ANAEROBIC TREATMENT OF LIGNOCELLULOSIC MATERIAL FOR ENERGY GENERATION

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ABSTRACT

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Lignocellulosic material is a renewable and sustainable feedstock for the conversion to energy products. The anaerobic treatment breaks down the lignocellulosic material by a community of various microorganisms to produce energy. This treatment, often referred to as anaerobic digestion, has long been adopted by countries, such as Germany and Denmark, because of desirable waste management and energy recovery practices. A novel approach with anaerobic digestion is to use it as a pretreatment method to generate a feedstock that is beneficial for a biofuel production. In this report, raw corn stover was digested with swine manure taken from Michigan State University in 0.5 L reactors. Digestion performance and solid fiber quality were measured and assessed on five different ratios of corn stover to swine manure. Biogas and solid fiber were collected over a period of 60 days. The remaining fiber after digestion was further pretreated using optimized dilute alkali conditions (2% sodium hydroxide, 130°C, and 2 hours), enzymatically hydrolyzed on a 5% dry basis. Ethanol concentration was calculated based upon the glucose production from the fiber of each ratio. Mass and energy balances were evaluated to determine which ratio would be most beneficial for adoption into a biorefinery. The stover-to-manure ratio of 40:60 generated the most energy at 3.4 MJ kg^{-1}dry raw feed, which was at most a 30% increase in total net energy compared to the other reactor ratios. The ratio effectively produced ethanol and methane at 41 and 101g kg^{-1} dry raw feed, respectively. Using anaerobic digestion as an energy producer and as a feedstock generator for biorefinery processing can contribute to solving energy problems that are prevalent in this country.

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Go Green.

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Introduction

Fossil fuels such as coal, oil, and natural gas have played an influential role in the development of human society. These fuels are used every day for commuting, heating, cooking, lighting purposes, etc. Presently, most fossil fuels are used for transportation purposes, which accounts for approximately 72% of the daily oil consumption. In the United States alone, Americans consume roughly 20 million barrels of oil daily. For a global perspective, the overall consumption of oil is approximately 85 million barrels a day (United States Energy Information Agency, 2008). This makes the United States the highest consumer of oil in the world, consuming over 20% of the total daily production. With only containing 4.5% of the world's population, the United States consumes 25% of the overall energy that is produced daily.

Although these fuels have been successful in the advancement of human society, there are certain problems that are associated. These problems include depleting oil reserves, high consumption rates, environmental and political concerns (Cherubini & Jungmeier, 2010). Human health has also been associated with fossil fuel consumption. Problems such as premature death, respiratory failure, and asthma attacks are closely linked to coal-burning power plants. Another major concern is the creation greenhouse gas emissions (GHG) that are produced from the combustion of these fossil fuels, which are associated with pollution and acid rain. Some typical GHGs include nitrogen oxides (NO_x), sulfur oxides (SO_x), carbon dioxide (CO_2), and methane (CH_4). Methane emissions are largely attributed to agricultural and industry wastes operations (Mata-Alvarez, Mace, & Llabres, 2000). Some estimations claim that the agricultural sector accounts approximately 10-12% of the total anthropogenic annual emissions of CO_2 equivalents (Scialabba & Muller-Lindenlauf, 2010). Even land-use change for human activity has a profound

impact on environmental emissions (Cherubini & Jungmeier, 2010). Political instability in the middle-east also influences market values of crude oil products (Banerjee et al., 2010). This leads consumers to continually see instability in gasoline and utility prices, which can contribute to stifling economic activity, which is present in the U.S.

Long-term economic and environmental issues have created much research into the advancement of renewable energy and fuels (Kumar, Barrett, Delwiche, & Stroeve, 2009). With the high abundance of plant material on this planet, the conversion of lignocellulosic plant material can be a solution as a renewable energy source. At the federal level, the United States government has even set a goal to displace 30% of its petroleum usage from biomass technologies by 2022 (Perlack et al., 2005). State governments are also taking approaches to solve energy problems too. Specifically in Michigan, the state passed its first renewable energy bill in 2008 that will require 10% of electricity to be generated from renewable sources by the year 2015 (Granholm & Cherry Jr., 2008). According to the United States Department of Agriculture (USDA) and Department of Energy's (DoE) billion ton study, the United States has about 1.2 billion dry tons of lignocellulosic material that can be used as feedstock for conversion to energy applications, meeting the 30% displacement of petroleum usage (2005). Technologies, such as anaerobic digestion and lignocellulosic bioethanol production, are emerging as viable renewable energy sources that can effectively utilize organic plant material (Jorgensen, Kristensen, & Felby, 2007; Mata-Alvarez et al., 2000).

Anaerobic digestion (AD) is the natural biological conversion that consumes organic materials, including plant biomass, to produce biogas, which can be used as a renewable energy source. The biogas produced is mainly composed of methane and carbon dioxide. Organic wastes used as AD feedstocks come from agriculture, food processing, and drinking

manufacturing in the form of solids or liquids (Callaghan, Wase, Thayanithy, & Forster, 1999). Communities of anaerobic microorganisms utilize the polysaccharides in the organic wastes, as well as other proteins and lipids present, to produce biogas. AD biogas can be refined and combusted to produce electricity for combined heating and power systems. The remaining slurry after the AD process can be separated into its solid and liquid phases for further utilization. Solid AD fibers are currently used for animal bedding, but more research is being applied to convert it into liquid fuels like bioethanol (Z. Yue, Teater, Liu, MacLellan, & Liao, 2010). Liquid portions from the AD system contains high amounts of nitrogen and phosphorus, which can have additional benefits as being used as an effective fertilizer or nutrient feedstock for other bioenergy crops such as algae, or used for land applications for crop production (San, Preston, & Ly, 2003; Z. Yue et al., 2010).

The following sections to be further discussed include an assessment of the biological polymers within various bioenergy feedstocks and a brief review of compositional analysis methods used that determines lignocellulosic components. An in-depth evaluation of the AD process will also be discussed, along with determining how it can more effectively be used as a renewable bioenergy resource in the future. Biorefineries that focus on the conversion of plant material to energy will be important as society looks for more renewable forms of energy.

Literature Review

1.1 Lignocellulosic Plant Biomass

Plants have changed the planet. Evidence is seen with every breath a person takes from the oxygen produced by photosynthetic plants. This interesting, complex, multicellular, organism is able to harvest solar energy and chemically-store it into carbohydrate-based polymers (Sarkar, Bosneaga, & Auer, 2009). For survival, plants have evolved into complex structures with various chemical mechanisms to resist microbial and animal consumption (Himmel et al., 2007; Mosier et al., 2005; Sarkar et al., 2009). Plant structures even account for environmental factors, such as wind and soil compaction (Lee, Marcus, & Knox, 2011). There are three major components that create the primary and secondary walls in vascular plants: cellulose, hemicellulose, and lignin. These materials in the plant cell walls are commonly referred to as lignocellulosic biomass and are the polymers used for bioconversion processes (Jorgensen et al., 2007; Theander, 1991). Each component is interconnected with each other throughout the primary and secondary cell wall making it a highly recalcitrant material (Himmel et al., 2007). Lignocellulosic materials are also composed of proteins and other extractives, but are not typically used for chemical and fuel production. Some examples of extractives are resin acids, fatty acids, sterols, and flavonoids.

1.2 Polymers in Bioenergy Conversion

Cellulose is the most abundant material on the planet (Baurhoo, Ruiz-Feria, & Zhao, 2008), and accounts for most of the polysaccharides found in the plant cell walls; approximately 30-90% of all wall polysaccharides (Lee et al., 2011). Cellulose also makes up about 30-50% of the entire cell wall material (Pauly & Keegstra, 2008). It is a β-1,4-glucan polymer with a highly crystalline structure (Himmel et al., 2007; Lee et al., 2011; Mosier et al., 2005; Pauly & Keegstra, 2008; Yarbrough, Himmel, & Ding, 2009). This polymer is an elongated microfibril

that is composed of 36 individual elementary fibrils connected through hydrogen bonding (Himmel et al., 2007). The current "general" plant cell wall model depicts the cellulosic microfibrils in an organized crossing-scaffold giving strength to the primary wall (Sarkar et al., 2009). Cellulose microfibrils are formed in the plasma membrane of the plant cell and are distributed to the primary and secondary cells walls.

Hemicellulose is a more heterogeneous structure composed of a variety of β-1,4-xylan and β-1,4-galactan backbones, uronic acids, side-branched acetyl and free carboxylic groups (Lee et al., 2011; McMillan, 1994; Taherzadeh & Karimi, 2008). This polymer is formed in the golgi apparatus by glycosyltransferases that consume the nucleotide sugar substrates produced within the plant cell. The amount of hemicellulose in the cell wall is approximately 25-35% (Pauly & Keegstra, 2008). As opposed to the structure of cellulose, hemicellulose is more weak, more random, and more amorphous, making it easily degradable (Taherzadeh & Karimi, 2008). This can explain why high concentrations of hemicellulose are witnessed in the secondary instead of the primary cell wall. Although hemicellulose has a lower tensile strength than other components in the plant, it is still believed to be one of the main load-bearing structures in the cell walls of most land plants (Sarkar et al., 2009).

Lignin is the second most abundant material on the plant and has been studied over the last two centuries (Baurhoo et al., 2008). The composition of lignin accounts for approximately 20-30% of all cell walls (Pauly & Keegstra, 2008). Lignin is heterogeneous with a basic assembly of a phenolic aromatic, a C3 carbon chain, and a hydroxyl functional group acting as the only reacting site (Kobayashi, Abe, & Dusek, 2010; United States Department of Energy, 2002). Lignin is composed of three residue monolignols: hydroxyphenyl (H), guaiacyl (G), and syringyl (S). There are three different alcohols that are precursors to monolignols: courmaryl,

coniferyl, and sinapyl alcohols, respectivetly. These monolignols are formed through the cinnamate pathway that converts phenylalanine, synthesized in the chloroplasts, into individuals H, G, S polymers. These polymers are then transfer to the secondary cell wall where they polymerize.

The structure of lignin helps to protect the more vulnerable carbohydrates from microbes, fungi, and insects (Baurhoo et al., 2008; Taherzadeh & Karimi, 2008). Along with providing vascular plants structural integrity, as with the cellulose and hemicellulose, lignin also has hydrophobic qualities to repel water. Commercial lignin is usually produced as a by-product from the pulping and bio-ethanol industries (Kobayashi et al., 2010). Because of its complex structure, most lignin residues are combusted for combined heat and power systems. Some research has been performed on purified lignin and its possible health benefits as a feed additive for monogastric animals (Baurhoo et al., 2008).

1.3 Bioenergy Feedstocks and Conversion

Common bioenergy feedstocks include switchgrass, poplar, wheat straw, miscanthus, and corn stover. These 2nd generation bioenergy crops have been identified for energy conversion based upon their high abundance, favorable lignocellulosic composition, and non-competitiveness to food crops, like corn and soybeans. Higher quantities of cellulose and hemicellulose are desirable in the bioenergy conversion, although the composition of these crops may differ based upon region, weather, soil type, harvesting, and storage practices (Gnansounou & Dauriat, 2010).

The polysaccharides are desirable because of their ability to be converted to mono-sugars (Hodge, Karim, Schell, & McMillian, 2008). This is usually referred to the 'sugar platform' (Jorgensen et al., 2007). Conversion to monomeric sugars is usually done with acids or enzymes for hydrolysis, and subsequently fermented by a number of microorganisms; predominately from a *Saccharomyces* species. Enzymatic hydrolysis is more favorable than with the use of acids because of its less-corrosive impact to the environment, although the use of enzymes is one of the most expensive costs in the conversion operation. The fermentation products consist of numerous chemicals and fuels, for example carboxylic acids and ethanol (Jorgensen et al., 2007).

1.4 Compositional Fiber Analysis

As previously mentioned, not all of the biomass components are used for chemical and fuel production. The main components used in the biotechnology industry are the structural carbohydrates and lignin. Knowing the composition of the fiber can help engineers develop mass and energy balances to design unit operations, as well as help predict production yields (Templeton, Scarlata, Sluiter, & Wolfrum, 2010). Compositional analysis can further be used to compare the nutrient value of animal feed and dietary fiber content in human food, but more importantly used in comparing bioenergy feedstocks and efficiency of biomass-to-energy processes (Moxley & Zhang, 2007; J. B. Sluiter, Ruiz, Scarlata, Sluiter, & Templeton, 2010).

Fiber composition has long been determined from a high concentrated sulfuric acid hydrolysis. The lineage of the different methods dates back to over a hundred years ago with the determination of lignin in wood. Johan Peter Klason was given credit for initially using a high, 72%, concentrated acid in 1906 (J. B. Sluiter et al., 2010). Methods further developed for different purposes include analyzing wood sugar and herbaceous lignin in the 1930's, animal feed and a human dietary fiber in the 1950's, and applications to biofuels and chemicals in the 1980's (J. B. Sluiter et al., 2010).

There are several procedures that have been predominately used for compositional fiber analysis today; the Uppsala, National Renewable Energy Laboratory (NREL), and Van Soest methods (Moxley & Zhang, 2007). The Uppsala method was developed from Theander, at the Sweden University Agricultural Sciences in Uppsala, to compare plant materials for biofuel feedstocks (J. B. Sluiter et al., 2010). NREL later adopted their method on the basis of the Uppsala procedure. These two methods are based upon a two-step hydrolysis, first to break the crystalline structure of the material and secondly to convert the structural carbohydrates into

monomeric sugars that can be measured from analytical laboratory equipment. This may include a high-performance liquid chromatography (HPLC) or gas chromatography (GC) equipment. Lignin is often measured gravimetrically because of its inability to be broken down by the concentrated sulfuric acid.

The van Soest method uses detergent solutions, neutral and acid, followed by an acid wash to extract individual components from the biomass sample through filtration (Vansoest, 1965). The neutral and acid detergents are able to solubilize extractives and hemicellulose from the samples, respectively. An acid wash is employed to degrade the cellulose component, leaving only the lignin residues. Calculations on specific amounts of each polysaccharide and lignin can then be performed based upon the gravimetric weights of the samples after each process.

Problems arise with the accuracy of these methods. The procedures employed to analyze the composition of the biomass are empirical and rely on the precision of whoever performs the test (Templeton et al., 2010). NREL has even studied the variability involved in the fiber composition by comparing over a hundred samples with other laboratories and references. Moxley and Zhang proposed a modified NREL method because of low yields seen with hemicellulose in various feedstocks; corn stover, switch grass, wheat straw, and hybrid poplar. Other work is continually being done to assess the impacts of reactions conditions on fiber results, such as reaction time, temperature, and acid concentration (Liao, Liu, Wen, Frear, & Chen, 2007). Proteinaceous contents within the lignocellulosic material also react with the carbohydrates through non-enzymatic browning reactions, further causing inaccurate compositional results.

1.5 Anaerobic Digestion

Anaerobic digestion (AD) is a natural, biological, process that breakdowns complex organic material by a community of microorganisms. This community is a synergistic collection of Archaea and bacteria, which are some of the oldest evolutionary microorganisms on the planet (Lubken, Gehring, & Wichern, 2010). This process occurs, with the absence of oxygen, and predominately produces methane and carbon dioxide gases (Hamilton, 2009). Other trace amounts of gases are produced in the process as well, such as hydrogen sulfide and ammonia (Y. Q. Lin, Wang, & Wang, 2010). AD is a multistage process that consists of four major steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Tchobanoglous, Burton, & Metcalf & Eddy., 1991).

The purpose of the hydrolysis step is to take the complex organic material that is composed of proteins, lipids, and carbohydrates, and convert them into a more soluble compounds such as amino acids, fatty acids, and sugars (Hamilton, 2009). Degradation of the organic material is primarily from polysaccharide-degrading enzymes that are produced from the bacteria community. Typical enzyme producing microbial species include a *Clostridium spp.*, *Baciillus spp.*, *Pseudomonas spp.*, and *Escherichia spp.* (Toerien & Hattingh, 1969). The more soluble substances are precursors for the acidogenesis step where they are further refined. In acidogenesis, the soluble organic molecules are transformed into a combination of acetic acid, volatile fatty acids (VFAs), hydrogen and carbon dioxide by microorganisms commonly referred to as acidogens. Acetogenesis then converts any remaining VFAs and higher organic acids into more suitable carbon sources like acetic acid and carbon dioxide (Y. Q. Lin et al., 2010). Methanogenesis, executed by methanogenic microorganisms called methanogens, consumes the refined carbon sources and produces methane gas. Common methane producing species include

a *Methanococcus spp.*, *Methanobaccilus spp.*, and a *Methanobacterium spp*. (Toerien & Hattingh, 1969). Methane is produced from two groups: one that uses acetate as a carbon source, and the other uses hydrogen and carbon dioxide as an electron donor and acceptor, respectively (Y. Q. Lin et al., 2010).

1.6 Anaerobic Digestion Benefits

There is a strong interest in AD technology because of the capabilities of organic waste disposal, odor control, and energy production (Cantrell, Ducey, Ro, & Hunt, 2008). AD is also beneficial in the destruction of pathogenic organisms, which is a common concern in organic waste disposal (San et al., 2003). In terms of manure management, the AD process reduces the volume size of the organic material anywhere between 50-90% (Lansing, Martin, Botero, da Silva, & da Silva, 2010). This can benefit operations that may have limited space, or provide more land available for business expansion. Products from AD also have the ability to contribute to the transportation fuel and chemical intermediate industries (Cantrell et al., 2008)

Although AD technology is being more heavily researched, its adoption in the United States to actual operation has been relatively low compared to other countries like Germany and Austria (Mata-Alvarez, Dosta, Mace, & Astals, 2011). This is largely due to high capital cost and low revenue from biogas production. One approach to enhance biogas production, thus increasing profits, is co-digestion. Anaerobic co-digestion (AcoD) refers to the mixture of two or more substances that complement the AD treatment to increase the production of biogas (Mata-Alvarez et al., 2011). AcoD has showed to have increased methane concentrations in biogas production by as much as 200%, depending upon operations conditions (Murto, Bjornsson, & Mattiasson, 2004). Although carbohydrates and protein are easily digestible, the addition of higher-fat materials, like food grease, into the digestion process has the ability to increase methane yields because of the oxidation state of the carbon in fats (Lansing et al., 2010; Zitomer & Adhikari, 2005). AcoD has also shown to be beneficial in balancing C:N ratios with certain feedstocks that can lead to a stable digestion process, especially with the digestion of swine manure that has lower C:N ratios around 6-8 (Wu et al., 2010).

1.7 Anaerobic Digestions Factors

AD is a living process. With that, it is important to provide the system with a proper amount of substrate to keep a healthy microbial community for biogas production. Without any substrate, the microorganisms will not be able to grow and reproduce. The AD process is often referred to be rate-limiting because of the different microorganisms that make up the community. Acidogens reproduce at a faster rate than most methanogens, creating a less desirable environment for methane production because of a decrease pH. This can lead to an accumulation of acid into the system, inevitably leading to processing failure (Murto et al., 2004; Omil, Mendez, & Lema, 1995). Therefore, the pH must be controlled by either using alkali or adjusting carbon: nitrogen (C: N) ratio in the feed.

Other factors that can affect the microbial system include organic solid loading, hydraulic retention time (HRT), solid retention time (SRT), temperature, carbon to nitrogen ratio and inhibitory inorganic compounds (Hamilton, 2009; San et al., 2003). HRT and SRT refer to how long liquid and solid materials, respectively, are in the digester system. Calculations for HRT and SRT are based upon a fixed volume. Common HRT's for digestion operation are from 10-40 days. Temperature is important in maintaining a healthy microbial culture with a range from 35-55 °C. Anaerobic microorganisms also require an optimum pH from neutral to slightly basic around 6.5-7.5. Proper C:N ratios can also provide the community with carbon for energy and nitrogen for growth. Ranges of C:N ratios are from 25-50, or even as high as 70 depending upon the employed feedstocks (Burton & Turner, 2003a, 2003b; C. Y. Lin & Lay, 2004; Z. Yue et al., 2010). Problems with inhibitory substances can cause anaerobic systems to fail. Inhibitors may include antibiotics, sulfates, sulfides and salts (Hamilton, 2009). Ammonia is another concern for the system, especially dealing with swine manure (Hansen, Angelidaki, & Ahring, 1998). The

ammonia compound is produced from the degradation of nitrogenous material, usually proteins and urea (Garcia & Angenent, 2009). Methanogens are less tolerant to high concentrations of ammonia compared to Acidogens, causing a decrease in methane production (Chen, Cheng, & Creamer, 2008).

1.8 Research Outlook

There are many problems associated with the consumption of fossil fuels. With the depletion and negative environmental impacts of these fuels, energy must be supplied by renewable resources. A solution is to concentrate on the conversion of plant material because of its abundance on this planet. Researchers continue to develop new technologies and approaches to convert the organic material into value-added products. Research must continue in order to make lignocellulosic biomass conversion as well as technology, such as AD, more commercially attractive in the United States. Other European countries, like Germany, have been able to utilize AD systems from plant material and organic wastes, but there are still many unknowns in understanding the complex process. The continuation of new ideas for efficient utilization of plant material and organic wastes ultimately contributes to the goal of producing energy from renewable sources.

The purpose of this work is to:

1. To optimize biogas production from the anaerobic co-digestion of agricultural residues, specifically corn stover and swine manure.
2. To identify if anaerobic co-digestion treatment of corn stover and swine manure can generate a valuable feedstock for conversion to bioenergy.
3. To assess lignocellulosic interactions that take place during compositional analysis and there effects on carbohydrate conversion.
4. To determine if the fiber analysis method from NREL is sufficient to track structural carbohydrate changes in protein-enriched feedstocks that are generated from anaerobic co-digestion.

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Chapter Two

A Manuscript

MacLellan, J., Chen, R., Zhong, Y., Kraemer, R., Liu, Y., Liao, W., 2012. Anaerobic Bacterial Treatment of Lignocellulosic Material for Energy Generation.

Chapter 2

2.1. Abstract

A biorefinery approach to generating energy and other high-valued products is going to play an important role in the future. Energy generation from waste materials is an important component for the future success of such sustainable operations. Five different reactor ratios of corn stover to swine manure were analyzed for anaerobic digestion performance and fiber quality into a biorefinery concept. With a working volume of 0.50 L, ratio 40:60 showed to produce the most biogas at a hydraulic retention time (HRT) of 20 days. The reactor ratio of 40:60 generated 245 mL kg^{-1}dry raw feed day^{-1} of biogas and 709 g of residual solid fibers kg^{-1}dry raw feed. The residual solid fibers after digestion were collected and pretreated with a 2% sodium hydroxide solution, and subsequently hydrolyzed with enzymes. Ethanol production was then calculated based upon the glucose production from the enzymatic hydrolysis of pretreated solid fiber. Digester ratio 40:60 was able to produce 101 g kg^{-1} and 41 g kg^{-1} dry raw feed of methane and ethanol, respectively. The net energy generated from reactor ratio 40:60 was calculated at 3.4 MJ kg^{-1} dry raw feed. Based on the two energy products, ratio 40:60 achieved a 30% increase of net energy output compared to the other reactor combinations and proving to be most beneficial for a biorefinery.

2.2 Introduction

Fuels like crude oil, natural gas, and coal have played an important part in the advancement of human society. With the intent to reduce foreign oil dependence, create new jobs, and limit pollution emissions, society is looking for new and effective ways to generate energy (Vispute & Huber, 2008). An often overlooked way to generate energy is through biological routes. One proven type of biological technology is anaerobic digestion (AD) that converts organic material into biogas. European countries like Germany and Denmark have long utilized this conversion of waste to energy, but AD hasn't become fully adopted in North America due to failed start-ups and poor system maintenance. This is an attractive practice due to its waste treatment capabilities and its energy recovery, which can help improve rural economics (Y. Chen, Cheng, & Creamer, 2008; Vispute & Huber, 2008). Other benefits include the reduction of odor and pathogens, and the preservation of plants nutrients (Cantrell, Ducey, Ro, & Hunt, 2008; Zhu, 2000). Organic matter is also reduced from 50-90% (Lansing, Martin, Botero, da Silva, & da Silva, 2010). Co-digestion of animal manures with supplemented crop residues is also making AD technology even more attractive (Wu, Yao, Zhu, & Miller, 2010). This is typically due to synergistic effects within the microbial community that increase biogas and methane yields (Mata-Alvarez, Mace, & Llabres, 2000).

There have been recent studies on the co-digestion of swine manure with agro-residues. Wu et al. studied the impact on co-digestion of swine manure with corn stocks, oat straw and wheat straw, and it showed to have a positive effect on biogas production. This was largely attributed to increasing the carbon to nitrogen ratio within the digesting reactors. Research was also performed by Lansing et al. with swine manure and cooking grease showing increased

energy production as much as 124%. Both studies were able to achieve methane concentrations at approximately 68%.

An area of study that is often overlooked is in the utilization of the residual solids remaining after digestion for energy production. This is owing to the materials recalcitrant property and low nutrient value (Tambone, Genevini, D'Imporzano, & Adani, 2009). Recent investigations, though, have concluded that biological treatment of agricultural waste, like dairy manure, still have important components that can be effective in a biorefinery concept; referring to the remaining carbohydrates and lignin portions (S. Chen et al., 2005; Z. Yue, Teater, Liu, MacLellan, & Liao, 2010).

The focus of this work was to incorporate co-digestion technology with swine manure and raw corn stover. Digestion performance as well as fiber quality for liquid fuel production was analyzed. Raw corn stover was combined at five different ratios with swine manure as the feed. Biogas accumulation and content were factors used to analyze the digestion performance. Fiber quality was examined by assessing the changes in fiber composition throughout the process and analyzing glucose production from enzymatic hydrolysis. Mass and energy balances were performed on the energy products to provide further insights for effective bioenergy generation.

2.3 Materials and Methods

2.3.1 Feedstocks

The swine manure used for the experiments was taken from the Swine Teaching and Research Center at Michigan State University. Hogs were fed with a mixture corn, soybean meal (SBM), and Start A300 Base manufactured from Provimi North America, Inc. Manure was collected in December of 2011, as well as February of 2012, and stored in a -20°C freezer until use. Corn Stover was harvested and collected in 2009 from a private farm in Muir, MI. Raw corn stover was then milled through a 2mm screen using a Schutte Buffalo hammer mill (Model No. WA-6-H). Samples were then collected and dried at 105°C for approximately 24 hours. Composition of the feedstocks can be seen in Table 2.1. Fiber composition was measured using the Laboratory Analytical Procedure (LAP) developed by Sluiter et. al at the national renewable energy laboratory (NREL) (2008). Elemental microanalysis of carbon and nitrogen were analyzed by Atlantic Microlabs, located in Norcross, GA.

2.3.2 Bacterial Reactor Systems

Five different ratios of corn stover to swine manure were used as feeds to feed the anaerobic reactors; 20:80, 40:60, 50:50, 60:40, 80:20. The composition of each feed was calculated and presented in Table 2.2. All reactors contained a working volume of 0.50 L, with a headspace of approximately 0.25 L. The initial headspace was purged with nitrogen for exactly 30 seconds. Each reactor was based on 5% total solids (TS) and a hydraulic retention time (HRT) of 20 days. Duplicates were created for each ratio, which generated a total of 10 reactors. The reactors were shook on a New Brunswick Scientific, Innova 2000 platform shaker, set at 150 revolutions per minute (rpm). Rubber septa caps were used to contain produced biogas, where it can be penetrated to measure daily gas production. The biogas production was measured using a

water displacement method. Feeding of reactors was performed every other day using a Plas Lab (Lansing, MI) Automatic Atmosphere Chamber. The chamber was purged with a medical grade specialty gas composed of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. A palladium catalyst heater was used to make the chamber completely anaerobic; suffice for feeding the anaerobic bacterial systems. Fresh feed was made every 20 days and stored in a refrigerator at 4°C. The pH for all systems was not to go below a value of 6.70, and was controlled by a 5 wt% sodium hydroxide (NaOH) solution.

2.3.3 Dilute Alkali Pretreatment

After 60 days, the solid fiber was collected from each reactor using an Allegra X-12R centrifuge. Pretreatment conditions were adopted by Teater et. al who optimized pretreatment parameters on anaerobically digested fiber of dairy manure (2011). The pretreatment parameters were fixed at 5% dry matter, with 2% NaOH at 130°C for 2 hours. Treated samples were centrifuged and rinsed using de-ionized water. Wet solid samples were stored in a freezer at -20°C. Solid residue and filtrate were taken for the analysis of mono-sugars, dry matter, and fiber content.

2.3.4 Enzymatic Hydrolysis

Wet alkali-pretreated fiber samples (2 g dry matter) and de-ionized water were mixed to a total mass of 40 g into a 125 ml shake flask, which makes the solid concentrations of 5% (w/w). All mixed samples were autoclaved before adding enzymes. Cellulase (ACCELLERASE 1500, Genencor, Rochester, NY) at loading of 26 FPU/g dry substrate was used to perform a 72 hour hydrolysis. The flasks were shook at 150 rpm, and the reaction temperature was 50°C. After 72 hours, aliquots were heated to 100°C for 5 min to inhibit enzyme activity. The liquid samples

were filtered into HPLC vials with Millex-GS 0.22 μm membrane for analysis of glucose and other monomeric sugars such as xylose, arabinose, and galactose.

The overall glucose conversion, xylose conversion and sugar concentrations after enzymatic hydrolysis were used as an indicator of fiber quality. The equation for the overall glucose conversion [%] is: overall glucose conversion [%] = ((substrate dry matter after pretreatment [g] * glucose concentration after enzymatic hydrolysis [g/L] * volume of enzymatic hydrolysate [L])/(substrate dry matter before pretreatment [g] * hydrolysis substrate dry matter [g] * initial raw feedstock cellulose content [%] * 1.11)) * 100. The equation for the overall glucose conversion [%] is: overall xylose conversion [%] = ((substrate dry matter after pretreatment [g] * xylose concentration after enzymatic hydrolysis [g/L] * volume of enzymatic hydrolysate [L])/(substrate dry matter before pretreatment [g] * hydrolysis substrate dry matter [g] * initial raw feedstock hemicellulose content [%] * 1.14)) * 100.

2.3.5 Analytical Methods

Glucose and other mono-sugars were determined using a Shimazdu high-performance liquid chromatography (HPLC) system equipped with a Bio-rad Aminex HPX-87P analytical column, Micro Guard de-ashing column, and a refractive index detector. The mobile phase was degassed Millepore water with a flow rate of 0.6 mL min^{-1}. An oven temperature was set at 80°C for the analytical column, while the de-ashing was placed outside of the oven at a room temperature of 22°C. High purity standards including glucose (Catalog Number: 49158), xylose (Catalog Number: 95729), galactose (Catalog Number: 48259), arabinose (Catalog Number: 10840), and mannose (Catalog Number: 63582) were purchased Sigma (St. Louis, MO).

Methane, carbon dioxide, and hydrogen sulfide content was measured using an SRI 8610C gas chromatography system. Helium was used as a carrier gas with pressure set at 21 pounds per square inch (psi). The system was equipped with a thermal conductivity detector and kept at a constant temperature of 150°C. An injection volume of 3 mL was used with only 100 µL accepted from the instrument.

2.4 Results and Discussion

2.4.1 Anaerobic Digestion Performance

Biogas production is a key parameter to evaluate digestion performance. Figure 2.1 demonstrated the total accumulated biogas production of all ratios during the duration of the experiment. From ascending order (stover-to-manure ratio 20:80 through 80:20), the total biogas volume, in liters (L), generated was 15.2, 18.3, 16.2, 15.8, 14.4, respectively. Initially, each ratio shows a lag phase for approximately one HRT (20 days), where the microbial community became adjusted to the new environmental conditions. During this period, pH of the digesters was continuously dropping, and NaOH had to be added daily to bring pH back to approximately 6.7. The higher the stover-to-manure ratio was, the longer the digester took to achieve a stable pH. After culturing for more than 20 days, the pH of all reactors remained fairly stable above 6.7 and little NaOH was used. Under the stabilized culture condition, the digester with stover-to-manure ratio of 40:60 showed to be more advantageous than the others by producing the most biogas of approximately 18.3 L. As depicted in Figure 3.1.1, the other four ratios were generally grouped together, reaching no more than 16 L. Ratios containing the highest amount of either swine manure or corn stover (i.e stover-to-manure ratios of 20:80 and 80:20) appeared to generate less desirable amount of biogas. Figure 2.2a shows the average daily biogas produced per gram of organic loading over the entire 60 days. Intuitively, the stover-to-manure ratio of

40:60 is shown to have the highest amount of gas produced. This can be credited to an optimum C:N ratio of approximately 17:1, which coincides within the optimum range provided from Sievers and Brune for swine waste digestion (1978). Figure 2.2b provides a breakdown of the biogas production rate for individual HRT's. The largest amount of biogas activity is observed from the transition from the 1^{st} to the 2^{nd} HRT. During the second HRT stover-to-manure ratio 40:60 showed the highest production rate at approximately 330 mL g^{-1} day^{-1}, further demonstrating the benefits the optimum C:N ratio. The biogas production rates within the 3^{rd} HRT begin to level off and mature, with ratios 20:80 and 40:60 being significantly ($P<0.05$) different from the remaining ratios. The smaller portions of corn stover added into the anaerobic systems seem to have provided the necessary nutrient supply to the microbial community. Providing a shorter HRT to the anaerobic systems may address the leveling off effect of the biogas production rates, and offer increased biogas activity benefits.

Operational factors also influenced the digestion performance. The reactors containing larger amount of corn stover were more difficult to mix, leaving a large portion of solids at the top of the working digestion volume. This is often referred to as the scum layer. Consequentially, ratio 40:60 provided to be acceptable mixture of feedstocks to maintain uniformity for microbial consumption, based upon its achievement of producing the largest volume of biogas. It also can be inferred that proper mixing was vital in the initial stages of digestion for the disbursement of alkali solution to maintain reactor pH and for more efficient mass and heat transfer for conversion of solids to gas (Z. B. Yue, Teater, MacLellan, Liu, & Liao, 2011). Reactor ratios with larger portions of corn stover took longer to reach a stabilized pH, which also affected biogas generation.

Biogas composition of each digesters can be seen in Table 2.3. There are noticeable trends with all three gases measured from each individual digesters. The ratios with the lower corn stover amounts observed higher methane and hydrogen sulfide concentrations, and lower carbon dioxide content, as opposed to ratios with higher corn stover portions. A decreasing trend of methane content with the increase in C:N ratio was also seen with Hills (1979). The stover-to-manure ratio of 40:60 had produced the most amount of biogas, but didn't observe an increase in methane content compared to ratio 20:80, which had the highest methane content at 66.2%. As suggested from Backus et al., methane productivity is dependent upon both influent C:N ratios and HRTs (1988). This may infer that the further extending the HRT of the 40:60 stover-to-manure ratio may be able to improve the methane content in the biogas.

H_2S is considered a nuance gas with no energy potential. The sulfide gas needs to be cleaned from the accumulated biogas before further conversion into combined heating and power systems. Less concentrations of H_2S are highly desirable. A sudden drop can be seen with H_2S content, which is as high as ≈ 1900 ppm with ratio 20:80 and as low as ≈ 50 ppm at ratio 80:20. This is attributed largely to the decreasing amount of nitrogen-rich swine manure in the feed. Although it was not characterized in this work, sulfur-containing amino acids, such as cysteine and methionine, could have been reduced and promoted H_2S production from the microbial community (Drennan & Distefano, 2010).

2.4.2 Fiber Quality

The compositions of digested fiber and pretreated digester fiber for all five stover-to-manure ratios were listed in Table 2.4. The table clearly shows a reduction in structural carbohydrates, both cellulose and hemicellulose, in the digested fiber compared to initial raw feed. Cellulose content seemed to decrease more than hemicellulose during the digestion process.

Among the five ratios, the stover-to-manure ratio of 40:60 had cellulose and hemicellulose reductions of 10.1 % and 2.9 %, respectively, which were signficantly ($P<0.05$) higher than other four ratios. The result of fiber degradation was consistent with the gas production of the digestion. More carbohydrates degraded in the stover-to-manure ratio of 40:60 led to more gas production.

As previously reported from Yue et. al (2011), hemicellulose was the only structural carbohydrate showing a decrease in content from the mono-digestion system of dairy manure. From that same study, the cellulose content actually showed to increase in content, by as much as 64%. The difference between both studies is largely due to the difference in feedstock recalcitrant characteristics; raw dairy cow feces and swine manure supplemented with corn stover. The corn stover added into the system had not been previously digested compared to the lignocellulosic fiber that was present within the dairy manure, which had already been digested within the ruminant prior to AD. This meaning that the amorphous regions within the raw corn stover feedstock were actively targeted and more easily consumed by the microorganisms in the 0.50 L reactors, explaining the reduction of both cellulose and hemicellulose in the digested fiber. Because of the additional carbon that was added with the swine manure, it is suggested that there was a beneficial change to the microbial system to breakdown both cellulose and hemicellulose portions. A more thorough investigation into the microbial community would be necessary, and is ongoing, to identify key polysaccharide degradating organisms.

Meanwhile, Dilute alkali pretreatment further enhanced carbohydrate composition in the digested fiber via lowering lignin amount from the disruption of ester bonds cross-linked in the cell wall matrix and removal of acetyl groups (Kumar et al., 2009; Taherzadeh & Karimi, 2008). The compostions of pretreated digested fiber were listed in Table 2.4. The enzymatic hydrolysis

of the pretreated fibers represented a very good conversion of carbohydrates in the digested fiber to mono-sugars (Tables 2.4 and 2.5). Naturally, as the corn stover portion in the raw feed increased and cellulose and hemicellulose in the feed correspondingly increased, more glucose and xylose were produced. Therefore, the largest amount of glucose and xylose produced was from the stover-to-manure ratio of 80:20 with 25.4 g L^{-1} and 11.2 g L^{-1}, respectively. the stover-to-manure ratio of 40:60 was able to produced 17.3 g L^{-1} and 6.2 g L^{-1} of glucose and xylose, respectively. However, in terms of fiber quality comparison of different stover-to-manure ratios, overall carbohydrate conversion was a key indicator (Figure 2.3). For both glucose and xylose, ratio 40:60 achieved the highest overall conversions, which peaked at 83.7% and 38.7%, respectively. There is a clear magnitude difference between the overal glucose and xylose conversion. This comes directly from the employment of a dilute alkali pretreatment on the residual digested fiber, which removes a large portion of the hemicellulose (Teater et al., 2011). The overall glucose conversion was roughly 5-10% higher in comparison from the work from Teater at the same pretreatment and enzyme loading conditions. An increase can be attributed again to the difference in feedstock characteristics for the digestion process, as well as the C:N nutrient source for the bacterial consortia. After the peak at ratio 40:60, there is a sudden drop in carbohydrate conversions; demonstrating an achieved optimum corn stover to swine manure ratio in terms of fiber quality. This establishes that ratio of 40:60 was most utilized by the microbial system and produced a feedstock fiber quality that is more beneficial for a biorefinery application

2.4.3 Mass and Energy Balances

A mass balance was performed on the digestion configuration with three selected stover-to-manure ratios; 20:80, 40:60, and 80:20. This was intended to compare the ratio of 40:60 with both extreme ratios with low and high corn stover amounts. Mass and energy balance analysis further demonstrated the advantage of the stover-to-manure ratio of 40:60. The mass balance can be seen in Table 2.6. The xylose that was produced from the enzymatic hydrolysis was recycled back into the reactors as an additive to enhance biogas production. An assumption of 1g of xylose was equivalent to 1g of chemical oxygen demand (COD) reduced to produce 0.350 L of methane gas. Methane generated from xylose was 3, 5, and 6 g kg^{-1}dry raw feed for the stover-to-manure ratios of 20:80, 40:60, and 80:20, respectively. Overall, the ratio of 40:60 generated the highest methane of 101 g kg^{-1} dry raw feed. Ethanol production from the residual fiber showed a large escalation on the stover-to-manure from 20:80 to 40:60, and was from 27 to 41 g kg^{-1} dry raw feed. The increase wasn't as abrupt from 40:60 to 80:20; a difference of only 8 g kg^{-1} dry raw feed.

An energy balance was calculated on the energy products and shown in Table 2.7. The energy balance clearly shows that the stover-to-manure ratio 40:60 is more favorable for adoption into a biorefinery, by producing 3.4 MJ kg^{-1} dry raw feed. The largest portion of the net energy output is from the increase in biogas amount because of the optimum C:N ratio of approximately 17:1. An increase of as high as 30% of net energy output was achieved with the optimal 40:60 corn stover to swine manure ratio compared to the other reactors analyzed.

2.5 Conclusion

Biorefineries are going to play an important role in the future by generating energy and other high-valued products. Co-digestion of corn stover and swine manure can successfully contribute by producing valuable energy products of methane and ethanol. This work concluded that the stover-to-manure ratio of 40:60 was the optimal feed ratio for the process in terms of digester performance and fiber quality. Based upon the total net energy output, the ratio of 40:60 showed an increase of at least 30% compared to other ratios. Increased biogas production largely contributed to the energy output, but ethanol production also contributed over 14%, showing that the process is able to produce a quality feedstock for further bioconversion. Both entities could provide to be beneficial for future biorefinery operations.

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Chapter Three

A Manuscript

MacLellan, J., Chen, R., Kraemer, R., Liu, Y., Liao, W., 2012. Revisiting Carbohydrate Analysis of Lignocellulosic Biomass

Chapter 3

3.1 Abstract

Interactions of lignocellulosic components during dietary fiber analysis were investigated using the highly adopted compositional analysis procedure from the National Renewable Energy Laboratory (NREL). Synthetic feedstock samples were used to study the effects of lignin/protein, cellulose/protein, and xylan/protein on carbohydrate conversion in a completely randomized design (CRD). With disregarding structural influence in the synthetic samples, lignin and protein components were the most significant ($P<0.05$) factors on glucan conversion. Xylan conversion was consistent and unaffected by content variation through the synthetic analysis. The statistical analysis further concluded that 0.9 ± 0.1 g/g cellulose in original sample can be achieved by maintaining protein content of less than 10 wt%. Validation of observed relationships from the synthetic feedstocks was studied using real lignocellulosic feedstocks: corn stover, poplar, and alfalfa. Neutral detergent (ND) solution was used to extract raw protein from real feedstocks so known protein amounts, in the form of peptone, could be added. Compositional analysis was performed on ND-washed samples with known lignin: protein ratios: 1:1, 3:1, and 5:1. A general verification was drawn from the analysis and observed similar observations, excluding two exceptions of cellulose conversion of poplar under higher protein content and xylan conversion of alfalfa under higher protein content. This showed that structural influences have a dominant role in carbohydrate analysis. The results further show that a substrate-specified method for carbohydrate analysis of different lignocellulosic materials could be developed according to their protein and lignin contents.

3.2 Introduction

Compositional fiber analysis has been extensively used to provide useful data on lignocellulosic materials. Fiber data is comprehensively utilized by the agricultural and paper pulping industries, and is becoming more adopted with emerging biobased products and technologies (Moxley & Zhang, 2007). Traditionally, fiber has been analyzed to measure lignin and carbohydrate contents in various plant materials, as well as to estimate nutritional value in animal feed and human food (Moxley & Zhang, 2007; J. B. Sluiter et al., 2010). Within the last couple of decades, as more interest has been focused into fuels derived from plant materials, compositional analysis of feedstocks has helped engineers compare potential bioenergy feedstocks and measure efficiencies within conversion processes (J. B. Sluiter et al., 2010). Regardless of how the information is inferred upon, current biotechnology applications use compositional methods to characterize the lignocellulosic materials by describing its potential resource quality (Roberts & Rowland, 1998).

Two compositional methods have emerged as protocols for analyzing components in lignocellulosic materials; dietary fiber analysis, based upon the Uppsala method and employed by the National Renewable Energy Laboratory (NREL), and the detergent fiber analysis based upon solution extraction developed from van Soest. One of the main challenges experienced within these methods are the severe complexity of the plant cell wall linkages. Primary and secondary plant cell walls have evolved to become chemically and physically non-uniform as a defense mechanism to degradation, which is the current approach of these two methods. Another issue is that these analyses provide only empirical information and depend heavily on how the method is run (Templeton et al., 2010). A way to better understand the interactions between plant cell walls components are desirable and could lead to more accurate compositional results.

Research has expanded upon these compositional analysis methodologies to develop more accurate ways to quantify carbohydrates in lignocellulosic biomass. An example can be seen with a modified NREL method suggested by Moxley and Zhang to use milder acid concentrations to yield more accurate xylan concentrations (2007). It has also been proposed to develop standard neutral detergent procedures when applying detergent fiber analysis, leading to more precise estimation of cellulose and hemicellulose portions (P.J van Soest, Robertson, & Lewis, 1991). Some studies have even developed models that try to correlate the two compositional methods together to provide quicker compositional data on certain biomass feedstocks (Wolfrum, Lorenz, & deLeon, 2009). But most of this work has been on methodology employment and with little emphasis on understanding true chemical interactions that play an important role in identifying structural carbohydrates.

With a focus on the dietary fiber analysis procedure from NREL, this current work looked to delve into the chemical interactions of the plant cell wall components and their influence on carbohydrate conversion, specifically glucan and xylan. For instance, to the author's knowledge, there has been no set limit as to how much protein can be present before carbohydrate conversion is negatively affected. Synthetic feedstock was created by chemical compounds, mimicking natural biomass, to understand how different components influenced glucan and xylan concentrations. Consequential analysis was performed on actual biomass feedstocks of corn stover, poplar and alfalfa to generally validate observations from the synthetic samples.

3.3 Materials and Methods

3.3.1 Feedstocks

Commercial chemicals of Peptone (Sigma Cat No. P5905), Cellulose powder ~ 20 microns (Sigma Cat No. 310697), Xylan from beechwood (Sigma Cat No. X4252), and Lignin alkali (Sigma Cat No. 370959) were used to create "synthetic" biomass samples. Real lignocellulosic feedstocks of Corn Stover, Poplar, and Alfalfa were used to verify the experimental results from "synthetic" biomass. Corn Stover was harvested and collected in 2009 from a private farm in Muir, MI. Poplar was donated from the Crop and Soil Sciences Department at Michigan State University (MSU) and were acquired from Michigan State University's Forest Biomass Innovation Center in Escanaba, MI. Poplar hybrids were planted in 1998 at a uniform spacing of 8x8 feet and harvested in fall of 2009. Alfalfa sample was collected from the dairy farm at MSU and was harvested in 2011 at a private farm in Riverdale, MI. All feedstock samples were milled through a 2 mm screen using a Schutte Buffalo hammer mill (Model No. WA-6-H). Samples were then collected and dried at 105°C for approximately 24 hours. Their carbon and nitrogen contents were listed in Table 3.1.

3.3.2 Analytical Methods

Neutral detergent solution used for protein extraction on real biomass feedstocks. The solution was prepared according to van Soest (P. J. van Soest, 1965). One gram (g) of raw feedstock was mixed with 100 mL of neutral detergent solution, 0.5 g sodium sulfite, and 2 mL of decahydronathalene in a reflux apparatus. Samples were boiled for one hour and rinsed with 300 mL of boiling deionized water. Subsequently, samples were dried in an oven set at 105°C for approximately 24 hours.

Elemental microanalysis of carbon and nitrogen for the three feedstocks were analyzed by Atlantic Microlabs, located in Norcross, GA. Analysis was performed by combustion using automatic analyzers.

A Shimazdu high-performance liquid chromatography (HPLC), equipped with a Aminex HPX-87P carbohydrate column, a Micro-Guard de-ashing column, and a RID detector, was used to analyze monomeric sugar concentrations from the dietary fiber procedure samples. The mobile phase was degassed Millepore water with a flow rate of 0.6 mL/min. An oven temperature was set at 80°C for the analytical column, while the de-ashing was placed outside of the oven at a room temperature of 22°C. High purity standards including glucose (Catalog Number: 49158), xylose (Catalog Number: 95729), galactose (Catalog Number: 48259), arabinose (Catalog Number: 10840), and mannose (Catalog Number: 63582) were purchased Sigma (St. Louis, MO). HPLC methodology follows the laboratory analytical procedure (LAP) for "Determination of Structural Carbohydrates and Lignin in Biomass" from NREL (A. Sluiter et al., 2008).

3.3.3 Effects of xylan, glucan, and lignin on the concentrated acid carbohydrate analysis of synthetic feedstocks

The effects of lignin/protein, cellulose/protein, and xylan/protein on concentrated acid carbohydrate analysis were first evaluated by a completely randomized design (CRD). Two cellulose/protein ratios (2:1 and 6:1), three lignin/protein ratios (1:1, 3:1, and 5:1), and three xylan/protein ratios (1:1, 3:1, and 5:1) were used by the CRD to create a total of 18 experimental runs with triplicates (Table 3.2). The LAP of NREL was modified to take weight measurement accuracy into consideration. A total mass of 0.6 grams was used to perform the concentrated acid carbohydrate analysis as opposed to the 0.3 grams suggested from NREL. Doubling the mass of

the sample was necessary in order to maintain component measurement accuracy. This resulted in doubling concentrated acid and water volumes throughout the process to keep concentrations at each step the same as in the original procedure. Samples were filtered using Whatman No. 1 filter paper prior to HPLC analysis of glucose and xylose concentrations. The responses were to evaluate the effects of glucan and xylan conversions. Glucan conversion was calculated as: cellulose conversion = [glucose concentration (g/L) x reaction volume (L) x 0.90] /the amount of cellulose added in the synthetic feedstock (g). Xylan conversion was calculated as: xylan conversion = [xylose concentration (g/L) x reaction volume (L) x 0.88] /the amount of xylan added in the synthetic feedstock (g).

3.3.4 Validation of the observations on synthetic feedstocks using lignocellulosic feedstocks

Validation of observed relationships from the synthetic feedstocks was investigated using real lignocellulosic feedstocks: corn stover, poplar, and alfalfa. Corn stover was selected because it is an agricultural waste and is heavily researched as a viable renewable bioenergy resource. Poplar and alfalfa were selected for its woody biomass and protein properties, respectively. Since the absolute values of fiber composition of lignocellulosic feedstocks are unknown and many fiber components can react with each other during the concentrated acid hydrolysis (J. B. Sluiter et al., 2010), it is difficult to directly use lignocellulosic feedstocks to verify the findings from the analysis of synthetic feedstocks. Considering that protein is one of components in lignocellulosic material that has the most reactions with other contents (cellulose, hemicellulose, and lignin) and methods to remove it from biomass have been well established (A. Sluiter et al., 2008; P. J. van Soest, 1965), the effect of protein content on the composition analysis of real lignocellulosic materials was adopted to validate the observed relationship from the synthetic feedstocks. Neutral detergent (ND) solution was used to first wash off the soluble extracts and

protein. After drying, dietary fiber analysis was then performed on the ND-washed lignocellulosic feedstocks (without protein) to determine carbohydrate composition. Carbohydrate contents from the hydrolysis without interference from protein and other soluble extracts are considered as the closest approximation to the real carbohydrate contents. Thus, the ND-washed lignocellulosic feedstocks were used as the base feedstocks to construct the experimental feedstocks with different protein contents. According to the protein contents used for synthetic feedstocks, protein (in the form of peptone) was added into the ND-washed samples to make three levels of protein content for individual feedstocks (Table 3.5). The modified NREL analysis was then executed to verify the effects of protein on the carbohydrate analysis of real lignocellulosic materials.

3.3.5 Statistical analysis

The effects of cellulose/protein, xylan/protein, and lignin/protein ratios on cellulose and xylan conversions were analyzed by a General Linear Model (GLM). The Statistical Analysis System program 9.0 (SAS Institute, Inc., Cary, NC) was used to conclude ANOVA tables and evaluate the effects. In addition, a ranked list that presented the relative importance among component ratios on conversions was formed by a 2^3 factorial analysis. The ratios used for the factorial analysis were labeled as low or high in Table 3.2). The list is given by the left-to-right order of the spikes in the Pareto chart (Haaland, 1989).

Pair-wise comparison using the Statistical Analysis System program 9.0 was also conducted on both synthetic feedstocks and lignocellulosic feedstocks to identify significant differences among different samples.

3.4 Results and Discussion

3.4.1 Effects of xylan, cellulose, and lignin on cellulose hydrolysis of synthetic feedstocks

The changes of cellulose conversion under different compositions of synthetic feedstocks were presented in Table 3.2. The analysis of 2^3 factorial design of lignin/protein, cellulose/protein and xylan/protein ratios demonstrated that the lignin/protein ratio had the greatest influence (36.22% of the total effect) on cellulose conversion, followed by lignin/protein x cellulose/protein x xylan/protein, lignin/protein x cellulose/protein, cellulose/protein x xylan/protein, cellulose/protein, lignin/protein x xylan/protein, and xylan/protein (Fig. 3.1). The ANOVA analysis elucidated that the ratios and their two-way and three-way interactions except xylan/protein ratio had significant influences ($P<0.05$) on the cellulose conversion (Table 3.3). The Pareto chart further demonstrated that the ratios and their interactions related with lignin/protein had more than 81% of total effect (Fig. 3.1). It is apparent that lignin/protein ratios played important roles on cellulose conversion.

The effects of individual components (lignin, protein, cellulose, and xylan) on the cellulose conversion were correspondingly investigated to further delineate the relationship between cellulose conversion and composition of feedstock. 0.8 ± 0.1 g/g cellulose in original sample was taken as an acceptable cellulose conversion range where hydrolysis was considered having minor effect on the conversion. The data presented that decreased protein content generally led to increase of cellulose conversion (Fig. 3.2a). The samples with less than 10% protein yielded the cellulose conversions of greater than 0.8 g/g original cellulose. However, some samples with higher protein content (14.3, 11.1 and 11.1 % protein) also had the conversions greater than 0.8 g/g original cellulose. Comparing with other high protein samples with low conversions (less than 0.8 g/g original cellulose), the main difference was that the high

cellulose conversion samples with high protein content had higher lignin/protein ratios of 1:1, 3:1 and 5:1 than the low cellulose conversion sample with high protein content (Table 3.4). The result suggested that lignin and its acid-degraded compounds positively interact with protein and its acid-degraded compounds, reduce the availability of protein and its degraded compounds for condensation reactions between glucose and nitrogen compounds, and improve the efficiency of cellulose conversion. Meanwhile, changes of xylan and cellulose contents in the synthetic samples did not yield any trends of cellulose conversion (Fig. 3.2b), which indicated that cellulose and xylan contents are less important factors on cellulose conversion.

The effects of individual components and their ratios on cellulose conversion made clear that without considering the structure influence of fiber matrix, protein and lignin are the most important factors that influence the cellulose conversion of synthetic feedstocks. The statistical analysis further concluded that 0.9 ± 0.1 g/g cellulose in original sample can be achieved by maintaining protein content of less than 10 wt% or lignin/protein ratio of bigger than 3:1.

3.4.2 Effects of xylan, cellulose, and lignin on xylan hydrolysis of synthetic feedstocks

Unlike cellulose conversion, the xylan conversion in the synthetic samples were unaffected by either ratio or contents of protein, lignin, cellulose, and xylan. Figure 3.3 demonstrated consistent conversions at 1.0±0.1 g/g xylan in original sample. It has been reported that the reactivity of xylose in browning reaction is reduced 100 times under low pH conditions (pH<3) than high pH conditions (pH=7) (Apriyantono & Ames, 1993), while corresponding glucose reactivity in browning reaction is only reduced by 60% at low pH (pH<4) condition compared to high pH condition (pH>7) (Ames, Defaye, & Bates, 1997). The slow reaction rate of xylose browning reaction under concentrated acid conditions can be the reason that xylan conversion maintained relatively no change within the experimental conditions.

3.4.3 Effects of protein and lignin interaction on concentrated acid carbohydrate analysis of lignocellulosic feedstocks

Corn stover, alfalfa, and poplar were selected to validate the observed relationship from the synthetic feedstocks. ND-washed feedstocks were mixed with protein (peptone) at three different levels (both below and above 10 wt% protein). The cellulose, hemicellulose, and lignin contents of ND-washed and measured feedstocks were listed in Table 3.5. All three feedstocks had the same trend of decreasing cellulose conversion with increasing protein content (Fig. 3.4a). Under the higher protein contents of 21.2%, 22.8%, and 15.8% with respect to alfalfa, corn stover, and poplar, the cellulose conversion were 0.73, 0.79, and 0.88 g/g cellulose in ND-washed sample, respectively. The cellulose conversion of poplar was higher than 0.8 g/g cellulose in ND-washed sample, which was different from the results of hydrolysis of synthetic feedstocks. Compared to the herbaceous crop and crop residue (alfalfa and corn stover), the apparent reason is the structure difference, mainly on lignin-carbohydrate interaction.

Herbaceous crops and crop residues contains lignin/phenolics-carbohydrate complex via ferulic bridges between lignin and carbohydrates by ester-linked ferulic acids (Buranov & Mazza, 2008), while poplar forms the lignin-carbohydrate complex via benzyl ester, benzyl ether, and glycosidic linkages (Eriksson, Goring, & Lindgren, 1980). In order to completely release carbohydrates, the lignin-carbohydrate complex has to be degraded. Because benzyl ester, benzyl ether and glycosidic linkages are much stronger than a ferulic acid linkage, it was much slower to release carbohydrates converted into mono-sugars from poplar than herbaceous crops and crop residues during the hydrolysis. The less mono-sugars available in the reaction solution led to the less loss of sugars. Meanwhile, the xylan conversion from alfalfa under higher protein content (Fig 3.4b) was lower than 0.9±0.1 g/g xylan in the original sample, which was also different from the results of synthetic feedstocks. The possible reason might be the great buffering capacity that alfalfa has. It has been reported that acid loadings of 2.25% to treat alfalfa made a reaction solution with pH ~1.0 compared to the same pH with 1.5% acid for grasses (Dien et al., 2006).

3.5 Conclusion

This study demonstrated the different responses of cellulose and xylan to other fiber components and their interaction during fiber analysis. Xylan conversion appeared to be unaffected by either present lignin or protein components. Glucan conversion was heavily influenced by lignin and protein quantities. The statistical analysis concluded that lignocellulosic samples with either less than 10 wt% protein or more than 3:1 ratio of lignin/protein will most likely have the cellulose conversion at 0.9±0.1 g/g cellulose in the original sample. The statistical analysis also showed that different content levels of xylan, cellulose, protein, and lignin in the experimental ranges have no large effect on xylan conversion. These carbohydrate results on synthetic feedstocks were generally verified by the validation test using real lignocellulosic samples and known protein contents, excluding two exceptions of cellulose conversion of poplar under higher protein content and xylan conversion of alfalfa under higher protein content. The results could be used to modify the NREL method of carbohydrate analysis to a substrate-specified method for carbohydrate analysis of different lignocellulosic materials according to their protein and lignin contents.

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Chapter 4

4.1 Conclusion

As previously mentioned, fossil fuels have played an important in the advancement and growth of many societies. But with increasingly high consumption rates, as well as other environmental fears and regulations associated with fossil fuels, ways to generate cleaner and more sustainable energy are vital for present and future societies. This research work of using anaerobic treatment to produce energy and biorefining feedstock provides an approach to achieve a sustainable solution of bioenergy production.

Chapter 2 studied the effects of different mass ratios of feedstocks, corn stover to swine manure, on digestion performance and fiber quality. From this study the conclusions were:

- Co-digestion of corn stover and swine manure can successfully produce valuable energy products of methane and ethanol.
- Stover-to-manure ratio of 40:60 was the optimal feed ratio for the process in terms of digester performance and fiber quality.
- Total net energy output of the stover-to-manure ratio of 40:60 showed an increase of at least 30% compared to other four ratios.
- Ethanol production contributed over 14% of the total net energy output, showing that fiber produced from the digestion of corn stover and swine manure is a valuable feedstock for a biorefinery.

Chapter 3 investigated component interactions during compositional analysis. This work was an important contribution for accurately determining the carbohydrate composition from protein-enriched fiber produced from the anaerobic treatment of corn stover and swine manure, which has been proven to be a valuable feedstock for a biorefinery. The conclusions from this study were:

- Glucan conversion was heavily influenced by lignin and protein quantities.
- Statistical analysis concluded that lignocellulosic samples with either less than 10 wt% protein or more than 3:1 ratio of lignin/protein will most likely have the cellulose conversion at 0.9±0.1 g/g cellulose in the original sample.
- Statistical analysis also showed that different content levels of xylan, cellulose, protein, and lignin in the experimental ranges have no large effect on xylan conversion.
- Carbohydrate results on synthetic feedstocks were generally verified by the validation test using real lignocellulosic samples and known protein contents, excluding two exceptions of cellulose conversion of poplar under higher protein content and xylan conversion of alfalfa under higher protein content.
- Results could be used to modify the NREL method of carbohydrate analysis to a substrate-specified method for carbohydrate analysis of different lignocellulosic materials according to their protein and lignin contents.

There are a couple of recommendations for future contribution to this study. The first recommendation includes an investigation into different anaerobic treatment processing parameters, such as different HRTs and temperatures, which could lead to further bioenergy benefits. This study only incorporated one HRT and one operational temperature. As suggested from the chapter 2 discussion, extending the HRT for the ratio of 40:60 may increase methane

content in biogas production. The second recommendation includes a thorough analysis into the microbial community within the digestion reactors. Key insights into specific polysaccharide degrading microorganisms may lead to further processing optimizations within the anaerobic treatment. As technology becomes more advanced and testing becomes easier and inexpensive, microbial analysis will be vital in order to understand the full complexity of the anaerobic systems.

APPENDIX

APPENDIX A

Table 2.1 Feedstock Characteristics

	Swine Manure	Raw Corn Stover
Carbon	37.7%	45.4%
Nitrogen	3.8%	0.4%
Cellulose	8.0%	36.3%
Hemicellulose	9.0%	22.0%
Lignin	23.8%	18.6%
Dry Matter	4.0%	92.0%

Table 2.2 Raw Feed Characteristics of each Reactor Ratio

Stover:Swine	**20:80**	**40:60**	**50:50**	**60:40**	**80:20**
Carbon*	39.3%	40.8%	41.6%	42.3%	43.9%
Nitrogen*	3.2%	2.5%	2.1%	1.8%	1.1%
Cellulose*	13.7%	19.3%	22.1%	25.0%	30.6%
Hemicellulose*	11.6%	14.2%	15.5%	16.8%	19.4%
Lignin*	22.8%	21.7%	21.2%	20.7%	19.6%

** Calculated based on reactor ratios and raw feedstocks characteristics*

Table 2.3 Average Gas Composition in Bacterial Systems

Stover:Swine	CH_4[a]	$C0_2$[a]	H_2S[b]
20:80	66.2	31.4	1871
40:60	61.7	35.1	1457
50:50	61.4	35.3	561
60:40	60.0	37.5	489
80:20	58.0	39.5	53

[a] *- denotes values as a percentage (%)*

[b] *- denotes values in parts per million (ppm)*

[*] *- duplicates were used and the average value was presented*

Table 2.4 Changes in Fiber Content from Dilute Alkaline Pretreatment

	Raw Feed Characteristics[a]			Digested Fiber			Pretreated Digested Fiber		
Stover:Swine	Cellulose	Hemicellulose	Lignin	Cellulose	Hemicellulose	Lignin	Cellulose	Hemicellulose	Lignin
20:80	13.7%	11.6%	22.8%	7.8%	9.0%	24.0%	26.8%	13.3%	8.8%
40:60	19.3%	14.2%	21.7%	10.9%	11.2%	26.9%	35.7%	17.4%	6.1%
50:50	22.1%	15.5%	21.2%	13.6%	14.0%	22.1%	41.4%	19.8%	6.9%
60:40	25.0%	16.8%	20.7%	14.9%	13.9%	26.0%	44.4%	20.8%	6.3%
80:20	30.6%	19.4%	19.6%	21.4%	18.4%	25.2%	49.8%	22.4%	5.8%

[a] *- Data of each ratio was calculated based on composition of raw swine manure and corn stover*

[*] *- Duplicates were used and the average value was presented*

Table 2.5 Glucose and Xylose Production from Enzymatic Hydrolysis

Stover:Swine	Glucose[a,*]	Xylose[a,*]
20:80	13.5	4.6
40:60	17.3	6.2
50:50	18.8	8.3
60:40	22.2	9.4
80:20	25.4	11.1

[a] *- Sugar production is presented in concentrations (g L^{-1})*

[*] *- Duplicates were used and the average value was presented*

Table 2.6 Mass balance of energy products from three specific feedstock reactors

	Reactor 20:80	Reactor 40:60	Reactor 80:20
Methane production[a,b]	88	101	77
Ethanol production[a,c]	27	41	49

[a] *– Results are reported as g kg^{-1} dry raw feed.*

[b] *– Data incorporates the recirculation of xylose in digestion system. Assumption of 1g xylose = 1g COD reduced = 0.350L methane. Xylose production for ratios 20:80, 40:60, and 80:20, are 3.2, 5.0, and 6.2 g kg^{-1} dry raw feed, respectively.*

[c] *– Conversion of 50% was assumed from glucose produced in enzymatic hydrolysis.*

Table 2.7 Energy balance from three specific feedstock reactors [a-c]

		Reactor 20:80	Reactor 40:60	Reactor 80:20
Anaerobic Digestion				
	Energy Input[d]	-2100	-2100	-2100
	Energy Output[e]	4422	5039	3848
Ethanol Production				
	Energy Input[f]	-431	-648	-772
	Energy Output	761	1143	1362
Net Energy Balances		**2652**	**3434**	**2338**

[a] *– Results are reported at kJ kg^{-1} dry raw feed.*

[b] *– Energy balance was calculated based on 1 kg of dry raw feed. Input and output energy are negative and positive, respectively.*

[c] *– The higher heating values of methane gas and ethanol are 50 MJ kg^{-1} and 28 MJ kg^{-1}, respectively.*

[d] *- Specific heat for the raw feed was 4.2 kJ kg^{-1}C^{-1}. Average temperature of digester influent for a typical cold weather climate is assumed 10°C. The operation temperature for the anaerobic digester is 35°C.*

[e] *– The energy output for anaerobic digestion was calculated by the heating value of methane (kJ g^{-1}) multiplied by methane production (g kg^{-1} dry raw feed).*

[f] *– The energy required for ethanol production was 15.88 MJ kg^{-1} ethanol (Piccolo & Bezzo, 2009).*

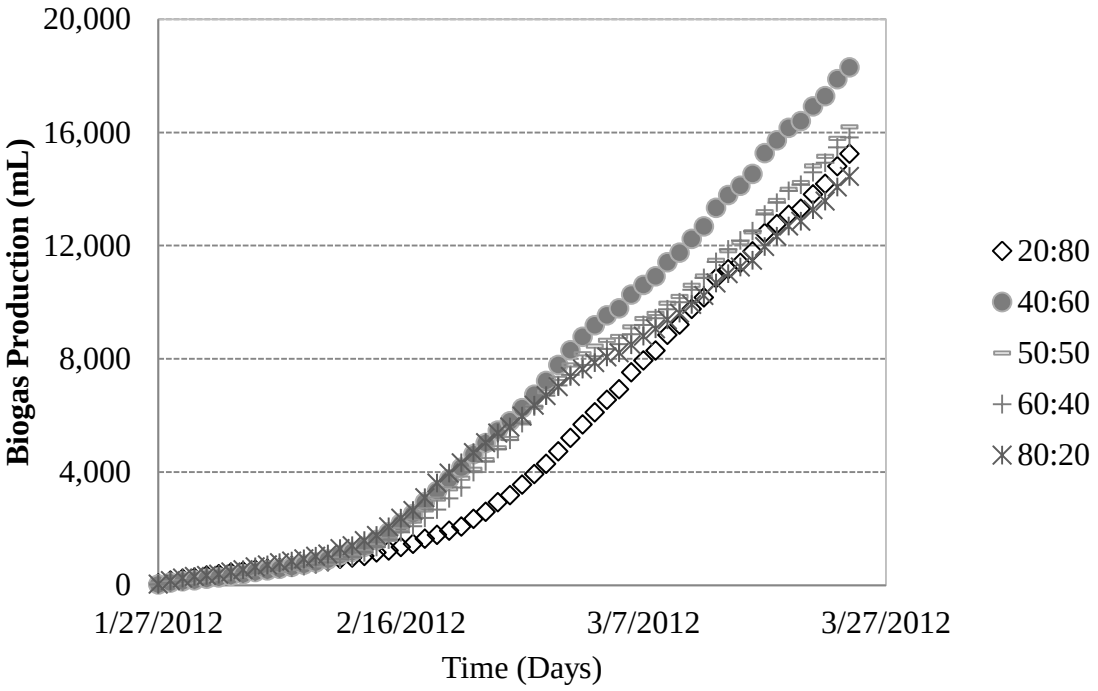
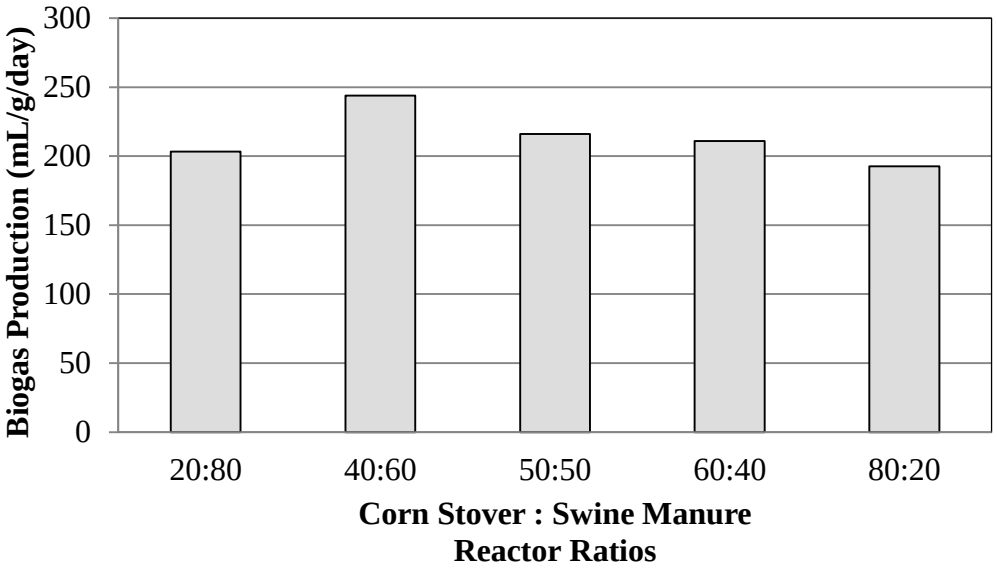


Figure 2.1 Total Accumulated Biogas from Raw Corn Stover Bacterial Systems

a.



b.

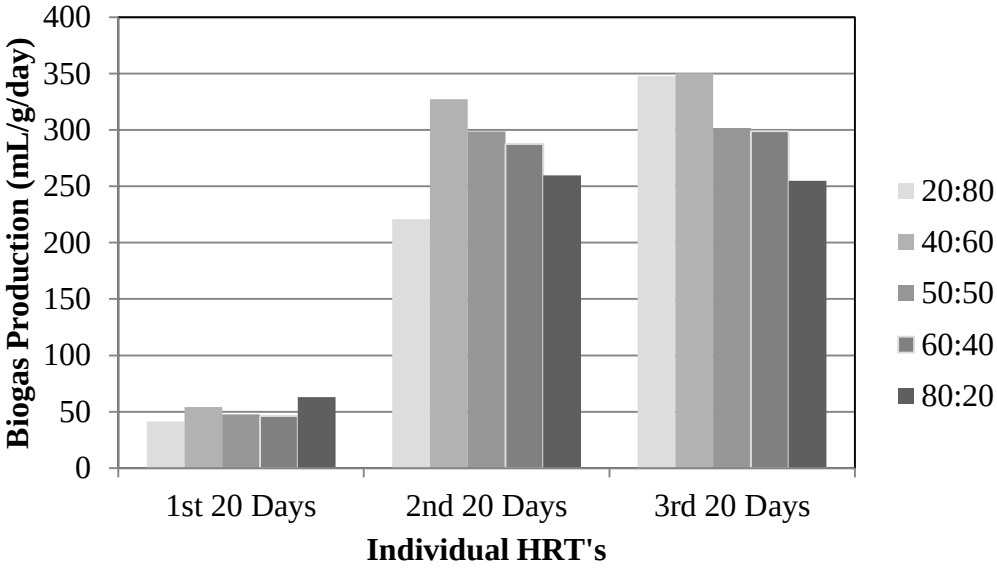
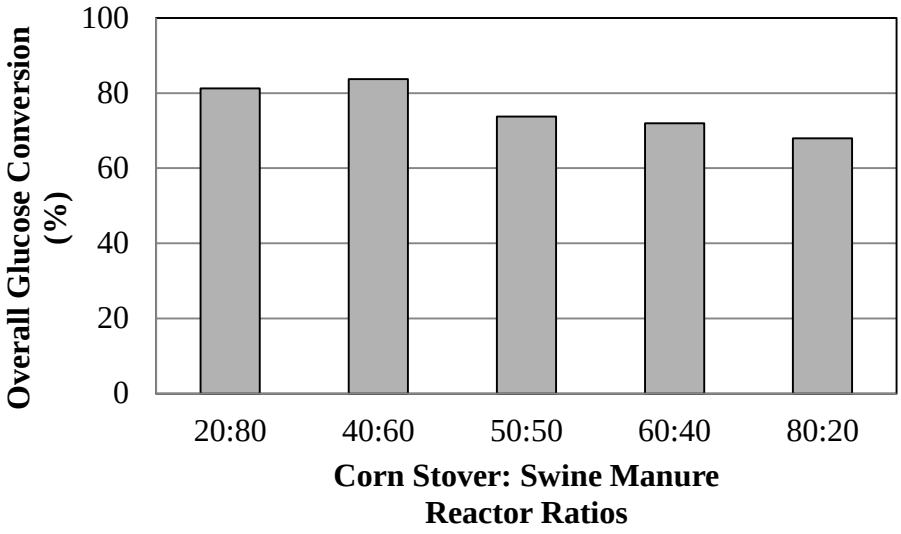


Figure 2.2 Biogas Production Rate per Organic Loading from Anaerobic Treatment Systems**.** a: Overall Biogas Production Rate (60 days). b: Biogas Production Rates during Individual HRTs

a.



b.

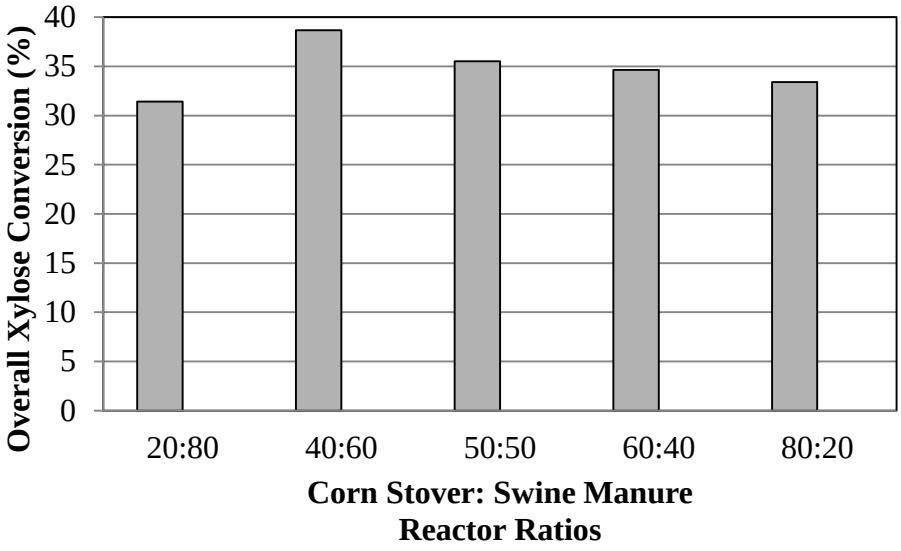


Figure 2.3 Overall Carbohydrate Conversions. a: Overall Glucose Conversion. b: Overall Xylose Conversion

APPENDIX B

Table 3.1 Carbon and nitrogen contents of raw feedstocks

	Carbon content (wt%)	Nitrogen content (wt%)
Corn stover	45.4	0.4
Poplar	47.9	0.2
Alfalfa	41.7	2.2

Table 3.2 Experimental results based on a completely randomized design of protein:cellulose:xylan:lignin ratios

Ratio [a]			Content in the sample				Conversion (%) [b]	
Cellulose:Protein	Xylan:Protein	Lignin:Protein	Protein (%)	Cellulose (%)	Xylan (%)	Lignin (%)	Glucan	Xylan
2:1	1:1	1:1	20.0	40.0	20.0	20.0	77.7 ± 3.29	96.8 ± 2.3
6:1	1:1	1:1	11.1	66.7	11.1	11.1	61.5 ± 0.36	102.4 ± 0.3
2:1	3:1	1:1	14.3	28.6	42.9	14.3	55.8 ± 2.75	93.1 ± 0.9
6:1	3:1	1:1	9.10	54.5	27.3	9.10	89.7 ± 0.58	96.4 ± 0.7
2:1	5:1	1:1	11.1	22.2	55.6	11.1	62.2 ± 5.43	97.2 ± 2.2
6:1	5:1	1:1	7.70	46.2	38.5	7.70	85.9 ± 1.25	101.6 ± 0.1
2:1	1:1	3:1	14.3	28.6	14.3	42.9	91.6 ± 0.72	105.9 ± 0.3
6:1	1:1	3:1	9.10	54.5	9.10	27.3	90.2 ± 0.98	103.6 ± 0.3
2:1	3:1	3:1	11.1	22.2	33.3	33.3	89.3 ± 0.11	99.1 ± 0.5
6:1	3:1	3:1	7.70	46.2	23.1	23.1	90.0 ± 2.34	104.0 ± 1.1
2:1	5:1	3:1	9.10	18.2	45.5	27.3	89.8 ± 2.26	98.8 ± 1.2
6:1	5:1	3:1	6.70	40.0	33.3	20.0	89.1 ± 1.08	100.9 ± 0.6
2:1	1:1	5:1	11.1	22.2	11.1	55.6	91.7 ± 0.79	102.6 ± 0.4
6:1	1:1	5:1	7.7	46.2	7.7	38.5	91.3 ± 0.96	105.3 ± 3.6
2:1	3:1	5:1	9.10	18.2	27.3	45.5	91.5 ± 0.67	99.7 ± 0.6
6:1	3:1	5:1	6.70	40.0	20.0	33.3	90.8 ± 0.29	102.7 ± 0.6
2:1	5:1	5:1	7.70	15.4	38.5	38.5	92.3 ± 3.00	104.4 ± 0.7
6:1	5:1	5:1	5.90	35.3	29.4	29.4	86.0 ± 1.35	103.7 ± 0.1

a. *Ratios are based on the weight.*

b. *The data are the average of three replicates with standard deviation at α=0.05*

Table 3.3 Analysis of variance (ANOVA) for ratios and their interaction on glucan conversions of synthetic feedstocks from completely randomized design

Parameter	Degree of freedom	Mean square	F-value	P-value
Lignin:Protein	2	0.19	471.06	<0.0001
Cellulose:Protein	1	0.018	41.53	<0.0001
Xylan:Protein	2	0.00012	0.29	0.7526
Lignin:Protein x Cellulose:Protein	2	0.0353	83.55	<0.0001
Lignin:Protein x Xylan:Protein	4	0.0022	5.20	0.0021
Cellulose:Protein x Xylan:Protein	2	0.0347	82.26	<0.0001
Lignin:Protein x Cellulose:Protein x Xylan:Protein	4	0.0359	85.09	<0.0001
Error	36	0.0004		
Total	53			

* Complete ANOVA analysis can be seen in the Appendix A section of this document

Table 3.4 Effects of protein and lignin on cellulose conversion of synthetic feedstocks under high protein content (greater than 10 wt%)[a]

Content in the synthetic feedstocks (wt%)				Ratio			Cellulose conversion (%) [b]
Protein	Lignin	Cellulose	Xylan	Lignin:Protein	Cellulose:Protein	Xylan:Protein	
20.0	20.0	40	20	1:1	2:1	1:1	77.7 ± 3.29
14.3	14.3	28.6	42.9	1:1	2:1	3:1	55.8 ± 2.75
11.1	11.1	66.7	11.1	1:1	6:1	1:1	61.5 ± 0.36
11.1	11.1	22.2	55.6	1:1	2:1	5:1	62.2 ± 5.43
14.3	**42.9**	28.6	14.3	**3:1**	2:1	1:1	**91.6 ± 0.72**
11.1	**33.3**	22.2	33.3	**3:1**	2:1	3:1	**89.3 ± 0.11**
11.1	**55.6**	22.2	11.1	**5:1**	2:1	1:1	**91.7 ± 0.79**

a. *Ratios are based on the weight.*

b. *The data are the average of three replicates with standard deviation at α=0.05*

Table 3.5 Validation of carbohydrate analysis using different lignocellulosic feedstocks[a]

Feedstocks	Treatment		Cellulose (wt%)	Xylan (wt%)	Lignin (wt%)
	Protein content (wt%)	Ratio of protein:lignin			
Alfalfa	NDF-washed (no protein)	0	38.6	13.0	21.2
	4.2	1:5	32.1	11.8	20.6
	7.1	1:3	31.9	11.7	20.6
	21.2	1:1	28.1	10.3	21.9
Poplar	NDF-washed (no protein)	0	34.3	13.6	22.8
	4.6	1:5	32.7	13.2	21.7
	7.6	1:3	31.9	12.8	21.2
	22.8	1:1	30.0	13.3	22.3
Corn stover	NDF-washed (no protein)	0	34.9	26.2	15.8
	3.2	1:5	30.7	24.7	11.7
	5.3	1:3	29.1	25.9	11.6
	15.8	1:1	27.6	25.7	12.3

a. The data are the average of two replicates.

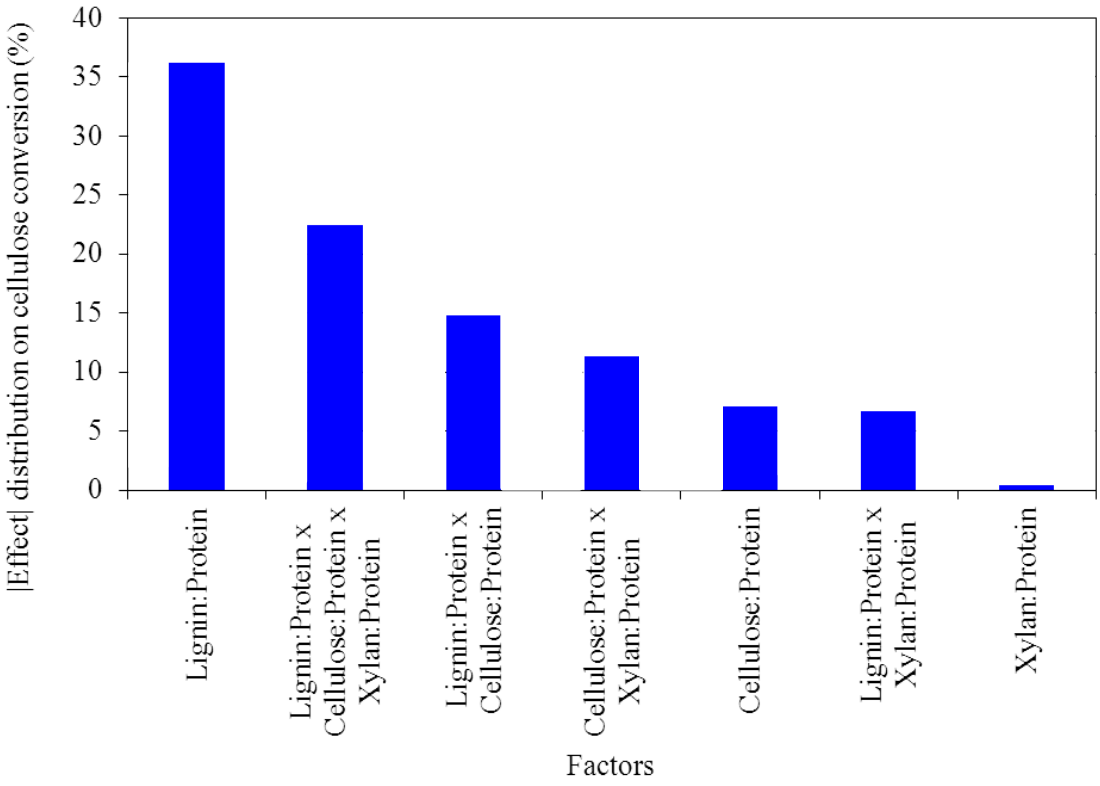
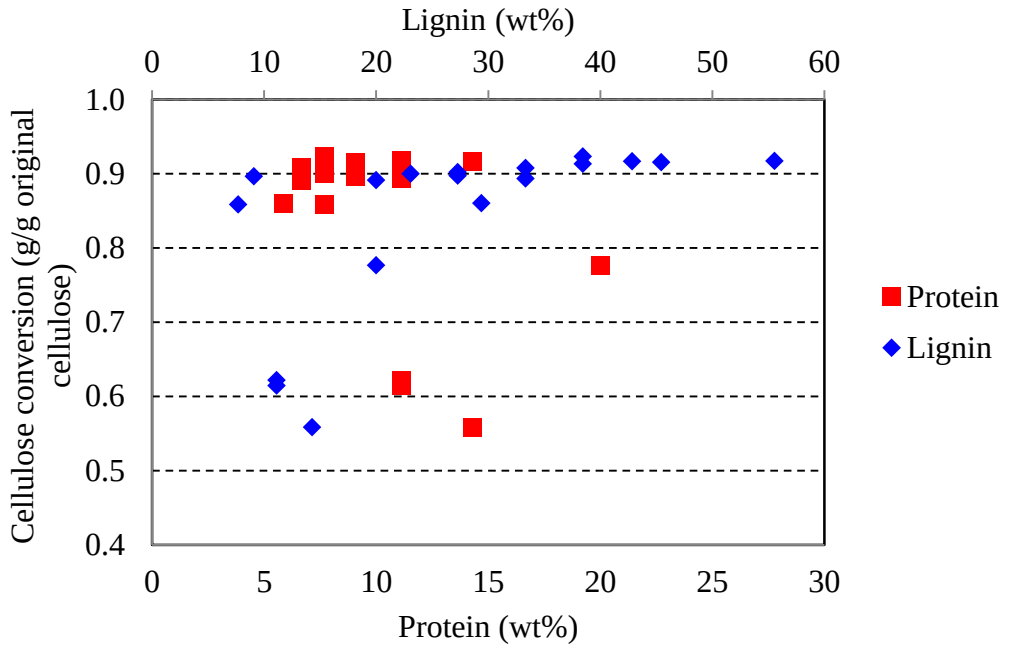
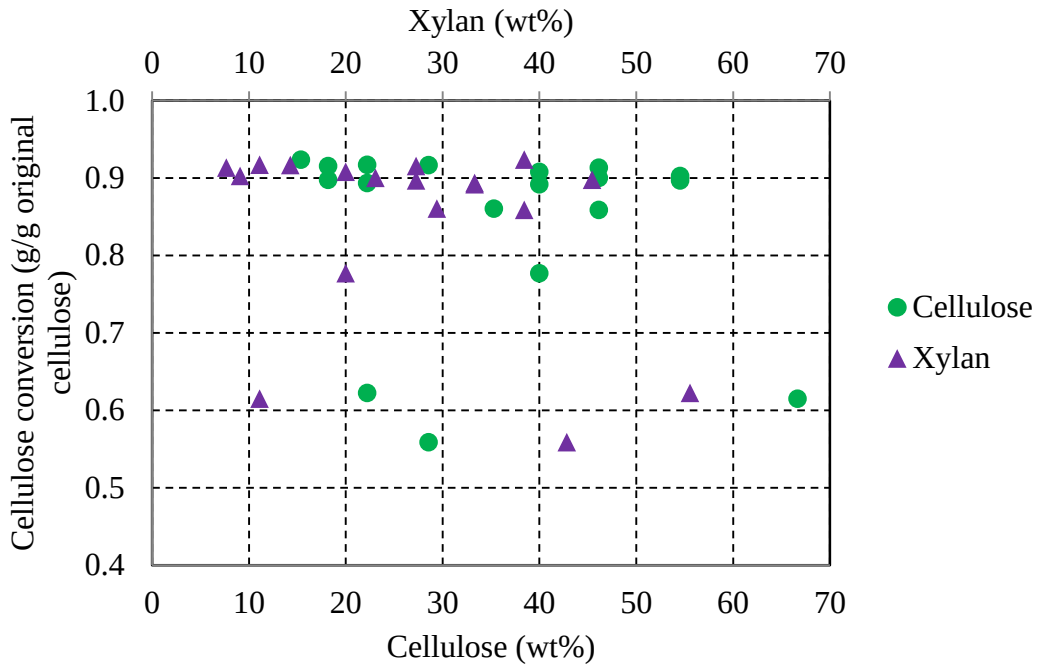


Figure 3.1 Pareto charts of effects of ratios of lignin/protein, cellulose/protein, and xylan/protein on cellulose conversion of synthetic feedstocks

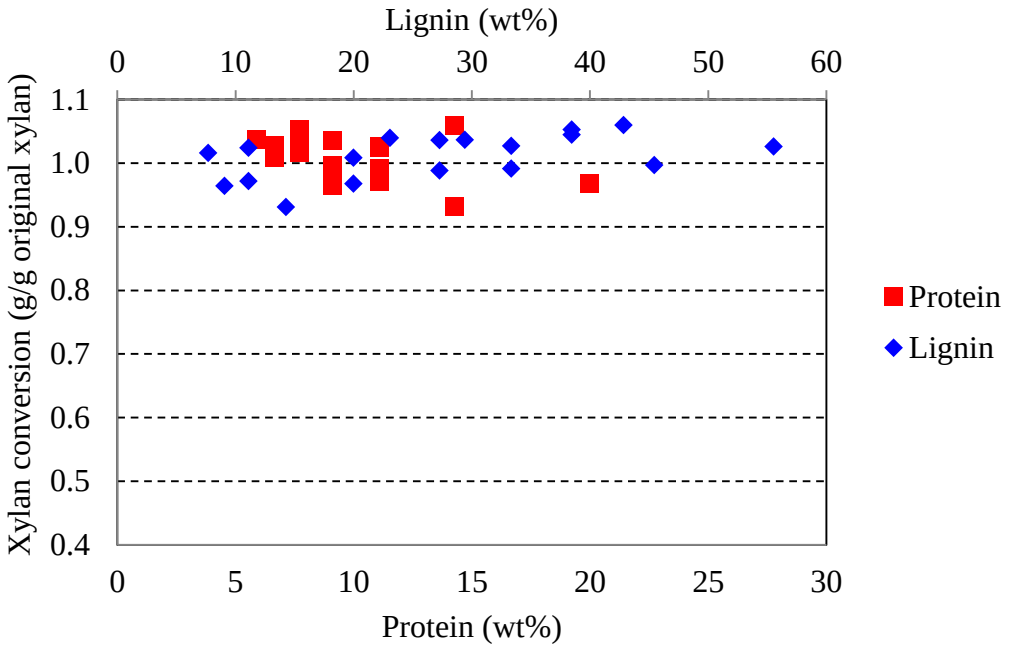


a. Effects of protein and lignin

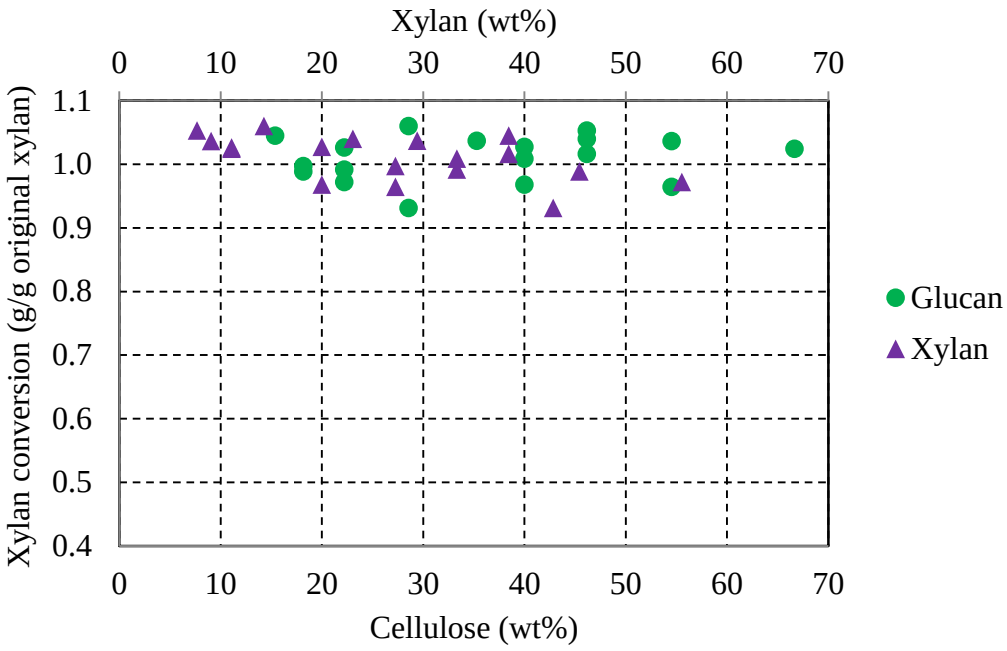


b. Effects of cellulose and xylan

Figure 3.2 Effects of protein, lignin, cellulose, and xylan contents on cellulose conversion of synthetic feedstocks

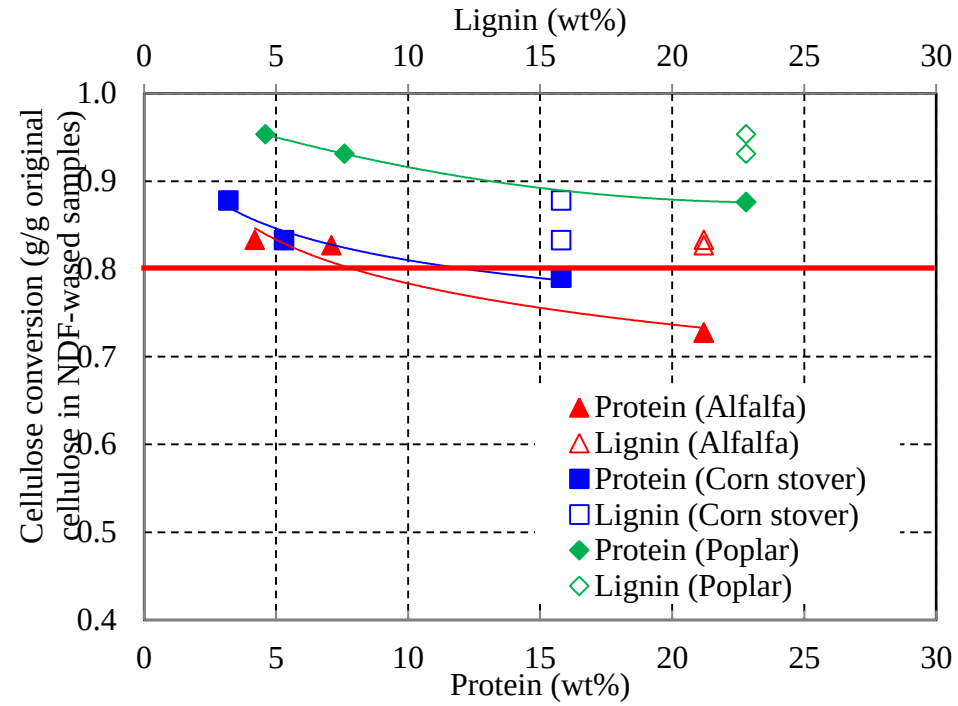


a. Effects of protein and lignin

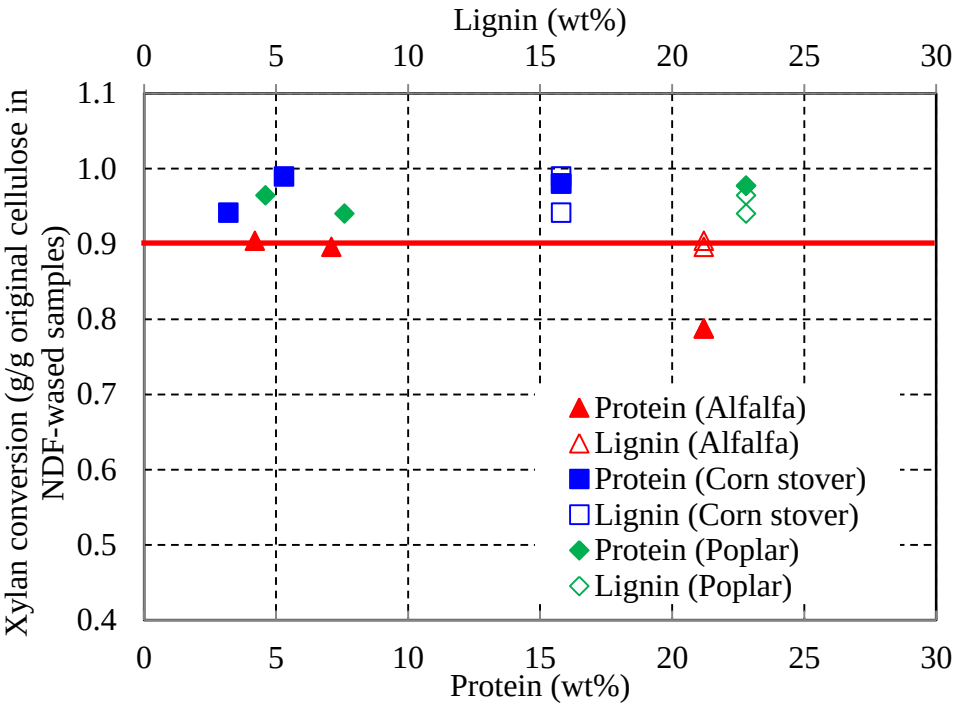


b. Effects of cellulose and xylan

Figure 3.3 Effects of protein, lignin, cellulose, and xylan on xylan conversion of synthetic feedstocks



a. Cellulose conversion



b. Xylan conversion

Figure 3.4 Effects of protein and lignin on carbohydrate conversion of lignocellulosic feedstocks

APPENDIX C

Statistical Analysis Code

Class Level Information

Class	Levels	Values
G	2	2 6
X	3	1 3 5
L	3	1 3 5

Glu 0.5319 0.5566 0.5704 0.5868 0.6118 0.6133 0.6166 0.6186 0.6787 0.7432 0.7774
0.8089 0.845 0.8502 0.8525 0.8658 0.8703 0.8715 0.8728 0.873 0.883 0.8877
0.8894 0.8909 0.8919 0.8925 0.8937 0.894 0.9032 0.9036 0.9041 0.9047 0.9076
0.9078 0.9079 0.9082 0.9086 0.9096 0.9102 0.9104 0.9113 0.912 0.9124 0.9149
0.9151 0.9161 0.9166 0.9213 0.9232 0.9244 0.9253 0.9344 0.9461

Number of Observations Read	54
Number of Observations Used	54

The GLM Procedure

Dependent Variable: Glu

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	0.70824148	0.04166126	98.65	<.0001
Error	36	0.01520388	0.00042233		
Corrected Total	53	0.72344536			

R-Square	Coeff Var	Root MSE	Glu Mean
0.978984	2.439442	0.020551	0.842433

Source	DF	Type I SS	Mean Square	F Value	Pr > F
G	1	0.01753923	0.01753923	41.53	<.0001
X	2	0.00024201	0.00012101	0.29	0.7526
G*X	2	0.06948041	0.03474021	82.26	<.0001
L	2	0.39788721	0.19894360	471.06	<.0001
G*L	2	0.07057096	0.03528548	83.55	<.0001
X*L	4	0.00877981	0.00219495	5.20	0.0021
G*X*L	4	0.14374185	0.03593546	85.09	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
G	1	0.01753923	0.01753923	41.53	<.0001
X	2	0.00024201	0.00012101	0.29	0.7526
G*X	2	0.06948041	0.03474021	82.26	<.0001
L	2	0.39788721	0.19894360	471.06	<.0001
G*L	2	0.07057096	0.03528548	83.55	<.0001
X*L	4	0.00877981	0.00219495	5.20	0.0021
G*X*L	4	0.14374185	0.03593546	85.09	<.0001

The GLM Procedure

Level of	----------Glu----------		
G	N	Mean	Std Dev
2	27	0.82441111	0.13733721
6	27	0.86045556	0.09104245

Level of	----------Glu----------		
X	N	Mean	Std Dev
1	18	0.83997778	0.11639898
3	18	0.84514444	0.13277023
5	18	0.84217778	0.10660543

Level of	----------Glu----------		
L	N	Mean	Std Dev
1	18	0.72108889	0.13529957
3	18	0.90009444	0.01506334
5	18	0.90611667	0.02485159

The GLM Procedure

Least Squares Means

	H0:LSMean1=Standard	H0:LSMEAN=0	LSMean2	
G	Glu LSMEAN	Error	Pr > |t|	Pr > |t|
2	0.82441111	0.00395498	<.0001	<.0001
6	0.86045556	0.00395498	<.0001	

		Standard	LSMEAN	
X	Glu LSMEAN	Error	Pr > |t|	Number
1	0.83997778	0.00484384	<.0001	1
3	0.84514444	0.00484384	<.0001	2
5	0.84217778	0.00484384	<.0001	3

Least Squares Means for effect X

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Glu

i/j	1	2	3
1		0.4556	0.7499
2	0.4556		0.6675
3	0.7499	0.6675	

G	X	Glu LSMEAN	Standard Error	LSMEAN Pr > |t|	Number
2	1	0.87000000	0.00685022	<.0001	1
2	3	0.78900000	0.00685022	<.0001	2
2	5	0.81423333	0.00685022	<.0001	3
6	1	0.80995556	0.00685022	<.0001	4
6	3	0.90128889	0.00685022	<.0001	5
6	5	0.87012222	0.00685022	<.0001	6

The GLM Procedure

Least Squares Means

Least Squares Means for effect G*X

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Glu

i/j	1	2	3	4	5	6
1		<.0001	<.0001	<.0001	0.0026	0.9900
2	<.0001		0.0133	0.0372	<.0001	<.0001
3	<.0001	0.0133		0.6614	<.0001	<.0001
4	<.0001	0.0372	0.6614		<.0001	<.0001
5	0.0026	<.0001	<.0001	<.0001		0.0027
6	0.9900	<.0001	<.0001	<.0001	0.0027	

L	Glu LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	0.72108889	0.00484384	<.0001	1
3	0.90009444	0.00484384	<.0001	2
5	0.90611667	0.00484384	<.0001	3

Least Squares Means for effect L

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Glu

i/j	1	2	3
1		<.0001	<.0001
2	<.0001		0.3852
3	<.0001	0.3852	

G	L	Glu LSMEAN	Standard Error	LSMEAN Pr > |t|	Number
2	1	0.65227778	0.00685022	<.0001	1
2	3	0.90240000	0.00685022	<.0001	2
2	5	0.91855556	0.00685022	<.0001	3

The GLM Procedure

Least Squares Means

G	L	Glu LSMEAN	Standard Error	LSMEAN Pr > |t|	Number
6	1	0.78990000	0.00685022	<.0001	4
6	3	0.89778889	0.00685022	<.0001	5
6	5	0.89367778	0.00685022	<.0001	6

Least Squares Means for effect G*L

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Glu

i/j	1	2	3	4	5	6
1		<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001		0.1041	<.0001	0.6370	0.3739
3	<.0001	0.1041		<.0001	0.0389	0.0145
4	<.0001	<.0001	<.0001		<.0001	<.0001
5	<.0001	0.6370	0.0389	<.0001		0.6738
6	<.0001	0.3739	0.0145	<.0001	0.6738	

X	L	Glu LSMEAN	Standard Error	LSMEAN Pr > |t|	Number
1	1	0.69553333	0.00838978	<.0001	1
1	3	0.90935000	0.00838978	<.0001	2
1	5	0.91505000	0.00838978	<.0001	3
3	1	0.72750000	0.00838978	<.0001	4
3	3	0.89646667	0.00838978	<.0001	5
3	5	0.91146667	0.00838978	<.0001	6
5	1	0.74023333	0.00838978	<.0001	7
5	3	0.89446667	0.00838978	<.0001	8
5	5	0.89183333	0.00838978	<.0001	9

The GLM Procedure

Least Squares Means

Least Squares Means for effect X*L

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Glu

i/j	1	2	3	4	5	6	7	8	9
1		<.0001	<.0001	0.0107	<.0001	<.0001	0.0006	<.0001	<.0001
2	<.0001		0.6338	<.0001	0.2848	0.8594	<.0001	0.2178	0.1485
3	<.0001	0.6338		<.0001	0.1260	0.7644	<.0001	0.0913	0.0582
4	0.0107	<.0001	<.0001		<.0001	<.0001	0.2903	<.0001	<.0001
5	<.0001	0.2848	0.1260	<.0001		0.2143	<.0001	0.8671	0.6985
6	<.0001	0.8594	0.7644	<.0001	0.2143		<.0001	0.1605	0.1067
7	0.0006	<.0001	<.0001	0.2903	<.0001	<.0001		<.0001	<.0001
8	<.0001	0.2178	0.0913	<.0001	0.8671	0.1605	<.0001		0.8256
9	<.0001	0.1485	0.0582	<.0001	0.6985	0.1067	<.0001	0.8256	

G	X	L	Glu LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
2	1	1	0.77650000	0.01186493	<.0001	1
2	1	3	0.91650000	0.01186493	<.0001	2
2	1	5	0.91700000	0.01186493	<.0001	3
2	3	1	0.55843333	0.01186493	<.0001	4
2	3	3	0.89320000	0.01186493	<.0001	5
2	3	5	0.91536667	0.01186493	<.0001	6
2	5	1	0.62190000	0.01186493	<.0001	7
2	5	3	0.89750000	0.01186493	<.0001	8
2	5	5	0.92330000	0.01186493	<.0001	9
6	1	1	0.61456667	0.01186493	<.0001	10
6	1	3	0.90220000	0.01186493	<.0001	11
6	1	5	0.91310000	0.01186493	<.0001	12
6	3	1	0.89656667	0.01186493	<.0001	13
6	3	3	0.89973333	0.01186493	<.0001	14
6	3	5	0.90756667	0.01186493	<.0001	15
6	5	1	0.85856667	0.01186493	<.0001	16
6	5	3	0.89143333	0.01186493	<.0001	17
6	5	5	0.86036667	0.01186493	<.0001	18

The GLM Procedure

Least Squares Means

Least Squares Means for effect G*X*L

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Glu

i/j	1	2	3	4	5	6	7	8	9
1		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001		0.9764	<.0001	0.1735	0.9465	<.0001	0.2650	0.6877
3	<.0001	0.9764		<.0001	0.1647	0.9230	<.0001	0.2528	0.7095
4	<.0001	<.0001	<.0001		<.0001	<.0001	0.0006	<.0001	<.0001
5	<.0001	0.1735	0.1647	<.0001		0.1948	<.0001	0.7992	0.0812
6	<.0001	0.9465	0.9230	<.0001	0.1948		<.0001	0.2941	0.6392
7	<.0001	<.0001	<.0001	0.0006	<.0001	<.0001		<.0001	<.0001
8	<.0001	0.2650	0.2528	<.0001	0.7992	0.2941	<.0001		0.1329
9	<.0001	0.6877	0.7095	<.0001	0.0812	0.6392	<.0001	0.1329	
10	<.0001	<.0001	<.0001	0.0019	<.0001	<.0001	0.6647	<.0001	<.0001
11	<.0001	0.3997	0.3836	<.0001	0.5950	0.4378	<.0001	0.7810	0.2167
12	<.0001	0.8406	0.8175	<.0001	0.2434	0.8933	<.0001	0.3587	0.5471
13	<.0001	0.2426	0.2312	<.0001	0.8421	0.2700	<.0001	0.9559	0.1199
14	<.0001	0.3244	0.3103	<.0001	0.6993	0.3577	<.0001	0.8949	0.1687
15	<.0001	0.5977	0.5775	<.0001	0.3976	0.6448	<.0001	0.5523	0.3547
16	<.0001	0.0014	0.0013	<.0001	0.0463	0.0017	<.0001	0.0261	0.0005
17	<.0001	0.1439	0.1363	<.0001	0.9167	0.1624	<.0001	0.7198	0.0656
18	<.0001	0.0019	0.0018	<.0001	0.0582	0.0023	<.0001	0.0333	0.0006

Least Squares Means for effect G*X*L

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Glu

i/j	10	11	12	13	14	15	16	17	18
1	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001	0.3997	0.8406	0.2426	0.3244	0.5977	0.0014	0.1439	0.0019
3	<.0001	0.3836	0.8175	0.2312	0.3103	0.5775	0.0013	0.1363	0.0018
4	0.0019	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
5	<.0001	0.5950	0.2434	0.8421	0.6993	0.3976	0.0463	0.9167	0.0582
6	<.0001	0.4378	0.8933	0.2700	0.3577	0.6448	0.0017	0.1624	0.0023
7	0.6647	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
8	<.0001	0.7810	0.3587	0.9559	0.8949	0.5523	0.0261	0.7198	0.0333
9	<.0001	0.2167	0.5471	0.1199	0.1687	0.3547	0.0005	0.0656	0.0006
10		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
11	<.0001		0.5201	0.7390	0.8839	0.7509	0.0134	0.5252	0.0174
12	<.0001	0.5201		0.3310	0.4309	0.7435	0.0025	0.2048	0.0033
13	<.0001	0.7390	0.3310		0.8514	0.5163	0.0297	0.7614	0.0377
14	<.0001	0.8839	0.4309	0.8514		0.6434	0.0191	0.6239	0.0246
15	<.0001	0.7509	0.7435	0.5163	0.6434		0.0060	0.3427	0.0079
16	<.0001	0.0134	0.0025	0.0297	0.0191	0.0060		0.0579	0.9152
17	<.0001	0.5252	0.2048	0.7614	0.6239	0.3427	0.0579		0.0723
18	<.0001	0.0174	0.0033	0.0377	0.0246	0.0079	0.9152	0.0723	