy of 2.2 MJ/kg dry feedstock.

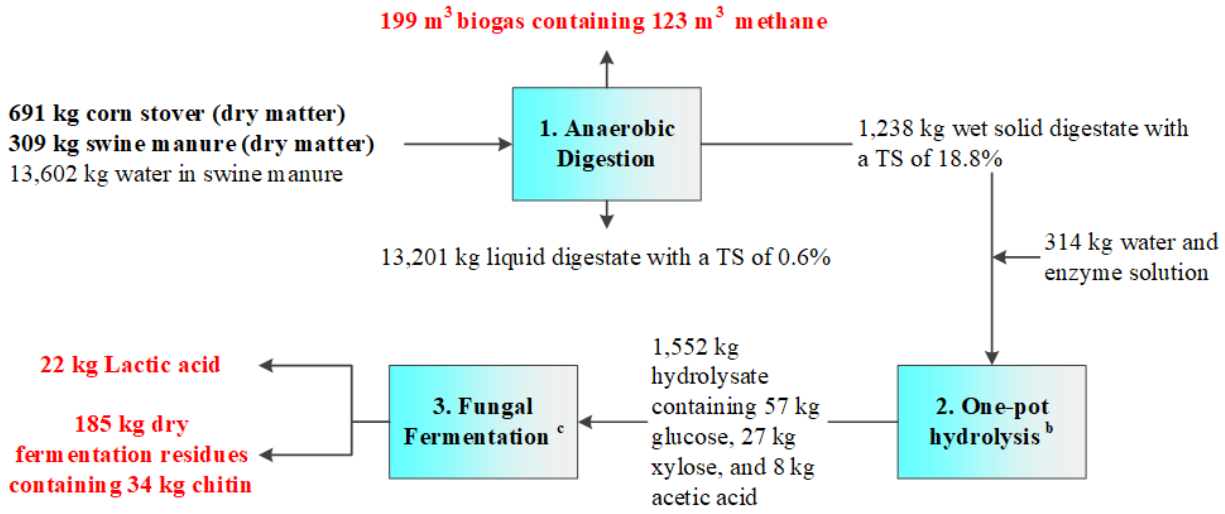


Figure 11. Mass balance flow of the integrates system based on a system processing 1,000 kg dry feedstock per day

Table 7. Energy balance of the integrated system

Energy Balance	AD [c]	One-pot hydrolysis [d]	Fungal fermentation [e]
Energy input (MJ/kg dry feedstock)	-1.6	-0.5	-0.09
Energy output (MJ/kg dry feedstock)	4.4	-	-
Net energy (MJ/kg dry feedstock)	2.8	-0.5	-0.09
Overall net energy (MJ/kg dry feedstock)	**2.2**		

a. All inputs are assigned "-", and all outputs are assigned "+".
b. Data were calculated and adjusted based on 1 kg dry feedstock.
c. Total amount of feed to the proposed process is 1 kg dry matter. The TS in the mixed feed is 6.85%. The methane gas of 123 L is produced from the AD of the mixed feed. The low heating value of methane of 35.8 KJ/L methane. The specific heat is 4.2 kJ/kg°C. A year-round average temperature of feed is assumed to be 15ºC. The operational temperature for the AD is 35ºC. Besides the heat for maintaining the temperature of the AD, ten percent of energy in methane is set as the parasitic energy to power the equipment for the AD.
d. The electricity consumption of the one-pot process is 0.56 kWh-e/kg dry solid digestate. 1 kg dry mixed feedstock produces 0.233 kg dry solid digestate [22].
e. The fungal fermentation conditions are 72 hours of the culture time, 200 rpm of the agitation, 0.5 vvm of the aeration, and 30ºC of the culture temperature. The energy demand of fungal fermentation is 0.06 MJ/kg fermentation broth[65].

CHAPTER 3. CONCLUSIONS AND FUTURE WORK

CONCLUSIONS

The chemical production industry currently is heavily reliant on fossil fuels and energy-intensive processes. This means that that chemical production has a big impact on the environment, so new technologies deriving chemicals from cleaner sources are needed. A largely untapped source is biomass from plants. To address these problems a new mechano-biocatalytic process was designed. The feedstocks are corn stover and swine manure. These feedstocks undergo, anaerobic digestion, ball mill and fungal fermentation. After these steps lactic acid and chitin (a precursor to glucosamine) are produced. Therefore, this study will test all three components of this system to determine feasibility of this new conversion process.

This study shows the proposed process is indeed feasible. Anaerobic digestion was shown to reach steady-state condition by the end of four HRT's. It was shown that digestion time does have a significant effect on biogas production, fiber composition and microbial analysis. The microbial community was shown to significantly change throughout the process. An example of this change can be seen in Figure 9C, as digestion time increased so did the relative frequency of *Methanosarcinaceae*. Cellulose and xylan removal were also shown to increase during the experiment. Despite this high removal of carbohydrates, the ball mill and enzymatic hydrolysis were still able to reach high titer and conversion. Fungal fermentation by *Rhizopus oryzae* was shown to produce significant concentrations of lactic acid when using washed solid digestate. However, it was unable to produce lactic acid using unwashed solid digestate. Using the solid fermentation residues, it was determined that chitin reached concentrations of 20.07 g/L, meaning glucosamine production is feasible. Finally, mass and energy analysis showed this process has the capabilities of being an energy-positive process with overall net energy gain of

2.2 MJ/kg dry feedstock. These results elucidate that the proposed process is a feasible and productive process that warrants further investigation.

FUTURE WORK

The proposed process is still very much in its infancy. Overall, more work needs to be done to fully understand the dynamics of the process. The effect of anaerobic digestion on the recalcitrant structure of the biomass needs to be better understood. Also, effects of increased concentrations of biomass in anaerobic digestion need to be studied. For fermentation the nutrient characteristics of the hydrolysate need to be further analyzed to determine the effect it has on fermentation. Also, a full life cycle analysis needs to be performed to fully understand its effects on the environment. Finally, more work needs to be done to understand the challenges of scaling up this process to an industrial scale. If higher total solids in the mechano-biocatalytic can be achieved higher concentrations of glucose can be achieve, leading to higher concentrations of products. Also, more work to improve the conversion efficiency of the process. Research should also be conducted investigating fermentation using different organisms. Different products could be produced to further increase the profit margins of this process. Alternative chemicals include, shikimic acid, muconic acid and vanillin. This future work may also be applied to other environmentally friendly chemical production processes in the continuing pursuit of a cleaner chemical production industry.

APPENDIX

TABLES AND FIGURES

Table 8. Taxonomy of Anaerobic Digestion

row.names	Domain	Phylum	Class	Order	Family	Genus	Species
Frequency1	Archaea	Archaea_unclassified	Archaea unclassified	Archaea_unclassified	Archaea_unclassified	Archaea_unclassified	Archaea_unclassified
Frequency2	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium	beijingense
Frequency3	Archaea	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcinaceae_unclassified	Methanosarcinaceae_unclassified
Frequency4	Bacteria	Bacteria_unclassified	Bacteria unclassified	Bacteria_unclassified	Bacteria_unclassified	Bacteria_unclassified	Bacteria_unclassified
Frequency5	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetales_unclassified	Actinomycetales_unclassified	Actinomycetales_unclassified
Frequency6	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	Streptomycetaceae_unclassified	Streptomycetaceae_unclassified
Frequency7	Bacteria	Bacteroidetes	Bacteroidetes unclassified	Bacteroidetes_unclassified	Bacteroidetes_unclassified	Bacteroidetes_unclassified	Bacteroidetes_unclassified
Frequency8	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidales_unclassified	Bacteroidales_unclassified	Bacteroidales_unclassified
Frequency9	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Marinilabiaceae	Ruminofilibacter	xylanolyticum
Frequency10	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Porphyromonadaceae_unclassified	Porphyromonadaceae_unclassified
Frequency11	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Petrimonas	sulfuriphila
Frequency12	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagales_unclassified	Cytophagales_unclassified	Cytophagales_unclassified
Frequency13	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacteriaceae_unclassified	Flavobacteriaceae_unclassified
Frequency14	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacteriaceae_unclassified	Sphingobacteriaceae_unclassified
Frequency15	Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Chitinophagaceae_unclassified	Chitinophagaceae_unclassified
Frequency16	Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	Anaerolinaceae_unclassified	Anaerolinaceae_unclassified
Frequency17	Bacteria	Cyanobacteria	Cyanobacteria unclassified	Cyanobacteria_unclassified	Cyanobacteria_unclassified	Cyanobacteria_unclassified	Cyanobacteria_unclassified
Frequency18	Bacteria	Cyanobacteria	Chloroplast	Chlorophyta	Chlorophyta_unclassified	Chlorophyta_unclassified	Chlorophyta_unclassified
Frequency19	Bacteria	Firmicutes	Firmicutes unclassified	Firmicutes_unclassified	Firmicutes_unclassified	Firmicutes_unclassified	Firmicutes_unclassified

Table 9 Continued

Frequency20	Bacteria	Firmicutes	Bacilli	Bacilli_unclassified	Bacilli_unclassified	Bacilli_unclassified	Bacilli_unclassified
Frequency21	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillales_unclassified	Bacillales_unclassified	Bacillales_unclassified
Frequency22	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillales_unclassified	Lactobacillales_unclassified	Lactobacillales_unclassified
Frequency23	Bacteria	Firmicutes	Clostridia	Clostridia_unclassified	Clostridia_unclassified	Clostridia_unclassified	Clostridia_unclassified
Frequency24	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiales_unclassified	Clostridiales_unclassified	Clostridiales_unclassified
Frequency25	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	Clostridium_unclassified
Frequency26	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospiraceae_unclassified	Lachnospiraceae_unclassified
Frequency27	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Defluviitalea	saccharophila
Frequency28	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcaceae_unclassified	Peptostreptococcaceae_unclassified
Frequency29	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Clostridium	ruminantium
Frequency30	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcaceae_unclassified	Ruminococcaceae_unclassified
Frequency31	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Clostridium	Clostridium_unclassified
Frequency32	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Sporobacter	termitidis
Frequency33	Bacteria	Firmicutes	Clostridia	Thermoanaerobacterales	Thermoanaerobacteraceae	Thermoanaerobacteraceae_unclassified	Thermoanaerobacteraceae_unclassified
Frequency34	Bacteria	Planctomycetes	Planctomycetia	Planctomycetia_unclassified	Planctomycetia_unclassified	Planctomycetia_unclassified	Planctomycetia_unclassified
Frequency35	Bacteria	Proteobacteria	Proteobacteria unclassified	Proteobacteria_unclassified	Proteobacteria_unclassified	Proteobacteria_unclassified	Proteobacteria_unclassified
Frequency36	Bacteria	Proteobacteria	Alphaproteobacteria	Alphaproteobacteria_unclassified	Alphaproteobacteria_unclassified	Alphaproteobacteria_unclassified	Alphaproteobacteria_unclassified
Frequency37	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacteraceae_unclassified	Caulobacteraceae_unclassified
Frequency38	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	Brevundimonas_unclassified
Frequency39	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales_unclassified	Rhizobiales_unclassified	Rhizobiales_unclassified
Frequency40	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobiaceae_unclassified	Hyphomicrobiaceae_unclassified

Table 10 Continued

Frequency41	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacteriaceae_unclassified	Methylobacteriaceae_unclassified
Frequency42	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Phyllobacteriaceae_unclassified	Phyllobacteriaceae_unclassified
Frequency43	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	Xanthobacter	Xanthobacter_unclassified
Frequency44	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacteraceae_unclassified	Rhodobacteraceae_unclassified
Frequency45	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillales_unclassified	Rhodospirillales_unclassified	Rhodospirillales_unclassified
Frequency46	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acetobacteraceae_unclassified	Acetobacteraceae_unclassified
Frequency47	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Rhodospirillaceae_unclassified	Rhodospirillaceae_unclassified
Frequency48	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadales_unclassified	Sphingomonadales_unclassified	Sphingomonadales_unclassified
Frequency49	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonadaceae_unclassified	Sphingomonadaceae_unclassified
Frequency50	Bacteria	Proteobacteria	Betaproteobacteria	Betaproteobacteria unclassified	Betaproteobacteria_unclassified	Betaproteobacteria_unclassified	Betaproteobacteria_unclassified
Frequency51	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_unclassified	Burkholderiales_unclassified	Burkholderiales_unclassified
Frequency52	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Polyangiaceae_unclassified	Polyangiaceae_unclassified
Frequency53	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	Helicobacter	Helicobacter_unclassified
Frequency54	Bacteria	Proteobacteria	Gammaproteobacteria	Gammaproteobacteria unclassified	Gammaproteobacteria_unclassified	Gammaproteobacteria_unclassified	Gammaproteobacteria_unclassified
Frequency55	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacteriaceae_unclassified	Enterobacteriaceae_unclassified
Frequency56	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Xanthomonadaceae_unclassified	Xanthomonadaceae_unclassified
Frequency57	Bacteria	Verrucomicrobia	Verrucomicrobia_unclassified	Verrucomicrobia_unclassified	Verrucomicrobia_unclassified	Verrucomicrobia_unclassified	Verrucomicrobia_unclassified
Frequency58	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Verrucomicrobium	spinosum

ANAEROBIC DIGESTION STATISTICAL CODE

Biogas

```
## Statistical analysis
## Performance data of anaerobic digestion
## Wei Liao, October 30, 2020

# Loading Library and Tables ----------------------------------------------

library (MASS)
library(ggplot2)
library(grid)
library(gridExtra)
library(ggpubr)

# Installing the font package ---------------------------------------------
library(extrafont)
font_import() #It may take a few minutes to import.
loadfonts(device="win")

# PROGRAM TO PLOT BAR CHART WITH STANDARD DEVIATION ----------------------

#+++++++++++++++++++++++++
# Function to calculate the mean and the standard deviation
# for each group
#+++++++++++++++++++++++++
# data : a data frame
# varname : the name of a column containing the variable
#to be summariezed
# groupnames : vector of column names to be used as
# grouping variables
data_summary <- function(data, varname, groupnames){
  require(plyr)
  summary_func <- function(x, col){
    c(mean = mean(x[[col]], na.rm=TRUE),
      sd = sd(x[[col]], na.rm=TRUE))
  }
  data_sum<-ddply(data, groupnames, .fun=summary_func,
                  varname)
  data_sum <- rename(data_sum, c("mean" = varname))
  return(data_sum)
}


# ANALYSIS----------------------------------------------------------------
## the .txt file needs to be saved as the type of "Tab delimited".
```

```
##load biogas.txt
con <-file.choose(new = FALSE)
metadata <- read.table(con, header = T, row.names = 1)

## DEFINING FACTORS

metadata$HRT <- factor(metadata$HRT) ##Factor Statement


# 1. Effects of HRT on biogas production

## One-way ANOVA

# Daily biogas
fit1 <- aov(Daily_biogas~HRT, data = metadata)
summary(fit1)
Tukey1 <- TukeyHSD(fit1, conf.level=0.95) #Tukey multiple comparions
Tukey1
Daily_biogas_data <- data_summary(metadata, varname="Daily_biogas", groupnames="HRT")
Daily_biogas_data

# Daily methane
fit2 <- aov(Daily_methane~HRT, data = metadata)
summary(fit2)
Tukey2 <- TukeyHSD(fit2, conf.level=0.95) #Tukey multiple comparions
Tukey2
Daily_methane_data <- data_summary(metadata, varname="Daily_methane",
groupnames="HRT")
Daily_methane_data


#2. Plot

#Daily biogas production

Daily_biogas_production <- data_summary(metadata, varname="Daily_biogas",
                            groupnames=c("HRT"))

Daily_biogas_production$HRT=as.factor(Daily_biogas_production$HRT)
head(Daily_biogas_production)

box_1 <- ggplot(Daily_biogas_production, aes(x=HRT, y=Daily_biogas, fill=HRT)) +
  geom_bar(stat="identity", position=position_dodge(0.9), width=0.5)+
  geom_errorbar(aes(ymin=Daily_biogas-sd, ymax=Daily_biogas+sd), width=0.2,
position=position_dodge(0.9))+
  xlab("HRT")+
```

```
  ylab("Biogas production (mL/g VS loaded/day)") + ylim(0, 600)  + labs(title = "",
subtitle=NULL) +
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=20, family="Times New Roman"),
        axis.text.y=element_text(size=20, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.position="none")+
  scale_fill_manual(values=c("seagreen3", "red", "blue", "blue"))
box_1


#Daily methane production

Daily_methane_production <- data_summary(metadata, varname="Daily_methane",
                                groupnames=c("HRT"))

Daily_methane_production$HRT=as.factor(Daily_methane_production$HRT)
head(Daily_methane_production)

box_2 <- ggplot(Daily_methane_production, aes(x=HRT, y=Daily_methane, fill=HRT)) +
  geom_bar(stat="identity", position=position_dodge(0.9), width=0.5)+
  geom_errorbar(aes(ymin=Daily_methane-sd, ymax=Daily_methane+sd), width=0.2,
position=position_dodge(0.9))+
  xlab("HRT")+
  ylab("Methane production (mL/g VS loaded/day)") + ylim(0, 350)  + labs(title = "",
subtitle=NULL) +
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=20, family="Times New Roman"),
        axis.text.y=element_text(size=20, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.position="none")+
  scale_fill_manual(values=c("seagreen3", "red", "blue", "blue"))
box_2
```

Chemical Oxygen Demand and Nutrient

```
## Statistical analysis
## Performance data of anaerobic digestion
## Wei Liao, October 30, 2020

# Loading Library and Tables ----------------------------------------------

library (MASS)
library(ggplot2)
library(grid)
```

```
library(gridExtra)
library(ggpubr)

# Installing the font package ------------------------------------------
library(extrafont)
font_import() #It may take a few minutes to import.
loadfonts(device="win")

# PROGRAM TO PLOT BAR CHART WITH STANDARD DEVIATION ----------------------

#++++++++++++++++++++++++++
# Function to calculate the mean and the standard deviation
# for each group
#++++++++++++++++++++++++++
# data : a data frame
# varname : the name of a column containing the variable
#to be summariezed
# groupnames : vector of column names to be used as
# grouping variables
data_summary <- function(data, varname, groupnames){
  require(plyr)
  summary_func <- function(x, col){
    c(mean = mean(x[[col]], na.rm=TRUE),
      sd = sd(x[[col]], na.rm=TRUE))
  }
  data_sum<-ddply(data, groupnames, .fun=summary_func,
                  varname)
  data_sum <- rename(data_sum, c("mean" = varname))
  return(data_sum)
}


# ANALYSIS---------------------------------------------------------------

## the .txt file needs to be saved as the type of "Tab delimited".

##load CODandNutrients.txt
con <-file.choose(new = FALSE)
metadata <- read.table(con, header = T, row.names = 1)

## DEFINING FACTORS

metadata$HRT <- factor(metadata$HRT) ##Factor Statement


# 1. Effects of HRT on TS production
```

```
## One-way ANOVA

# Effluent COD
fit1 <- aov(COD~HRT, data = metadata)
summary(fit1)
Tukey1 <- TukeyHSD(fit1, conf.level=0.95) #Tukey multiple comparions
Tukey1
COD_data <- data_summary(metadata, varname="COD", groupnames="HRT")
COD_data

# Effluent TP
fit2 <- aov(TP~HRT, data = metadata)
summary(fit2)
Tukey2 <- TukeyHSD(fit2, conf.level=0.95) #Tukey multiple comparions
Tukey2
TP_data <- data_summary(metadata, varname="TP", groupnames="HRT")
TP_data

# Effluent TN
fit3 <- aov(TN~HRT, data = metadata)
summary(fit3)
Tukey3 <- TukeyHSD(fit3, conf.level=0.95) #Tukey multiple comparions
Tukey3
TN_data <- data_summary(metadata, varname="TN", groupnames="HRT")
TN_data

# COD removal
fit4 <- aov(COD_removal~HRT, data = metadata)
summary(fit4)
Tukey4 <- TukeyHSD(fit4, conf.level=0.95) #Tukey multiple comparions
Tukey4
COD_data <- data_summary(metadata, varname="COD_removal", groupnames="HRT")
COD_data

#2. Plot

#Effluent COD

COD_content <- data_summary(metadata, varname="COD",
                            groupnames=c("HRT"))

COD_content$HRT=as.factor(COD_content$HRT)
head(COD_content)

box_1 <- ggplot(COD_content, aes(x=HRT, y=COD, fill=HRT)) +
  geom_bar(stat="identity", position=position_dodge(0.9), width=0.5)+
```

```
  geom_errorbar(aes(ymin=COD-sd, ymax=COD+sd), width=0.2,
position=position_dodge(0.9))+
  xlab("HRT")+
  ylab("Effluent COD concentration (mg/L)") + ylim(0, 45000)  + labs(title = "", subtitle=NULL)
+
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=20, family="Times New Roman"),
        axis.text.y=element_text(size=20, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.position="none")+
  scale_fill_manual(values=c("seagreen3", "blue", "blue", "blue"))
box_1

#Effluent TP

TP_content <- data_summary(metadata, varname="TP",
                           groupnames=c("HRT"))

TP_content$HRT=as.factor(TP_content$HRT)
head(TP_content)

box_2 <- ggplot(TP_content, aes(x=HRT, y=TP, fill=HRT)) +
  geom_bar(stat="identity", position=position_dodge(0.9), width=0.5)+
  geom_errorbar(aes(ymin=TP-sd, ymax=TP+sd), width=0.2, position=position_dodge(0.9))+
  xlab("HRT")+
  ylab("Effluent TP concentration (mg/L)") + ylim(0, 1000)  + labs(title = "", subtitle=NULL) +
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=20, family="Times New Roman"),
        axis.text.y=element_text(size=20, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.position="none")+
  scale_fill_manual(values=c("seagreen3", "seagreen3", "red", "blue"))
box_2

#Effluent TN

TN_content <- data_summary(metadata, varname="TN",
                           groupnames=c("HRT"))

TN_content$HRT=as.factor(TN_content$HRT)
head(TN_content)

box_3 <- ggplot(TN_content, aes(x=HRT, y=TN, fill=HRT)) +
  geom_bar(stat="identity", position=position_dodge(0.9), width=0.5)+
```

```
  geom_errorbar(aes(ymin=TN-sd, ymax=TN+sd), width=0.2, position=position_dodge(0.9))+
  xlab("HRT")+
  ylab("Effluent TN concentration (mg/L)") + ylim(0, 2500)  + labs(title = "", subtitle=NULL) +
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=20, family="Times New Roman"),
        axis.text.y=element_text(size=20, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.position="none")+
  scale_fill_manual(values=c("blue", "blue", "blue", "blue"))
box_3

#COD removal

COD_removal_plot <- data_summary(metadata, varname="COD_removal",
                      groupnames=c("HRT"))

COD_removal_plot$HRT=as.factor(COD_removal_plot$HRT)
head(COD_removal_plot)

box_4 <- ggplot(COD_removal_plot, aes(x=HRT, y=COD_removal, fill=HRT)) +
  geom_bar(stat="identity", position=position_dodge(0.9), width=0.5)+
  geom_errorbar(aes(ymin=COD_removal-sd, ymax=COD_removal+sd), width=0.2,
position=position_dodge(0.9))+
  xlab("HRT")+
  ylab("COD removal (%)") + ylim(0, 100)  + labs(title = "", subtitle=NULL) +
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=20, family="Times New Roman"),
        axis.text.y=element_text(size=20, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.position="none")+
  scale_fill_manual(values=c("seagreen3", "blue", "blue", "blue"))
box_4
```

Total Solids

```
## Statistical analysis
## Performance data of anaerobic digestion
## Wei Liao, October 30, 2020

# Loading Library and Tables ----------------------------------------------

library (MASS)
```

```
library(ggplot2)
library(grid)
library(gridExtra)
library(ggpubr)

# Installing the font package ------------------------------------------
library(extrafont)
font_import() #It may take a few minutes to import.
loadfonts(device="win")

# PROGRAM TO PLOT BAR CHART WITH STANDARD DEVIATION ----------------------

#+++++++++++++++++++++++++
# Function to calculate the mean and the standard deviation
# for each group
#+++++++++++++++++++++++++
# data : a data frame
# varname : the name of a column containing the variable
#to be summariezed
# groupnames : vector of column names to be used as
# grouping variables
data_summary <- function(data, varname, groupnames){
  require(plyr)
  summary_func <- function(x, col){
    c(mean = mean(x[[col]], na.rm=TRUE),
      sd = sd(x[[col]], na.rm=TRUE))
  }
  data_sum<-ddply(data, groupnames, .fun=summary_func,
                  varname)
  data_sum <- rename(data_sum, c("mean" = varname))
  return(data_sum)
}


# ANALYSIS---------------------------------------------------------------

## the .txt file needs to be saved as the type of "Tab delimited".

##load TS-VS.txt
con <-file.choose(new = FALSE)
metadata <- read.table(con, header = T, row.names = 1)

## DEFINING FACTORS

metadata$HRT <- factor(metadata$HRT) ##Factor Statement
```

```
# 1. Effects of HRT on TS production

## One-way ANOVA

# Effluent TS
fit1 <- aov(TS~HRT, data = metadata)
summary(fit1)
Tukey1 <- TukeyHSD(fit1, conf.level=0.95) #Tukey multiple comparions
Tukey1
TS_data <- data_summary(metadata, varname="TS", groupnames="HRT")
TS_data

# Effluent VS
fit2 <- aov(VS~HRT, data = metadata)
summary(fit2)
Tukey2 <- TukeyHSD(fit1, conf.level=0.95) #Tukey multiple comparions
Tukey2
VS_data <- data_summary(metadata, varname="VS", groupnames="HRT")
VS_data

# TS removal
fit3 <- aov(TS_removal~HRT, data = metadata)
summary(fit3)
Tukey3 <- TukeyHSD(fit3, conf.level=0.95) #Tukey multiple comparions
Tukey3
TS_removal_data <- data_summary(metadata, varname="TS_removal", groupnames="HRT")
TS_removal_data

# VS removal
fit4 <- aov(VS_removal~HRT, data = metadata)
summary(fit4)
Tukey4 <- TukeyHSD(fit4, conf.level=0.95) #Tukey multiple comparions
Tukey4
VS_removal_data <- data_summary(metadata, varname="VS_removal", groupnames="HRT")
VS_removal_data


## Plot

#Effluent TS

TS_content <- data_summary(metadata, varname="TS",
                           groupnames=c("HRT"))

TS_content$HRT=as.factor(TS_content$HRT)
head(TS_content)
```

```
box_1 <- ggplot(TS_content, aes(x=HRT, y=TS, fill=HRT)) +
  geom_bar(stat="identity", position=position_dodge(0.9), width=0.5)+
  geom_errorbar(aes(ymin=TS-sd, ymax=TS+sd), width=0.2, position=position_dodge(0.9))+
  xlab("HRT")+
  ylab("Effluent TS content (%)") + ylim(0, 6)  + labs(title = "", subtitle=NULL) +
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=20, family="Times New Roman"),
        axis.text.y=element_text(size=20, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.position="none")+
  scale_fill_manual(values=c("seagreen3", "red", "blue", "blue"))
box_1

#Effluent VS

VS_content <- data_summary(metadata, varname="VS",
                           groupnames=c("HRT"))

VS_content$HRT=as.factor(VS_content$HRT)
head(VS_content)

box_2 <- ggplot(VS_content, aes(x=HRT, y=VS, fill=HRT)) +
  geom_bar(stat="identity", position=position_dodge(0.9), width=0.5)+
  geom_errorbar(aes(ymin=VS-sd, ymax=VS+sd), width=0.2, position=position_dodge(0.9))+
  xlab("HRT")+
  ylab("Effluent VS content (%)") + ylim(0, 5)  + labs(title = "", subtitle=NULL) +
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=20, family="Times New Roman"),
        axis.text.y=element_text(size=20, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.position="none")+
  scale_fill_manual(values=c("seagreen3", "red", "blue", "blue"))
box_2

#TS removal

TS_removal_plot <- data_summary(metadata, varname="TS_removal",
                           groupnames=c("HRT"))

TS_removal_plot$HRT=as.factor(TS_removal_plot$HRT)
head(TS_removal_plot)

box_3 <- ggplot(TS_removal_plot, aes(x=HRT, y=TS_removal, fill=HRT)) +
  geom_bar(stat="identity", position=position_dodge(0.9), width=0.5)+
```

```
  geom_errorbar(aes(ymin=TS_removal-sd, ymax=TS_removal+sd), width=0.2,
position=position_dodge(0.9))+
  xlab("HRT")+
  ylab("TS removal (%)") + ylim(0, 100)  + labs(title = "", subtitle=NULL) +
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=20, family="Times New Roman"),
        axis.text.y=element_text(size=20, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.position="none")+
  scale_fill_manual(values=c("seagreen3", "red", "blue", "blue"))
box_3

#VS removal

VS_removal_plot <- data_summary(metadata, varname="VS_removal",
                          groupnames=c("HRT"))

VS_removal_plot$HRT=as.factor(VS_removal_plot$HRT)
head(VS_removal_plot)

box_4 <- ggplot(VS_removal_plot, aes(x=HRT, y=VS_removal, fill=HRT)) +
  geom_bar(stat="identity", position=position_dodge(0.9), width=0.5)+
  geom_errorbar(aes(ymin=VS_removal-sd, ymax=VS_removal+sd), width=0.2,
position=position_dodge(0.9))+
  xlab("HRT")+
  ylab("VS removal (%)") + ylim(0, 100)  + labs(title = "", subtitle=NULL) +
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=20, family="Times New Roman"),
        axis.text.y=element_text(size=20, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.position="none")+
  scale_fill_manual(values=c("seagreen3", "red", "blue", "blue"))
box_4
```

Fiber Composition

```
## Statistical analysis
## Performance data of anaerobic digestion
## Wei Liao, October 30, 2020

# Loading Library and Tables ----------------------------------------------

library (MASS)
library(ggplot2)
```

```
library(grid)
library(gridExtra)
library(ggpubr)

# Installing the font package ------------------------------------------
library(extrafont)
font_import() #It may take a few minutes to import.
loadfonts(device="win")

# PROGRAM TO PLOT BAR CHART WITH STANDARD DEVIATION ----------------------

#+++++++++++++++++++++++++
# Function to calculate the mean and the standard deviation
# for each group
#+++++++++++++++++++++++++
# data : a data frame
# varname : the name of a column containing the variable
#to be summariezed
# groupnames : vector of column names to be used as
# grouping variables
data_summary <- function(data, varname, groupnames){
  require(plyr)
  summary_func <- function(x, col){
    c(mean = mean(x[[col]], na.rm=TRUE),
      sd = sd(x[[col]], na.rm=TRUE))
  }
  data_sum<-ddply(data, groupnames, .fun=summary_func,
                  varname)
  data_sum <- rename(data_sum, c("mean" = varname))
  return(data_sum)
}


# ANALYSIS---------------------------------------------------------------

## the .txt file needs to be saved as the type of "Tab delimited".

##load FiberComposition.txt
con <-file.choose(new = FALSE)
metadata <- read.table(con, header = T, row.names = 1)
## DEFINING FACTORS

metadata$HRT <- factor(metadata$HRT) ##Factor Statement


# 1. Effects of HRT on TS production
```

```
## One-way ANOVA

# Cellulose
fit1 <- aov(Cellulose~HRT, data = metadata)
summary(fit1)
Tukey1 <- TukeyHSD(fit1, conf.level=0.95) #Tukey multiple comparions
Tukey1
Cellulose_data <- data_summary(metadata, varname="Cellulose", groupnames="HRT")
Cellulose_data

# Xylan
fit2 <- aov(Xylan~HRT, data = metadata)
summary(fit2)
Tukey2 <- TukeyHSD(fit2, conf.level=0.95) #Tukey multiple comparions
Tukey2
Xylan_data <- data_summary(metadata, varname="Xylan", groupnames="HRT")
Xylan_data

# Cellulose removal
fit3 <- aov(Cellulose_removal~HRT, data = metadata)
summary(fit3)
Tukey3 <- TukeyHSD(fit3, conf.level=0.95) #Tukey multiple comparions
Tukey3
Cellulose_removal_data <- data_summary(metadata, varname="Cellulose_removal",
groupnames="HRT")
Cellulose_removal_data

# Xylan removal
fit4 <- aov(Xylan_removal~HRT, data = metadata)
summary(fit4)
Tukey4 <- TukeyHSD(fit4, conf.level=0.95) #Tukey multiple comparions
Tukey4
Xylan_removal_data <- data_summary(metadata, varname="Xylan_removal",
groupnames="HRT")
Xylan_removal_data


## Plot
#Cellulose
Cellulose_content <- data_summary(metadata, varname="Cellulose",
                                  groupnames=c("HRT"))

Cellulose_content$HRT=as.factor(Cellulose_content$HRT)
head(Cellulose_content)

box_1 <- ggplot(Cellulose_content, aes(x=HRT, y=Cellulose, fill=HRT)) +
```

```
  geom_bar(stat="identity", position=position_dodge(0.9), width=0.5)+
  geom_errorbar(aes(ymin=Cellulose-sd, ymax=Cellulose+sd), width=0.2,
position=position_dodge(0.9))+
  xlab("HRT")+
  ylab("Cellulose content in the effluent (% TS)") + ylim(0, 20)  + labs(title = "", subtitle=NULL)
+
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=20, family="Times New Roman"),
        axis.text.y=element_text(size=20, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.position="none")+
  scale_fill_manual(values=c("blue", "blue", "blue", "blue"))
box_1

#Xylan

Xylan_content <- data_summary(metadata, varname="Xylan",
                      groupnames=c("HRT"))

Xylan_content$HRT=as.factor(Xylan_content$HRT)
head(Xylan_content)

box_2 <- ggplot(Xylan_content, aes(x=HRT, y=Xylan, fill=HRT)) +
  geom_bar(stat="identity", position=position_dodge(0.9), width=0.5)+
  geom_errorbar(aes(ymin=Xylan-sd, ymax=Xylan+sd), width=0.2,
position=position_dodge(0.9))+
  xlab("HRT")+
  ylab("Xylan content in effluent (% TS)") + ylim(0, 20)  + labs(title = "", subtitle=NULL) +
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=20, family="Times New Roman"),
        axis.text.y=element_text(size=20, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.position="none")+
  scale_fill_manual(values=c("blue", "blue", "blue", "blue"))
box_2

#Cellulose_removal removal

Cellulose_removal_data <- data_summary(metadata, varname="Cellulose_removal",
                           groupnames=c("HRT"))

Cellulose_removal_data$HRT=as.factor(Cellulose_removal_data$HRT)
head(Cellulose_removal_data)
```

```
box_3 <- ggplot(Cellulose_removal_data, aes(x=HRT, y=Cellulose_removal, fill=HRT)) +
  geom_bar(stat="identity", position=position_dodge(0.9), width=0.5)+
  geom_errorbar(aes(ymin=Cellulose_removal-sd, ymax=Cellulose_removal+sd), width=0.2,
position=position_dodge(0.9))+
  xlab("HRT")+
  ylab("Cellulose removal (%)") + ylim(0, 70)  + labs(title = "", subtitle=NULL) +
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=20, family="Times New Roman"),
        axis.text.y=element_text(size=20, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.position="none")+
  scale_fill_manual(values=c("blue", "blue", "red", "blue"))
box_3

#Xylan removal

Xylan_removal_data <- data_summary(metadata, varname="Xylan_removal",
                        groupnames=c("HRT"))

Xylan_removal_data$HRT=as.factor(Xylan_removal_data$HRT)
head(Xylan_removal_data)

box_4 <- ggplot(Xylan_removal_data, aes(x=HRT, y=Xylan_removal, fill=HRT)) +
  geom_bar(stat="identity", position=position_dodge(0.9), width=0.5)+
  geom_errorbar(aes(ymin=Xylan_removal-sd, ymax=Xylan_removal+sd), width=0.2,
position=position_dodge(0.9))+
  xlab("HRT")+
  ylab("Xylan removal (%)") + ylim(0, 70)  + labs(title = "", subtitle=NULL) +
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=20, family="Times New Roman"),
        axis.text.y=element_text(size=20, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.position="none")+
  scale_fill_manual(values=c("seagreen3", "red", "blue", "blue"))
box_4
```

MICROBIAL ANALYSIS STATISTICAL ANALYSIS

Metagenomic Analysis

```
## Metagenomic analysis
## Co-digestion of swine manure and corn stover
## Part A
## Wei Liao, August 9, 2020
```

```
# Loading Library and Tables ----------------------------------------------

# Install "phyloseq" package
# source ('http://bioconductor.org/biocLite.R')
# biocLite('phyloseq')

library(vegan)
library(phyloseq)
library (MASS)
library(ggplot2)
library(grid)
library(gridExtra)
library(ggpubr)

library(extrafont)
font_import() #It may take a few minutes to import.
loadfonts(device="win")

## the .txt file needs to be saved as the type of "Tab delimited".

#Gene frequency data from QIIME2

## Choose data files -----

#Choose the Frequency_Table.txt
con <- file.choose(new = FALSE)
Frequency_Table <- read.table(con, header = T, row.names = 1)
#Choose the Frequency_Table_taxanomy.txt
con1 <-file.choose(new = FALSE)
Frequency_Table_taxonomy <- read.delim(con1, header = T, row.names = 1)

## Alpha Diversity -----
#Create a matrix object with the data frame
t.Frequency.table <- t(Frequency_Table)  # Transpose the data
class(t.Frequency.table) # Check the class of the table
#Alpha diversity analysis indexes
#Shannon
H <- diversity(t.Frequency.table, index = "shannon", MARGIN = 1, base = exp(1))
#Simpson
D <- diversity(t.Frequency.table, "simpson", MARGIN = 1, base = exp(1))
#Inverse Simpson
iD <- diversity(t.Frequency.table, "inv")
#Pielou's evenness
J<-H/log(specnumber(t.Frequency.table))
#List all indexes
IN <- cbind(H,D,iD,J)
```

```
IN
write.csv(IN, "diversity.csv")

#Plot H, D, iD, and J
plot(H)
plot(D)
plot(iD)
plot(J)

#Estimate Chao1 and ACE
estimateR(t.Frequency.table)

## ANOVA for Alpha Diversity -----

#Using the H, D, iD, and J data to generate "alphadiversity.txt" to run ANOVA
#Choose alphadiversity.txt

con2 <-file.choose(new = FALSE)
alphadiversity <- read.table(con2, header = T, row.names = 1)

#Define factors for alpha diversity
alphadiversity$HRT <- factor(alphadiversity$HRT)

# Normality
shapiro.test(alphadiversity$H)
shapiro.test(alphadiversity$D)
shapiro.test(alphadiversity$iD)
shapiro.test(alphadiversity$J)

#ANOVA of H index
Hfit <- aov(H ~ HRT, data = alphadiversity)
summary(Hfit)

#ANOVA of J index
Jfit <- aov(J ~ HRT, data = alphadiversity)
summary(Jfit)

## Plots of H and J -----

box_1 <- ggboxplot(alphadiversity, x = "HRT", y = "H")+xlab("HRT") +
  ylab("Shannon's Index (H)") +
  theme(legend.position="right",
        axis.text.x = element_text(size = 12, family="Times New Roman"),
        axis.title.x = element_text(size =14, family="Times New Roman"),
        axis.title.y = element_text(size = 14, family="Times New Roman"),
        axis.text.y = element_text(size = 12, family="Times New Roman"),
```

```
      legend.text = element_text(size = 12, family="Times New Roman"),
      legend.title = element_text(size = 14, family="Times New Roman"),
      plot.title= element_blank())
box_1

box_2 <- ggboxplot(alphadiversity, x = "HRT", y = "J")+ xlab("HRT") +
  ylab("Pielou's Index (J)") +
  theme(legend.position="right",
      axis.text.x = element_text(size = 12, family="Times New Roman"),
      axis.title.x = element_text(size = 14, family = "Times New Roman"),
      axis.title.y = element_text(size = 14, family="Times New Roman"),
      axis.text.y = element_text(size = 12, family="Times New Roman"),
      legend.text = element_text(size = 12, family="Times New Roman"),
      legend.title = element_text(size = 14, family="Times New Roman"),
      plot.title= element_blank())
box_2

## Rarefaction -----

col <- c("black", "darkred", "forestgreen", "orange", "blue", "yellow", "hotpink")
lty <- c("solid", "dashed", "longdash", "dotdash")
pars <- expand.grid(col = col, lty = lty, stringsAsFactors = FALSE)
head(pars)
ra <- rarecurve(t.Frequency.table, step = 20, col =col,lty = lty, cex = 0.6) # curve of rarefication
rad <- rad.lognormal(t.Frequency.table) # Rank of Abundance
rad1 <- plot(rad, xlab = "Rank", ylab = "Abundance") # Plotting the rank

## Beta diversity -----

beta <- vegdist(t.Frequency.table, binary = TRUE)
pcoa.obj <- capscale(t.Frequency.table ~ 1, distance = "bray")
plot(pcoa.obj) #plot the PcoA plot
text(scores(pcoa.obj)$sites[,1], scores(pcoa.obj)$sites[,2]) # change of the labes
labels=row.names(t.Frequency.table)

## Vegan Tools -----

#Rank Indexes study
rankindex(scale(t.Frequency.table), t.Frequency.table, c("euc", "man", "bray", "jac", "kul"))

#monMDS
vare.mds <- metaMDS(t.Frequency.table, trace = FALSE)
vare.mds
plot(vare.mds, type ="t")
stressplot(vare.mds)
```

```
metaNMDS <- metaMDS(t.Frequency.table, distance = "bray",
                    k = 2, trymax = 20, engine = c("monoMDS"),
                    wascores = TRUE, expand = TRUE, trace = 1, plot = FALSE) #Run the NMDS
stressplot(metaNMDS)

ordiplot(metaNMDS, type = "n")
orditorp(metaNMDS, display = "species", choices = c(1,2), air = 1)

## Dendogram -----

distance <-vegdist(t.Frequency.table, method="euclidean") ## Production of Distance Matrix
cluster <- hclust(distance, method="complete", members = NULL) ## Production of Hierarchical
Cluster Production
tree_m <- plot(cluster, xlab = "Samples", sub = NULL, main ="Dendogram")

range(distance)
rect.hclust(cluster, k = 2, border = "red")
grp <- cutree(cluster, k = 2)
```

Domain, Phylum and Family Analysis

```
## Metagenomic analysis
## Co-digestion of swine manure and corn stover
## Part B ANOVAs
## Wei Liao, August 9, 2020

# Install "phyloseq" package
# source ('http://bioconductor.org/biocLite.R')
# biocLite('phyloseq')

## Load libraries -----

 library(vegan)
  library(phyloseq)
  library (MASS)
  library(ggplot2)
  library(grid)
  library(gridExtra)
  library(ggpubr)
  library(extrafont)
  font_import() #It may take a few minutes to import.
  loadfonts(device="win")

## Import data files -----
```

```
  #Choose Frequency_Table_Percentage_ANOVAs.txt (change gene frequency to percentage)
  con <- file.choose(new = FALSE)
  Frequency_Table <- read.table(con, header = T, row.names = 1)
  #Choose Frequency_Table_taxonomy.txt
  con1 <-file.choose(new = FALSE)
  Frequency_Table_taxonomy <- read.delim(con1, header = T, row.names = 1)


## Phyloseq -----
  Full_Frequency <- cbind.data.frame(Frequency_Table, Frequency_Table_taxonomy)
  Frequency <- otu_table(Frequency_Table,taxa_are_rows = TRUE) #Frequency table
production for phyloseq
  TAX <- tax_table(as.matrix(Frequency_Table_taxonomy)) #Taxanomy production for
phyloseq
  physeq <- phyloseq(Frequency, TAX) #physeq document production
  physeq0 <- tax_glom(physeq, taxrank=rank_names(physeq)[7], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  tax_table(physeq0)

## Overall abundances for Domain, Phylum, Class, Order, and Family ---------

  #Domain Abundance
  physeqa <-tax_glom(physeq, taxrank=rank_names(physeq)[1], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  tablea <- otu_table(physeqa)
  tablea
  write.csv(tablea, "domain.csv")

  #Phylum Abundance
  physeqa1 <-tax_glom(physeq, taxrank=rank_names(physeq)[2], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  tablea1 <- otu_table(physeqa1)
  tablea1
  write.csv(tablea1, "Phylum.csv")

  #Class Abundance
  physeqa2 <-tax_glom(physeq, taxrank=rank_names(physeq)[3], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  tablea2 <- otu_table(physeqa2)
  tablea2
  write.csv(tablea2, "Class.csv")

  #Order Abundance
  physeqa3 <-tax_glom(physeq, taxrank=rank_names(physeq)[4], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  tablea3 <- otu_table(physeqa3)
```

```
  tablea3
  write.csv(tablea3, "Order.csv")

  #Family Abundance
  physeqa4 <-tax_glom(physeq, taxrank=rank_names(physeq)[5], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  tablea4 <- otu_table(physeqa4)
  tablea4
  write.csv(tablea4, "Family.csv")

  #Genus Abundance
  physeqa5 <-tax_glom(physeq, taxrank=rank_names(physeq)[6], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  tablea5 <- otu_table(physeqa5)
  tablea5
  write.csv(tablea5, "Genus.csv")

## Abundance Plotbar Bacteria -----

  #Bacteria Abundance (Phylum)
  physeq2 <-subset_taxa(physeq, Domain== "Bacteria")
  physeq2_1 <-tax_glom(physeq2, taxrank=rank_names(physeq2)[2], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  table2_1 <- otu_table(physeq2_1)
  write.csv(table2_1, "bacterialPhylum.csv")

  #Bacteroidetes Abundance (Family)
  physeq3 <-subset_taxa(physeq, Phylum == "Bacteroidetes")
  physeq3_1 <-tax_glom(physeq3, taxrank=rank_names(physeq3)[5], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  table3_1 <- otu_table(physeq3_1)
  table3_1
  write.csv(table3_1, "BacteroidetesFamily.csv")

  #Firmicutes Abundance (Family)
  physeq4 <-subset_taxa(physeq, Phylum == "Firmicutes")
  physeq4_1 <-tax_glom(physeq4, taxrank=rank_names(physeq4)[5], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  table4_1 <- otu_table(physeq4_1)
  table4_1
  write.csv(table4_1, "FirmicutesFamily.csv")

  #Archaea Abundance (Family)
  physeq5 <-subset_taxa(physeq, Domain == "Archaea")
  physeq5_1 <-tax_glom(physeq5, taxrank=rank_names(physeq5)[5], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
```

```
table5_1 <- otu_table(physeq5_1)
table5_1
write.csv(table5_1, "ArchaeaFamily.csv")


## ANOVA on abundances of key microbial communities -----

#Choose metadata_PartB.txt (change gene frequency to percentage)
con2 <-file.choose(new = FALSE)
metadata <- read.table(con2, header = T, row.names = 1, fill = TRUE)

#Define factors for metadata
metadata$HRT <- factor(metadata$HRT)


#ANOVA Bacteria (Domain)
Bacteria <- aov(Bacteria ~ HRT, data = metadata)
summary(Bacteria)
Tukey1 <- TukeyHSD(Bacteria, conf.level=0.95) #Tukey multiple comparison
Tukey1 #Output Tukey results

#ANOVA Archaea (Domain)
Archaea <- aov(Archaea~ HRT, data = metadata)
summary(Archaea)
Tukey2 <- TukeyHSD(Archaea, conf.level=0.95) #Tukey multiple comparison
Tukey2 #Output Tukey results

 #ANOVA Euryarchaeota (Phylum)
Euryarchaeota <- aov(Euryarchaeota~ HRT, data = metadata)
summary(Euryarchaeota)
Tukey3 <- TukeyHSD(Euryarchaeota, conf.level=0.95) #Tukey multiple comparison
Tukey3 #Output Tukey results

#ANOVA Bacteria_unclassified (Phylum)
Bacteria_unclassified <- aov(Bacteria_unclassified ~ HRT, data = metadata)
summary(Bacteria_unclassified)
Tukey4 <- TukeyHSD(Bacteria_unclassified, conf.level=0.95) #Tukey multiple comparison
Tukey4 #Output Tukey results

#ANOVA Bacteroidetes (Phylum)
Bacteroidetes <- aov(Bacteroidetes ~ HRT, data = metadata)
summary(Bacteroidetes)
Tukey5 <- TukeyHSD(Bacteroidetes, conf.level=0.95) #Tukey multiple comparison
Tukey5 #Output Tukey results

#ANOVA Firmicutes (Phylum)
```

```
Firmicutes <- aov(Firmicutes ~ HRT, data = metadata)
summary(Firmicutes)
Tukey6 <- TukeyHSD(Firmicutes, conf.level=0.95) #Tukey multiple comparison
Tukey6 #Output Tukey results

#ANOVA Bacteroidetes_unclassified (Family)
Bacteroidetes_unclassified <- aov(Bacteroidetes_unclassified ~ HRT, data = metadata)
summary(Bacteroidetes_unclassified)
Tukey7 <- TukeyHSD(Bacteroidetes_unclassified, conf.level=0.95) #Tukey multiple
comparison
Tukey7 #Output Tukey results

#ANOVA Bacteroidales_unclassified (Family)
Bacteroidales_unclassified <- aov(Bacteroidales_unclassified ~ HRT, data = metadata)
summary(Bacteroidales_unclassified)
Tukey8 <- TukeyHSD(Bacteroidales_unclassified, conf.level=0.95) #Tukey multiple
comparison
Tukey8 #Output Tukey results

#ANOVA Porphyromonadaceae (Family)
Porphyromonadaceae <- aov(Porphyromonadaceae ~ HRT, data = metadata)
summary(Porphyromonadaceae)
Tukey9 <- TukeyHSD(Porphyromonadaceae, conf.level=0.95) #Tukey multiple comparison
Tukey9 #Output Tukey results

#ANOVA Firmicutes_unclassified (Family)
Firmicutes_unclassified <- aov(Firmicutes_unclassified ~ HRT, data = metadata)
summary(Firmicutes_unclassified)
Tukey10 <- TukeyHSD(Firmicutes_unclassified, conf.level=0.95) #Tukey multiple comparison
Tukey10 #Output Tukey results

#ANOVA Clostridiaceae (Family)
Clostridiaceae <- aov(Clostridiaceae ~ HRT, data = metadata)
summary(Clostridiaceae)
Tukey11 <- TukeyHSD(Clostridiaceae, conf.level=0.95) #Tukey multiple comparison
Tukey11 #Output Tukey results

#ANOVA Lachnospiraceae (Family)
Lachnospiraceae <- aov(Lachnospiraceae ~ HRT, data = metadata)
summary(Lachnospiraceae)
Tukey12 <- TukeyHSD(Lachnospiraceae, conf.level=0.95) #Tukey multiple comparison
Tukey12 #Output Tukey results

#ANOVA Peptostreptococcaceae (Family)
Peptostreptococcaceae <- aov(Peptostreptococcaceae ~ HRT, data = metadata)
summary(Peptostreptococcaceae)
```

```
Tukey13 <- TukeyHSD(Peptostreptococcaceae, conf.level=0.95) #Tukey multiple comparison
Tukey13 #Output Tukey results

#ANOVA Ruminococcaceae (Family)
Ruminococcaceae <- aov(Ruminococcaceae ~ HRT, data = metadata)
summary(Ruminococcaceae)
Tukey14 <- TukeyHSD(Ruminococcaceae, conf.level=0.95) #Tukey multiple comparison
Tukey14 #Output Tukey results



## Network -----

n <- make_network(physeq, max.dist =0.35)
plot_network(n, physeq, color = NULL, shape = NULL)
```

Plotting

```
## Metagenomic analysis
## Co-digestion of swine manure and corn stover
## Part B Plots
## Wei Liao, August 9, 2020
## Wei Liao, October 26, 2020 updated

# Install "phyloseq" package
# source ('http://bioconductor.org/biocLite.R')
# biocLite('phyloseq')

## Load libraries -----
library(vegan)
library(phyloseq)
library (MASS)
library(ggplot2)
library(grid)
library(gridExtra)
library(ggpubr)
library(extrafont)
loadfonts(device="win")

## Import data files -----
#Choose Frequency_Table_Percentage_Plot.txt (change gene frequency to relative frequency
(%))
con <- file.choose(new = FALSE)
Frequency_Table <- read.table(con, header = T, row.names = 1)
```

```
  #Choose Frequency_Table_taxonomy.txt
  con1 <-file.choose(new = FALSE)
  Frequency_Table_taxonomy <- read.delim(con1, header = T, row.names = 1)

## Phyloseq -----
  Full_Frequency <- cbind.data.frame(Frequency_Table, Frequency_Table_taxonomy)
  Frequency <- otu_table(Frequency_Table,taxa_are_rows = TRUE) #Frequency table
production for phyloseq
  TAX <- tax_table(as.matrix(Frequency_Table_taxonomy)) #Taxanomy production for
phyloseq
  physeq <- phyloseq(Frequency, TAX) #physeq document production
  physeq0 <- tax_glom(physeq, taxrank=rank_names(physeq)[7], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  tax_table(physeq0)

  # Plot
  p = plot_bar(physeq0, fill = "Class", facet_grid=Domain~Phylum) +
    xlab("") + ylab("Relative Frequency (%)") +
    geom_bar(color = "black", size = .1, stat = "identity", position = "stack")+
    theme(legend.position="right",
        axis.text.x = element_text(size = 11, family="Times New Roman", angle = 90, hjust = 1),
        axis.text.y = element_text(size = 11, family="Times New Roman"),
        axis.title.x = element_text(size = 12, family="Times New Roman"),
        axis.title.y = element_text(size = 12, family="Times New Roman"),
        legend.text = element_text(size = 11, family="Times New Roman"),
        legend.title= element_text(size = 12, family="Times New Roman"))
  p

## Overall abundances for Domain and Phylum -----
  #Abundance Plotbar Domain
  physeqa <-tax_glom(physeq, taxrank=rank_names(physeq)[1], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  tablea <- otu_table(physeqa)

  a = plot_bar(physeqa, fill = "Domain") +
    geom_bar(aes(color=Domain, fill=Domain), stat = "identity", position = "stack") +
    xlab("") + ylab("Relative Frequency (%)") +
    theme(legend.position="right",
        axis.text.x = element_text(size = 11, family="Times New Roman", angle = 90, hjust = 1),
        axis.text.y = element_text(size = 11, family="Times New Roman"),
        axis.title.x = element_text(size = 12, family="Times New Roman"),
        axis.title.y = element_text(size = 12, family="Times New Roman"),
        legend.text = element_text(size = 11, family="Times New Roman"),
        legend.title= element_text(size = 12, family="Times New Roman"))
  a
  a+scale_x_discrete(limits=c("Feed", "HRT1", "HRT2", "HRT3", "HRT4"),
```

```
                labels=c("Feed"="Feed","HRT1"="HRT 1", "HRT2"="HRT 2", "HRT3"="HRT 3",
"HRT4"="HRT 4")
                )

  #Abundance Plotbar Phylum
  physeqa1 <-tax_glom(physeq, taxrank=rank_names(physeq)[2], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  tablea1 <- otu_table(physeqa1)

  a1 = plot_bar(physeqa1, fill = "Phylum") +
    geom_bar(aes(color=Phylum, fill=Phylum), stat = "identity", position = "stack") +
    xlab("") + ylab("Relative Frequency (%)")  +
    theme(legend.position="right",
          axis.text.x = element_text(size = 11, family="Times New Roman", angle = 90, hjust = 1),
          axis.text.y = element_text(size = 11, family="Times New Roman"),
          axis.title.x = element_text(size = 12, family="Times New Roman"),
          axis.title.y = element_text(size = 12, family="Times New Roman"),
          legend.text = element_text(size = 11, family="Times New Roman"),
          legend.title= element_text(size = 12, family="Times New Roman"))
  a1
  a1+scale_x_discrete(limits=c("Feed", "HRT1", "HRT2", "HRT3", "HRT4"),
                labels=c("Feed"="Feed","HRT1"="HRT 1", "HRT2"="HRT 2", "HRT3"="HRT 3",
"HRT4"="HRT 4")
  )

  ## Stop at the Phylum level


## Abundance Plotbar Bacteria----------
  #Abundance Plotbar Bacteria (Phylum)
  physeq2 <-subset_taxa(physeq, Domain== "Bacteria")
  physeq2_1 <-tax_glom(physeq2, taxrank=rank_names(physeq2)[2], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  table2_1 <- otu_table(physeq2_1)

  c = plot_bar(physeq2_1, fill = "Phylum") +
    geom_bar(aes(color=Phylum, fill=Phylum), stat = "identity",position = "stack") +
    xlab("") + ylab("Bacteria relative Frequency (%)") +
    theme(legend.position="right",
          axis.text.x = element_text(size = 11, family="Times New Roman", angle = 90, hjust = 1),
          axis.text.y = element_text(size = 11, family="Times New Roman"),
          axis.title.x = element_text(size = 12, family="Times New Roman"),
          axis.title.y = element_text(size = 12, family="Times New Roman"),
          legend.text = element_text(size = 11, family="Times New Roman"),
          legend.title= element_text(size = 12, family="Times New Roman"))
  c
```

```
  c+scale_x_discrete(limits=c("Feed", "HRT1", "HRT2", "HRT3", "HRT4"),
                     labels=c("Feed"="Feed","HRT1"="HRT 1", "HRT2"="HRT 2", "HRT3"="HRT
3", "HRT4"="HRT 4")
  )

  #Abundance Plotbar Bacteroidetes (Family)
  physeq3 <-subset_taxa(physeq, Phylum == "Bacteroidetes")
  physeq3_1 <-tax_glom(physeq3, taxrank=rank_names(physeq3)[5], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  table3_1 <- otu_table(physeq3_1)
  table3_1

  d = plot_bar(physeq3_1, fill = "Family")+ geom_bar(aes(color=Family, fill=Family), stat =
"identity",position = "stack") +
    xlab("") + ylab("Bacteroidetes Relative Frequency (%)") +
    theme(legend.position="right",
          axis.text.x = element_text(size = 11, family="Times New Roman", angle =90, hjust = 1),
          axis.text.y = element_text(size = 11, family="Times New Roman"),
          axis.title.x = element_text(size = 12, family="Times New Roman"),
          axis.title.y = element_text(size = 12, family="Times New Roman"),
          legend.text = element_text(size = 11, family="Times New Roman"),
          legend.title= element_text(size = 12, family="Times New Roman"))
  d
  d+scale_x_discrete(limits=c("Feed", "HRT1", "HRT2", "HRT3", "HRT4"),
                     labels=c("Feed"="Feed","HRT1"="HRT 1", "HRT2"="HRT 2", "HRT3"="HRT
3", "HRT4"="HRT 4")
  )

  #Abundance Plotbar Firmicutes (Family)
  physeq5 <-subset_taxa(physeq, Phylum == "Firmicutes")
  physeq5_1 <-tax_glom(physeq5, taxrank=rank_names(physeq5)[5], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  table5_1 <- otu_table(physeq5_1)
  table5_1

  f = plot_bar(physeq5_1, fill = "Family")+ geom_bar(aes(color=Family, fill=Family), stat =
"identity",position = "stack") +
    xlab("") + ylab("Firmicutes Relative Frequency (%)") +
    theme(legend.position="right",
          axis.text.x = element_text(size = 11, family="Times New Roman", angle = 90, hjust = 1),
          axis.text.y = element_text(size = 11, family="Times New Roman"),
          axis.title.x = element_text(size = 12, family="Times New Roman"),
          axis.title.y = element_text(size = 12, family="Times New Roman"),
          legend.text = element_text(size = 11, family="Times New Roman"),
          legend.title= element_text(size = 12, family="Times New Roman"))
  f
```

```
  f+scale_x_discrete(limits=c("Feed", "HRT1", "HRT2", "HRT3", "HRT4"),
                     labels=c("Feed"="Feed","HRT1"="HRT 1", "HRT2"="HRT 2", "HRT3"="HRT
3", "HRT4"="HRT 4")
  )

  #Abundance Plotbar Archaea (Family)
  physeq6 <-subset_taxa(physeq, Domain == "Archaea")
  physeq6_1 <-tax_glom(physeq6, taxrank=rank_names(physeq6)[5], NArm=TRUE,
bad_empty=c(NA, "", " ", "\t"))
  table6_1 <- otu_table(physeq6_1)
  table6_1

  g = plot_bar(physeq6_1, fill = "Family")+ geom_bar(aes(color=Family, fill=Family), stat =
"identity",position = "stack") +
    xlab("") + ylab("Archaea Relative Frequency (%)") +
    theme(legend.position="right",
          axis.text.x = element_text(size = 11, family="Times New Roman", angle = 90, hjust = 1),
          axis.text.y = element_text(size = 11, family="Times New Roman"),
          axis.title.x = element_text(size = 12, family="Times New Roman"),
          axis.title.y = element_text(size = 12, family="Times New Roman"),
          legend.text = element_text(size = 11, family="Times New Roman"),
          legend.title= element_text(size = 12, family="Times New Roman"))
  g
  g+scale_x_discrete(limits=c("Feed", "HRT1", "HRT2", "HRT3", "HRT4"),
                     labels=c("Feed"="Feed","HRT1"="HRT 1", "HRT2"="HRT 2", "HRT3"="HRT 3",
"HRT4"="HRT 4")
```

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