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DEVELOPMENT OF A CELLULOSIC ETHANOL PRODUCTION PROCESS INTEGRATING ANAEROBIC DIGESTION WITH BIOREFINING

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Abstract

DEVELOPMENT OF A CELLULOSIC ETHANOL PRODUCTION PROCESS INTEGRATING ANAEROBIC DIGESTION WITH BIOREFINING

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Anaerobic digestion (AD) of animal manure is traditionally classified as a treatment to reduce the environmental impacts of odor, pathogens, and excess nutrients associated with animal manure. This report shows that AD also changed the composition of manure fiber and made it suitable as a cellulosic feedstock for ethanol production by increasing the cellulose content, reducing the particle size, and enhancing the digestibility. The solid digestate from an anaerobic digester (AD fiber) was assessed for ethanol production in this paper. AD fiber from two types of digesters was used in this study, a plug-flow reactor (PFR) and a completely stirred tank reactor (CSTR). Switchgrass and corn stover were used as controls for comparison to a more researched energy crop and agricultural residue. Dilute alkali and dilute acid pretreatment methods were compared for effectiveness of ethanol production. Using the most effective dilute alkali pretreatment conditions (2% sodium hydroxide, 130°C, and 2 h), enzymatic hydrolysis of 10% (dry basis) pretreated AD fiber from a plug flow reactor (PFR) produced 51 g/L glucose at a conversion rate of 90%. The ethanol fermentation on the hydrolysate had a 72% ethanol yield. The results indicated that 120 million dry tons of cattle manure available annually in the U.S. can generate 63 million dry tons of AD fiber that can produce more than 1.67 billion gallons of ethanol. Integrating AD with biorefining will make significant a contribution to cellulosic ethanol production.

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INTRODUCTION

Petroleum provides more energy in the United States than any other resource, about 37% of all the energy consumed or about 19.5 million barrels per day. Domestic petroleum reserves and production are limited at 8.5 million barrels per day; therefore almost 11 million barrels per day, or 56% of the petroleum consumed, are imported into the United States (United States Energy Information Administration, 2008). Long term economic, environmental and national security concerns over petroleum have motivated research into renewable domestic sources over the last three decades. Ethanol is the most important renewable fuel in terms of volume and market value. A record 10.75 billion gallons of corn-based ethanol was produced in the U.S. in 2009 (RFA 2009). Corn-based ethanol is the only commercial production system operating in the United States. Currently practiced technologies in the fuel ethanol industry utilize the fermentation of sugars from starch and sugar crops are relatively mature with little opportunity for process enhancements. While reducing the need for foreign oil, corn-based ethanol also diverts corn away from food markets, inevitably resulting in food-fuel competition (Koh and Ghazoul, 2008). Therefore alternative sources of feedstock are necessary to produce ethanol commercially without competing for food.

Lignocellulosic biomass, including agricultural residue, forest residue, dedicated energy crops, municipal solid waste, animal waste, etc., is a renewable resource considered to be a solution to the feedstock for ethanol problem. It has great potential for affordable ethanol production because it is less expensive than starch and sucrose crops, corn and sugarcane, and is available in large quantities. The USDA Billion Ton study indicated that with enhanced technology, 1.2 billion dry tons of lignocellulosic feedstock

can be used to produce 60 billion gallons of ethanol at 2030. However, the current available biomass for bio-fuel production is only 194 million dry tons per year. Animal manure, especially cattle manure, is included in the USDA billion ton study, but only 35 million dry tons is included as potential biomass to ethanol production. It has been estimated that a total of about 120 million dry tons of cattle manure are produced annually in the United States (USDA Economic Research Service, 1997). This represents a considerable amount of untapped biomass for bio-based energy production. In order to achieve sustainable development of animal production and bio-fuel industries, an integrated solution is necessary for animal manure management and cellulosic feedstock production.

Anaerobic digestion (AD) is a natural biological process that has been proven effective at converting wet organic biomass into energy in the form of biogas. The biogas produced consists mainly of methane and carbon dioxide, which can be combusted to produce relatively clean electricity. Anaerobic digestion also provide a wide array of benefits including, significant reduction of odors and flies associated with manure, greenhouse gas emissions are reduced, production of a relatively clean liquid effluent for fertilizer and irrigation, pathogens are reduced in the liquid and solid products, and nonpoint source pollution is substantially reduced (Burke, 2001). Even with all these benefits, AD is an underutilized resource mainly due to the economics. For most confined cattle operations, the high capital cost and the relatively low revenue from biogas methane production, make the currently available AD technology difficult to be adopted. The payback period on capital investment needs to be reduced for further adoption of the AD process worldwide. Utilization of the solid digestate from anaerobic digestion (AD

fiber) for lignocellulosic ethanol production has potential to greatly increase the economic feasibility of waste-to-energy production.

The goal of the current research is to develop a better understanding of the integrated process of anaerobic digestion and biorefining. The hypothesis is that ***anaerobically treated manure fiber (AD fiber) is capable of producing ethanol in a similar effectiveness to that of more researched agricultural residues and dedicated energy crops, corn stover and switchgrass.*** The composition of AD fiber and the effectiveness of various pretreatments on enhancing enzymatic digestibility and yeast fermentability of AD fiber must be assessed to determine if the carbohydrate content and conversion yields are large enough to generate a significant amount of ethanol effectively.

The composition of a material is of great importance in determining if the biomass is suitable for use as a fermentation feedstock. Most biomass is not fermentable without pretreatment to allow access to the sugars, because the potential fermentable sugars are in a polymeric form (polysaccharides). The polysaccharides are further bound in the plant cell walls by interactions between the polysaccharides as well as with various other non-carbohydrate constituents, mainly lignin. Ultimately, pretreatment is required to breakdown the polysaccharides into individual sugar units (monosaccharides), a form which the fermentative organisms will be able to utilize. The most commonly used fermentative organisms are yeast, or more specifically *Saccharomyces cerevisiae*, due to its robust nature and ability to ferment C-6 sugars (glucose mannose, and galactose). The main problem associated with *Saccharomyces cerevisiae* is that it cannot ferment C-5 sugars (xylose and arabinose). However, with the combination of anaerobic digestion and

biorefining, the C-5 sugars can be utilized in the anaerobic digester to by microorganisms to produce biogas. Therefore a genetically engineered microorganism is not required for effective fermentation.

To date, the process of obtaining monosaccharides from biomass has been a two-stage process whereby the first stage breaks down the biomass cell wall structure, and the second step depolymerizes the polysaccharides. Several forms of pretreatment have been investigated utilizing different types of biomass. Two predominant processes are dilute acid and dilute alkali pretreatment; each followed by enzymatic hydrolysis. The effectiveness of pretreating each raw material feedstocks varies depending on the pretreatment process and conditions. Therefore, each pretreatment method must be assessed for the effectiveness on enhancing the digestibility of AD fiber. The best pretreatment method and conditions can then be determined for the most effective utilization of AD fiber for ethanol production.

The following sections describe in detail the factors that must be addressed in the integration of anaerobic digestion and biorefining. These factors include; biomass composition, limitations of enzymatic digestibility, pretreatment processes including, anaerobic digestion, dilute acid, and dilute alkali, enzymatic hydrolysis, and fermentation. Assessing all these topics will provide a better understanding of using AD fiber as a feedstock for ethanol production.

LITERATURE REVIEW

1.1. Lignocellulose Fiber Characteristics

Lignocellulosic biomass is primarily composed of three types of polymers, cellulose, hemicellulose, and lignin, in addition to smaller amounts of pectin, protein, extractives and ash. Carbohydrates are the largest fraction (50-80% dry basis) of lignocellulosic biomass, which includes cellulose and hemicellulose (Zheng et al., 2009). Cellulose is a linear polysaccharide of D-glucose units connected by β-1,4-glycosidic bonds with a degree of polymerization of up to 10,000 or higher (McMillan, 1994; Jorgensen et al., 2007). Cellulose consists in a hierarchal structure of smaller at mechanistically stronger units (Subramanian et al., 2008). Hydrogen bonds pack the cellulose chains together into elementary fibrils, the basic unit of cellulose fiber, which are approximately 3nm in diameter (Ha et al., 1998). Elementary fibrils consist of 36 linear cellulose chains aggregated by both intra- and intermolecular hydrogen bonds (Jorgensen et. al., 2007). Microfibrils are composed of the elementary fibrils packed together with hydrogen bonds. These microfibrils are attached to each other by hemicelluloses, pectin, and lignin, and are associated in the form of bundles or macrofibrils (Taherzadeh and Karimi, 2008). This complex and highly crystalline structure makes cellulose resistant to biological and chemical treatments. Regions of a less organized, amorphous structure exist within the crystalline structure of native cellulose (Hendriks and Zeeman, 2008). These amorphous areas are most susceptible to enzymatic attack.

Hemicelluloses are shorter chain, amorphous polysaccharides of hexosans (mannan, galactan, and glucan), pentosans (xylan and arabinan), as well as uronic acids,

methoxyl, acetyl, and free carboxylic groups (McMillan, 1994). The dominant sugars in hemicellulose are xylan in hardwoods and agricultural residues, and glucomannan for softwood. Unlike cellulose, hemicelluloses have random, amorphous, and branched structures that offer little resistance to hydrolysis (Taherzadeh and Karimi, 2008). Removal of hemicellulose increases the porosity of biomass and therefore increases the accessibility of cellulose for enzymatic hydrolysis (Chandra et al., 2007). The degree of acetylation in hemicellulose is another important factor in enzymatic digestibility because lignin and acetyl groups are attached to hemicellulose and may hinder the reduction of carbohydrates (Chang and Holtzapple, 2000). It has been found that samples with the same amount of deacetylation produce the same sugar yields upon enzymatic hydrolysis. An increase in the degree of deacetylation increases the yield of sugars obtained from enzymatic hydrolysis with all other compositional factors held constant. For aspen wood, both acetyl group and lignin content were important barriers to effective enzymatic hydrolysis; however the xylan backbone was not (Kong et al.,1992).

Lignin, the most abundant non-polysaccharide fraction of lignocellulosic biomass, is an aromatic polymer constructed of three different phenylpropane units, p-coumaryl, coniferyl, and sinapyl alcohol (Hendriks and Zeeman, 2008). A protective covering is formed around cellulose by lignin and hemicellulose, which enhances structural strength to the biomass matrix. Lignin is the main component in the outer portion of the middle lamellae, effectively creating a seal at the outer edge of lignocellulosic fibers (McMillan, 1994). Structural support, resistance against microbial and oxidation stress, and impermeability are the main features of lignin. These functions, in addition to being non-water soluble and optically inactive, make lignin very difficult to degrade (Hendriks and

Zeeman, 2008). The ease of digestibility of lignocellulosic biomass is highly dependent on the lignin content, the most recalcitrant component of plant cell walls, which varies depending on biomass type. Generally, herbaceous plants and agricultural residues have the lowest lignin content (10-20%), whereas softwoods have the greatest lignin content (25-35%), with hardwoods (18-25%) in between (McMillan, 1994; Jorgensen et al., 2007). Lignin reduces the effectiveness of enzymatic hydrolysis of cellulose by acting as a physical barrier and also non-productively binding cellulase enzymes (Alvira et al., 2009). Various strategies have been studied to reduce the non-productive adsorption of lignin including alkali extraction and addition of protein, such as bovine serum albumin (BSA) (Yang and Wyman, 2006; Pan et al., 2005). In order to justify the additional cost of the additives, significant improvements in the enzymatic hydrolysis must be achieved (Alvira et al., 2009).

1.2. Substrate Factors Limiting Enzymatic Digestibility

Resistance to enzymatic attack is an intrinsic property of lignocellulosic materials. The goal of pretreatment is to alter these properties in order to enhance the enzymatic digestibility. Due to the variability in composition of lignocellulosic biomass, the best pretreatment method and condition for one feedstock can be completely ineffective for another (Taherzadeh and Karimi, 2008). The major substrate related factors limiting enzymatic hydrolysis include physical and chemical features. The physical features include cellulose crystallinity, surface area, porosity, particle size, and the degree of polymerization. As discussed in the previous section, the main chemical, structural features affecting enzymatic digestibility of cellulose are lignin content, hemicellulose content, and the degree of acetylation of hemicellulose (Zhu et al., 2008). The complexity

of the biomass matrix is reflected in the relationship between the structural and chemical features. Each factor's relative contribution to the native recalcitrance of biomass is still disputed (Zheng et al., 2009). The variability of these characteristics in different biomass indicates that the enzymatic digestibility is substrate and pretreatment specific (Mosier et al., 2005).

Cellulose crystallinity is the relative amount of the crystalline and amorphous regions within the microfibrils, with most of natural cellulose in the crystalline form (Taherzadeh and Karimi, 2008). The rate of digestibility of the amorphous regions by cellulase enzymes is greater compared to the less accessible crystalline regions. However, crystallinity alone will not prevent hydrolysis if sufficient enzyme is used (Mosier et al., 2005). The combination of lignin content and cellulose crystallinity had the greatest affects on digestibility. However, reduction of lignin was the most important parameter for effective digestion. At short hydrolysis periods (1 - 6 hours) low crystallinity was also required to increase the digestion rate, but at long periods (72 hours) crystallinity was not important when lignin content was low (Zhu et al., 2008). In some cases, pretreatment improved digestibility while it simultaneously increased the crystallinity of the cellulose region due to the reduction of the easily available amorphous region (Alvira et al., 2009).

Research has shown a good correlation between accessible surface area or pore volume, and enzymatic digestibility. Contact between the substrate and enzyme is necessary for biodegradation of the cellulose, therefore accessibility of the substrate is a major factor influencing the hydrolysis process (Alvira et al., 2009; Taherzadeh and Karimi, 2008). Lignocellulosic biomass has two types of surface area, internal and external. External surface area is associated with the size and shape of the particles,

which is also referred to as particle size. The capillary structure or porosity of cellulosic fibers makes up the internal surface area. The increase in accessible surface area during pretreatment is related to the removal of hemicellulose (Grous et al., 1986). Removal of lignin and increase in moisture content also increase the accessible surface area (Hendriks and Zeeman, 2009; Taherzadeh and Karimi, 2008).

The degree of polymerization, or the number of glycosyl residues per cellulose chain, has effects on digestibility, however the role is not definitely know. Reduction is cellulose chain length, increase in crystallinity, and hemicellulose and lignin removal are all interrelated for thermochemical pretreatments (Kumar et al., 2009). Determining the effects of a single structural feature is not yet possible due to cross effects of various features during pretreatment (Chang and Holtzapple, 2000).

1.3. Anaerobic Digestion

Anaerobic digestion (AD) of organic matter into biogas is a complex biological process regarded as taking place in two distinct phases – an acid-production phase and an acid-consumption phase (Munch et al., 1999). The conversion process consists or several independent, parallel, and consecutive reactions, in which microorganisms work synergistically to degrade organic matter into a mixture of methane and carbon dioxide gases (Noykova et al., 2002). These processes consist of six main stages: (1) hydrolysis of carbohydrates proteins and lipids into sugars, amino acids, and long-chain fatty acids; (2) fermentation of amino acids and sugars into volatile fatty acids; (3) acetogenesis of long-chained fatty acids into acetate and hydrogen; (4) anaerobic oxidation of intermediate products such as volatile fatty acids into acetate and hydrogen; (5) aceticlastic methanogenesis of acetate into methane by acid-utilizing methanogens; and

(6) hydrogenotrophic methanogenesis of hydrogen into methane by hydrogen-utilizing methanogens (Jeyaseelan, 1997; Myint et al., 2006; Noykova et al., 2002). A four-step biological process has also been described for the anaerobic degradation of organic matter with steps including; hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Chynoweth and Isaacson, 1987).

A wide variety of anaerobic digesters have been developed and implemented over the past fifty years. For cattle waste, the most important factor in determining the digester type to use is whether it can handle the solids loading of manure, while still meeting the goals of anaerobic digestion. The goals include; reduction in solids mass, reduction in odors, production of clean liquid effluent for recycle or land application, concentration of nutrients in solid digestate for storage or export, generate energy, and reduce pathogens (Burke, 2001).

The completely stirred tank reactor (CSTR) is the most commonly implemented type of anaerobic digester. Most CSTR digesters are heated with spiral flow heat exchangers, which apply hot water to one side of the spiral and anaerobic slurry to the other. Mesophilic operation is most common, with the thermophilic range employed where sufficient energy is available to heat the reactor. The advantage of a CSTR digester is that it is a proven technology that achieves reasonable conversion of solids to gas using cattle manure. The disadvantages are in the high cost of installation and the energy cost to mix the reactor. At the other end of the spectrum is the plug flow reactor (PFR), the least expensive of the digesters, which is also commonly used. Applications are limited to concentrated manure with minor amounts of sand and silt. Significant operational costs will be incurred if stratification occurs due to dilute waste or excess sand (Burke, 2001).

As compared to other waste treatment technologies, there are many advantages of treating biomass waste with anaerobic digestion, including; reduced biomass sludge compared with aerobic treatment; more effective removal of pathogens, especially in multi-stage digesters; minimal odor emissions; compliance with national waste policies; carbon neutral energy is produced as biogas, and solid digestate is produced with increased carbohydrate content (Ward et al., 2008).

There is currently a great potential pollution risk to the environment from the large amounts of animal manure and slurries produced by the animal production sector world-wide, if it is not managed optimally (Holm-Nielsen et al., 2009). An estimated 18% of all anthropogenic greenhouse gas emissions, measured in CO_2 equivalent, from the five major sectors for greenhouse gas reporting: energy industry, waste, land use, land use change and forestry, and agriculture, are produced from livestock activities. This encompasses 9% of anthropogenic CO_2, 35-40% of anthropogenic methane, 65% of anthropogenic nitrous oxide, and 64% of anthropogenic ammonia, from the world-wide animal production sector (Steinfeld et al., 2006). Anaerobic digestion offers a unique solution to prevent emissions of greenhouse gases and leaching of organic matter and nutrients, mainly nitrogen and phosphorous, to the natural environment (Holm-Nielsen et al., 2009).

It has been estimated that 120 million dry tons of cattle manure are produced annually in the United States on 67,000 dairy and 956,500 beef cattle farms. (USDA Economic Research Service, 1997; USDA National Agricultural Statistics Service, 2009). This is a large potential source of carbohydrates for ethanol production. By composition, cattle manure contains 22% (w/w dry basis) cellulose and 17% hemicellulose. This

immense amount of cellulosic residue has the capability of providing an economic stimulus to dairy and beef cattle farms while reducing the associated environmental liabilities (Liao et al., 2004). Through anaerobic digestion and other pretreatment processes, the cellulosic content of the fiber will increase considerably, making the resulting fiber an attractive feedstock for ethanol production. Currently, anaerobically digested fiber (AD fiber) is an underutilized resource, being used for animal bedding, soil amendment or fertilizer (Johnson et al., 2006; Gomez and Gonzalez, 1977), and possibly particle board (Spelter et al., 2008). Traditionally it has been regarded as too recalcitrant to be used for ethanol production (Tambone et al., 2009). However as with all lignocellulosic materials, the correct pretreatment of AD fiber will increase the cellulose content and the digestibility of the cellulose during enzymatic hydrolysis.

1.4. Dilute Acid Pretreatment

Dilute acid pretreatment was derived from concentrated acid hydrolysis, which had been a major technology for hydrolyzing lignocellulosic biomass for ethanol production. The concentrated acid hydrolysis was temporarily commercialized during World War II (Zheng et al., 2009). Due to its extremely toxic, hazardous, and corrosive nature, along with the need to recover and recycle the concentrated acid, the concentrated acid process has gradually been phased out of use. However, dilute acid pretreatment has received numerous research interests and is probably the most commonly applied chemical pretreatment method (McMillan, 1994). Recent processes use less severe conditions and achieve high xylan to xylose conversion yields. High xylose conversion is necessary for favorable process economics because xylan accounts for up to a third of the total carbohydrates in many lignocellulosic materials (Hinman et al., 1989).

Dilute acid pretreatment can be conducted with either short retention time (e.g. 5 min) and high temperature (e.g. 180°C) or longer retention time (e.g. 30-90 min) at lower temperatures (e.g. 120 – 140°C) (Taherzadeh and Karimi, 2008; Alvira et al., 2009). The effects of several different acids, including dilute sulfuric acid, dilute nitric acid, dilute hydrochloric acid, dilute phosphoric acid, and peracetic acid have been reported with dilute sulfuric acid being the most extensively studied because it is inexpensive and effective (Zheng et al., 2009).

Various lignocellulosic biomasses have been pretreated with dilute sulfuric acid to assess the effectiveness of the pretreatment, including: agricultural residues such as corn stover, corn fiber, corn cobs, sugar cane bagasse, cattle manure, and olive tree biomass, rice hulls, rye straw, peanut shells, cassava stalks, and potato peels (Torget et al., 1991; Esteghlalian et al., 1997; Wu and Lee, 1997; Varga et al., 2002; Lloyd and Wyman, 2005; Chen et al., 2009; Zhu et al., 2009; Grohmann and Bothast, 1997;; Silverstein el al., 2007; Martin et al., 2007; Liao et al., 2004; Liao et al., 2007; Cara et al., 2007; Sun and Cheng, 2005; Lenihan et al., 2009), short rotation herbaceous crops such as switchgrass, Bermuda grass, weeping lovegrass, Jose tall wheatgrass, and creeping wild rye (Torget et al., 1990; Esteghlalian et al., 1997; Chung et al., 2005; Jensen et al., 2009; Sun and Cheng, 2005; Zheng et al., 2007), short rotation woody crops such as poplar, sweetgum, silver maple, sycamore, black locust, aspen, balsam, athel, and eucaplytus wood (Torget et al., 1990; Esteghlalian et al., 1997; Chung et al., 2005; Torget et al., 1991; Jensen et al., 2009; Zheng et al., 2007), and autoclaved municipal organic solid wastes (Zheng et al., 2007).

The National Renewable Energy Laboratory (NREL) favors dilute sulfuric acid hydrolysis mainly due to the fact that 80 – 90% of the hemicellulose sugars are recoverable (Torget et al. 1991; Grohmann and Bothast, 1996), which can enhance the economics greatly with efficient pentose fermentation (Aden et al., 2002). Enhanced reactivity of cellulose to enzymes correlates with the removal of hemicellulose during dilute acid pretreatment of biomass with low lignin content (Torget et al., 1990). The percentage of xylose recovery has been used in several studies to optimize the pretreatment. Under optimized xylose recovery pretreatment conditions of 1.2% (w/w) sulfuric acid at 180°C, 90% cellulose to glucose conversion was achieved with pretreated switchgrass (Chung et al. 2005).

However, the most effective pretreatment conditions for enzymatic hydrolysis are not necessarily the conditions with the highest hemicellulosic sugars recovery. Dilute acid pretreated olive tree biomass had three separate optimal conditions; (170°C and 1% H_2SO_4) for maximum hemicellulose recovery (83%), (210°C and 1.4% H_2SO_4) for maximum enzymatic hydrolysis yield(76.5%), and (180°C and 1% H_2SO_4) for maximum total sugar recovery (75%) (Cara et al., 2007). This indicates that dilute acid pretreatment can be optimized under different conditions for hemicellulose sugar recovery, glucose recovery, or total sugar recovery. Dilute sulfuric acid pretreated cotton stalks resulted in almost complete xylan reduction (95.23%), but very low cellulose to glucose conversion during enzymatic hydrolysis (23.85%). Sodium hydroxide pretreatment of the same cotton stalks resulted in significantly increased cellulose conversion (60.8%), mainly due to the delignification (65.63%) (Silverstein et al., 2006). Dilute sulfuric acid treatment on corn stover removed 76.6% of hemicellulose but yielded only a 39.4% cellulose

conversion yield during enzymatic hydrolysis. This is in contrast to dilute sodium hydroxide pretreatment, which removed 73.9% of lignin and yielded an 81.2% cellulose conversion rate during enzymatic hydrolysis (Chen et al., 2009). This is because dilute acid pretreatment does not significantly impact lignin removal. High lignin content leads to increased enzyme consumption due to irreversible adsorption of cellulase enzymes to lignin, decreasing the cellulose conversion effectiveness of enzymatic hydrolysis (Wu and Lee, 1997; Yang and Wyman, 2008). This indicates that optimization of hemicellulose sugar recovery does not always result in optimal enzymatic hydrolysis effectiveness, and is more significant in substances with low lignin content. Biomass with high lignin content requires an additional or different pretreatment method to remove or disrupt the lignin prior to hydrolysis to achieve effective cellulose-to-glucose conversion.

1.5. Dilute Alkali Pretreatment

Dilute alkali pretreatment is an alternative to the more common dilute acid pretreatment. Soaking in alkali solutions, most notably sodium hydroxide (NaOH) has been used to pretreat lignocellulosic materials. Lignin content is a major factor in the efficacy of dilute alkali pretreatment. Hardwoods pretreated with dilute sodium hydroxide showed increasing efficacy with as lignin content decreased from 24 to 18%. However, no effect was observed for softwoods with lignin content of 26-35%. Increased efficacy was shown for agricultural residues as compared to hardwoods, in part, due to the lower lignin content of the residues (McMillan, 1994). Feedstocks with low lignin content such as agricultural residues, herbaceous crops, and hardwoods are most suitable for dilute alkali pretreatment.

Swelling occurs in lignocellulosic biomass pretreated with dilute sodium hydroxide causing a separation in the structural linkages between lignin and carbohydrates, a decrease in crystallinity, a decrease in the degree of polymerization, an increase in internal surface area, and disruption of the lignin structure (Fan et al., 1987). Saponification of the ester bonds crosslinking xylan hemicellulose and lignin is believed to be the mechanism of alkali pretreatment (Tarkow and Feist, 1969). Compared with acid and oxidative reagents, alkali pretreatment is the most effective at breaking the ester bonds and avoiding reduction of the hemicellulose polymers (Taherzadeh and Karimi, 2008). An increase in fiber saturation point is the most noticeable physical effect. The swelling capacity of cell walls in hardwoods treated with 1% NaOH, followed by washing, was doubled. This increase in fiber saturation point provides for improved enzyme-substrate interactions (Tarkow and Feist, 1969).

Alkali pretreatments have been effective both at ambient conditions for long reaction times and more severe conditions for shorter times. Dilute sodium hydroxide pretreatment was effective in improving the enzymatic digestibility of switchgrass over a range of temperatures (21, 50, 121°C) (Xu et al., 2010). The best reaction conditions for each temperature studied were (1.0% NaOH, 0.5 h at 121°C; 1.0% NaOH, 12 h at 50°C; 2.0% NaOH, 6 h at 21°C), the total reducing sugar yields were respectively, 425.4, 453.4, and 406.2 mg/g raw switchgrass.

Enzymatic digestibility has been enhanced for corn stover (Chen et al., 2009; Varga et al., 2002; MacDonald et al. 1983), wheat straw (Sun et al., 1995; Farid et al., 1983), sugar-cane bagasse (Fox et al., 1989; Farid et al., 1983), sunflower stalks and hulls (Sharma et al., 2002; Soto et al., 1994; Farid et al., 1983), switchgrass (Xu et al., 2010),

coastal Bermuda grass (Wang et al., 2009), cotton stalks (Silverstein et al., 2007), and hardwoods (Millet et al., 1976) using dilute sodium hydroxide pretreatment.

Dilute NaOH pretreatment was more effective on corn stover as compared to dilute acid, lime, and aqueous ammonia pretreatments. Pretreated corn stover with conditions of 120°C, 30 minutes, and 2% NaOH produced 36.1 g/L glucose and 81.2% conversion rate after enzymatic hydrolysis of 8% substrate concentration and enzyme loading of 20 FPU/g substrate(Chen et al. 2009).

1.6. Enzymatic Hydrolysis

The process of enzymatic hydrolysis of cellulose contains three main components, the cellulase adsorption onto the surface of the cellulose, the biodegradation of the cellulose to fermentable sugars, and desorption of the cellulase. Highly specific cellulase and hemicellulase enzymes (glycosylhydrolases) carry out the enzymatic hydrolysis of cellulose and hemicellulose. Of the more than 80 known glycosyl hydrolase familes, the catalytic domains of cellulase and hemicellulase are currently grouped into at least 15, and the substrate binding domains fall into 13 families (Rabinovich et al., 2002).

At least three major classes of enzymes are involved in the synergistic action of the enzymatic degradation of cellulose to glucose: exo-1,4-β-D-glucanases, endo-1,4-β-D-glucanases, and 1,4-β-D-glucosidases. Together these enzymes are usually called cellulase or cellulolytic enzymes (Wyman, 1996). Endo-1,4-β-D-glucanases hydrolyze internal β-1,4-glucosidic bonds in areas of low crystallinity in the cellulose chain creating free chain-ends. Exo-1,4-β-D-glucanases or cellobiohyrolases (CBH) move processively along the cellulose chain and cleave off cellobiose units (dimers of glucose) from the free chain-ends. The 1,4-β-D-glucosidases hydrolyze cellobiose to glucose and also cleave of

glucose units from cellooligosaccharides. By creating new accessible sites for each other, removing obstacles, and relieving product inhibition, these enzymes work synergistically together to hydrolyze cellulose (Jorgensen et al., 2007; Taherzadeh and Karimi, 2007).

Certain species of bacteria and fungi produce cellulases for the hydrolysis of lignocellulosic material. Several bacteria species such as *Clostridium, Cellumonas, Thermomonospora, Ruminococcus, Erwinia, Acetovibrio, Microbispora, Bacillus, Bacteriodes, and Strepomyces* are able to produce cellulases (Sun and Cheng, 2002; Taherzadeh and Karimi, 2007). Cellulolytic anaerobes such as *Clostridium thermocellum* and *Bacteroides cellulosolvens* produce cellulases with high specific activity; however they do not produce high enzyme concentrations. Since the anaerobes have very low growth rate and require anaerobic conditions, most commercial research has focused on fungi (Duff and Murray, 1996). Species of certain fungi such as *Tricoderma, Penicillium, Fusarium, Phanerochaete, Humicola, Aspergillus, Schizophyllum, Sclerotium rolfsii,* and *P. chrysosporium* are able to produce cellulases and hemicellulases (Fan et al., 1987; Duff and Murray, 1996; Rabinovich et al., 2002; Sun and Cheng, 2002; Taherzadeh and Karimi, 2007). Of all the cellulases produced by different microorganisms, cellulases produced by *Trichoderma ressei* and *T. viride* have been researched most extensively and are best characterized. *Trichoderma viride* is a valid species aggregate that is used for all unknown *Trichoderma* species; while *T. ressei* are developed from a single isolate (QM6a) (Zhang and Lynd, 2004). The *Trichoderma reesei* cellulase mixture consists of many glycosyl hydrolases, of which five β-1,4-endoglucanases, two β-1,4-exoglucanases, two xylanases, a β-D-glucosidase, an α-L arbinofluranosidase, an acetyl xylan esterase, a β-mannanase, and an α-glucuronidase have been sequenced (Vinzant et al., 2001).

The advantages of cellulase produced by *Trichoderma* include resistance of the enzyme to chemical inhibitors, a full complement production of cellulase, and stability under enzymatic hydrolysis conditions. However, suboptimal levels and low activity of β-glucosidases inhibit the effectiveness of *Trichoderma* cellulase. Conversely, *Aspergilli* are very efficient β-glucosidase producers. Increased efficiency was found in *Trichoderma* cellulose supplemented with extra β-glucosidases (Taherzadeh and Karimi, 2007). *Trichoderma spp.* are used for most commercially produced cellulases, with a few produced from *Aspergillus niger* (Zhang and Lynd, 2004).

1.7. Fermentation

Numerous microorganisms have been used for ethanol production, with *Saccharomyces cerevisiae* remaining as the primary species (Bai et al., 2008). High ethanol yields and productivities in addition to a remarkable ethanol tolerance make this species the most widely used process organism. These unusual properties are the result of adaptation to efficient ethanol production from glucose over thousands of years (Olsson and Hahn-Hagerdal, 1996). *Zymomonas mobilis* has also been researched extensively, with repeated claims by some researchers to possess superior characteristics compared to *Saccharomyces cerevisiae,* mainly higher conversion rate of glucose to ethanol (Bai et al., 2008). However, *Z. mobilis* has many drawbacks, mainly that pure glucose is needed for effective fermentation, which is impossible in the commercial ethanol industry, and the biomass generated cannot be used as animal feed, unlike *S. cerevisiae* (Bai et al., 2008). Both microorganisms are capable of efficiently fermenting glucose into ethanol; however neither is able to ferment pentoses such as xylose (Keshwani and Cheng, 2009).

Despite having a full xylose metabolic pathway, *S. cerevisiae* is unable to utilize xylose as a sole carbon source (Batt et al., 1986).

Other yeasts such as *Pichia stipitis, Candida shehate,* and *Pachysolen tannophilus* are known to ferment xylose into ethanol. Ethanol yields are significantly lower than glucose fermentation by *S. cerevisiae* though, which necessitates considerable improvement in xylose fermenting technology (Chu and Lee, 2007). Other problems associated with the commercial use of these native yeast strains include low ethanol tolerance, difficult optimization of fermentation parameters, and slow rate of fermentation (Dupreez, 1994).

Even though *Saccharomyces cerevisiae* is unable to utilize xylose for fermentation, the isomer of xylose, xylulose, can be fermented. Xylose can be converted to xylulose using xylose isomerase. However, the fermentation rate of xylulose is ten times less than that of glucose (Olsson and Hahn-Hagerdal, 1996). This approach is not cost effective, therefore over the past two decades; much research had focused on developing genetically engineered xylose-fermenting microorganisms, mainly *S. cerevisiae* (Saha, 2003). Recombinant *S. cerevisiae* strains are able to convert xylose to ethanol at near theoretical yields of 0.51 g g^{-1} but with low maximal productivities (Chu and Lee, 2007).

Due to the difficulties in fermenting pentose sugars into ethanol, another option of generating energy from hemicellulose is through the anaerobic digestion process. The efficient conversion of the sugars into methane makes it a very effective process. The conversion rate of hemicellulose reducing sugars, mainly xylose, is much greater than that of cellulose reducing sugars, mainly glucose. The integration of anaerobic digestion

and biorefining that incorporates yeast fermentation with *Saccharomyces cerevisiae* offers a very attractive option for total energy production. The majority of pentose sugars are utilized by microorganisms in the digester to generate methane, while most of the hexoses remain for yeast fermentation to ethanol. The higher heating value of methane is 55 MJ/Kg, which makes it an extremely attractive energy source. Methane from hemicellulose generates more energy (electricity energy) than ethanol from hemicellulose due to the relatively low conversion rate (80%) of pentoses to ethanol (Aden et al., 2002).

1.8. Issues in the development of fuel ethanol production from AD Fiber

The general issues that need to be addressed in the production of ethanol from AD fiber include; 1) whether the composition of the AD Fiber is sufficiently high in fermentable sugars, 2) determining an optimal pretreatment method for maximizing the digestibility of the sugars for enzymatic hydrolysis, 3) comparing the digestibility of AD fiber to other potential cellulosic ethanol feedstocks, agricultural residues (i.e., corn stover) and dedicated energy crops (i.e., switchgrass), and 4) assessing the ethanol yield from fermentation of the AD fiber hydrolysate.

1.9. Research Overview and Objectives

Anaerobic digestion is a proven process for converting manure and agricultural residues into methane for heat and power production. The liquid effluent has been land applied as fertilizer; however the AD fiber is currently limited to uses of low economical value, animal bedding and soil amendment. In light of the issues discussed in the previous sections, the general objective of this research is to investigate, at the laboratory scale, the use of AD fiber for the production of fuel ethanol. A flow diagram of the integrated anaerobic digestion and biorefining process is provided in Figure 1.1 below.

The processes inside the dashed line were the focus of the current research. The solid AD fiber is the starting point of the current research. The processes included in the study are acid and alkali pretreatment, enzymatic hydrolysis, and yeast fermentation. Distillation and dehydration of the green beer into pure ethanol was not included in this research. Combustion of the solid residues after enzymatic hydrolysis, containing mostly lignin, was also not addressed in this study. AD fiber composition, pretreatment, hydrolysis and fermentation are addressed with an emphasis on the effects of pretreatment on glucose yield and ethanol production.

The specific objectives for the project are:

- To characterize the chemical composition of raw AD fiber and pretreated AD fiber.
- To assess the effects of dilute acid and dilute alkali pretreatments on glucose concentrations and cellulose to glucose conversion yields of AD fiber after enzymatic hydrolysis, and ethanol concentration and conversion yields after fermentation.
- To hydrolyze the pretreated AD fiber using commercial cellulase enzymes to soluble monosaccharide components for use as fermentation feedstock.
- To ferment the released sugars to ethanol using *Saccharomyces cerevisiae* D5A.
- To compare the glucose and ethanol concentration and conversion yields of AD Fiber with switchgrass and corn stover using the same processes.
- Energy production and environmental impacts of AD fiber biorefining.

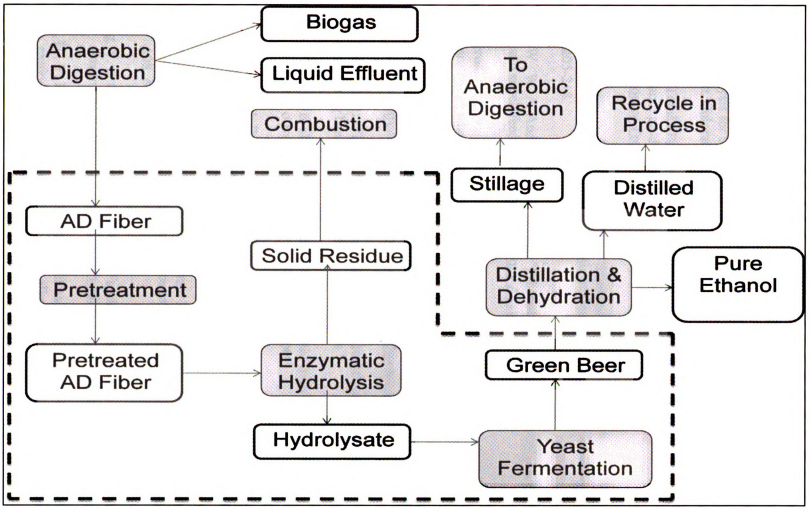

Figure 1.1. AD Fiber Process Flow Diagram

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Chapter Two

Teater, C., Yue, Z., MacLellan, J., Liu, Y., Wei, L., 2010. Assessing Solid Digestate from Anaerobic Digestion as Feedstock for Ethanol Production.

CHAPTER 2

2.1. Abstract

Ethanol production using solid digestate (AD fiber) from a Complete Stirred Tank Reactor (CSTR) anaerobic digester was assessed comparing to an energy crop of switchgrass, and an agriculture residue of corn stover. A complete random design was fulfilled to optimize the reaction conditions of dilute alkali pretreatment. Three reaction times (1, 2, 3 h), two temperatures (120, 130 °C), and four sodium hydroxide concentrations (0.5, 1, 2, 3 % w/w) were tested. The most effective dilute alkali pretreatment conditions for raw CSTR AD Fiber were 2% sodium hydroxide, 130 °C, and 3 hours. Under these pretreatment conditions the cellulose concentration of the AD Fiber was increased from 34 to 48%. Enzymatic hydrolysis of 10% (dry basis) pretreated AD fiber produced 49.8 g/L glucose, while utilizing 62.6% of the raw cellulose in the AD fiber. The ethanol fermentation on the hydrolysate had an 80.3% ethanol yield.

2.2. Introduction

A record 10.75 billion gallons of corn-based ethanol was produced in the U.S. in 2009 (RFA 2009).While reducing the need for foreign oil, corn-based ethanol also diverts corn away from food markets, inevitably resulting in food-fuel competition (Koh and Ghazoul, 2008). Lignocellulosic biomass from abundant and diverse non-food raw materials such as agricultural waste offers a better alternative for ethanol production. Cattle manure is a readily available lignocellulosic biomass capable of being converted into glucose and other fermentable mono-sugars. It has been estimated that a total of about 120 million dry tons of cattle manure are produced annually in the United States, which are from 67,000 dairy farms and 956,500 beef cattle producers that have approximately 95 million head of cattle evenly distributed across the country (USDA Economic Research Service, 1997; USDA National Agricultural Statistics Service, 2009). Currently, AD fiber is an underutilized resource, being used for animal bedding, soil amendment / fertilizer (Johnson, 2006; Gomez, 1977), and possibly particle board (Spelter, 2008). Traditionally it has been regarded as too recalcitrant to be utilized for ethanol production (Tambone, 2009). However as with all lignocellulosic materials, pretreatment of AD fiber increased the digestibility of the cellulose for enzymatic hydrolysis. The experimental data demonstrated that the solid effluent of anaerobic digestion (AD Fiber) is more suitable as a feedstock for ethanol production than raw manure. Also, dilute alkali pretreatment was determined to be a more effective process than dilute acid pretreatment due to the high alkalinity of AD Fiber.

Dilute sodium hydroxide at elevated temperature causes the swelling of lignocellulosic biomass, which leads to a separation in the structural linkages between

lignin and carbohydrates, a decrease in crystallinity, a decrease in the degree of polymerization, an increase in internal surface area, and disruption of the lignin structure (Fan et al. 1987). Saponification of the ester bonds crosslinking xylan hemicellulose and lignin is believed to be the mechanism of alkali pretreatment (Tarkow and Feist, 1969). Feedstocks with low lignin content such as agricultural residues, herbaceous crops, and hardwoods are most suitable for alkali pretreatment. Enzymatic digestibility has been enhanced for corn stover, wheat straw, sugar-cane bagasse, sunflower stalks, switchgrass, coastal Bermuda grass, cotton stalks, and hardwoods using sodium hydroxide pretreatment (Chen et al., 2009; Varga et al., 2002; MacDonald et al. 1983; Sun et al., 1995; Farid et al., 1983; Fox et al., 1989; Sharma et al., 2002; Soto et al., 1994;; Xu et al., 2010; Wang et al., 2009; Silverstein et al., 2007; Millet et al., 1976). Dilute sodium hydroxide pretreatment was more effective on corn stover as compared to dilute acid, lime, and aqueous ammonia pretreatments. Pretreated corn stover with conditions of 120°C, 30 minutes, and 2% sodium hydroxide produced 36.1 g/L glucose and 81.2% conversion rate after enzymatic hydrolysis of 8% substrate concentration and enzyme loading of 20 FPU/g substrate(Chen et al. 2009). Switchgrass pretreated with sodium hydroxide effectively improved the enzymatic digestibility at a variety of temperatures (Xu et al., 2010).

This study focused on an alkali pretreatment of AD fiber in order to conclude the most favorable pretreatment conditions to convert AD fiber into ethanol. The specific objectives of this study were to: (1) compare the suitability of AD Fiber from a completely stirred tank reactor (CSTR) for ethanol production with more commonly researched feedstocks; switchgrass and corn stover, and (2) statistically determine the

best dilute alkali pretreatment conditions; reaction time, reaction temperature, and sodium hydroxide concentration. The cellulose utilization efficiency and glucose concentration after enzymatic hydrolysis were used to determine the best pretreatment conditions. The equation for the cellulose utilization efficiency [%] is:

$$\text{Cellulose Utilization Efficiency [\%]} =$$

$$\frac{\text{Substrate DM after pretreatment [g]}}{\text{Substrate DM before pretreament [g]}}$$

$$* \frac{\text{Glucose Concentration after Enzymatic Hydrolysis } \left[\frac{\text{g}}{\text{L}}\right]}{\text{Hydrolysis Substrate DM [g]}}$$

$$* \frac{\text{Volume of hydrolyzate[L]}}{\text{Initial raw feedstock Cellulose Content} * 1.11} * 100$$

2.3. Materials and Methods

2.3.1. Fiber Samples

AD fiber samples were collected from a private dairy farm with 3,000 cattle. The CSTR anaerobic digester was operated at 40°C with a hydraulic retention time of 20 days. Switchgrass and Corn Stover were received from the Michigan State University Crop and Soil Science Teaching and Research Field Facility, and samples were air dried and grinded on-site using a grinder (Willey Mill, Standard Model No.3, Arthur H. Thomas, Philadelphia, PA) with 4mm size opening.

2.3.2. Alkali Pretreatment

The CSTR AD Fiber was pretreated using an autoclave (Brinkmann 2540M, Tuttnauer USA CO. Ltd, Hauppauge, NY) at three sodium hydroxide concentrations (1%, 2%, 3wt %), two retention times including warm-up time (2, 3 h), and two temperatures (120, 130 °C) using a CRD. Experimental results determined concentrations below one percent and retention times less than two hours to be ineffective for AD fiber (Results not shown). Switchgrass and corn stover were pretreated in the same autoclave at three sodium hydroxide concentrations (0.5%, 1%, 2%), two retention times (1, 2 h), and two temperatures (120, 130 °C) using a CRD. Additional pretreatments on switchgrass and corn stover were conducted with 3% sodium hydroxide under reaction conditions that showed increased glucose production with increased sodium hydroxide concentration. This excluded the more severe conditions that decreased glucose production with increased sodium hydroxide concentrations. Fiber concentration was fixed at 6% based on dry matter for all pretreatments. Pretreated mixture solutions were neutralized to pH values of 4.0-5.0 using a 20% sulfuric acid solution, then centrifuged and rinsed using de-ionized water. Wet solid samples were stored in a freezer at -20 °C. Solid residue was taken for analysis of dry matter and fiber content.

2.3.3. Enzymatic Hydrolysis

Wet solid samples (2 g dry matter) and de-ionized water were mixed with a total mass of 20 g into 125 mL shake flasks and autoclaved. Cellulase (ACCELLERASE™ 1500, Genencor, Rochester, NY, USA) at a loading of 26 FPU/g dry matter and autoclaved 0.05 M citrate buffer (pH = 4.8) were added to maintain identical initial dry matter concentration (5%) except for fiber type. Each flask was placed on a shaker at 140

rpm inside an incubator set at 50 °C. After 72 hours the aliquots were boiled for 5 minutes and filtered with Whatman (#1) filter paper. The filtrates 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. Enzymatic hydrolysis at 10% dry matter concentration was then performed on the most effectively pretreated samples. Wet solid samples (2 g dry matter), de-ionized water, and cellulase were mixed with a total mass of 20 g into 125 mL shake flasks. The solids and de-ionized water mixture was autoclaved prior to addition of cellulase. Cellulase was added at a loading of 52 FPU/g dry matter. The remaining procedure for the 10% solids was identical to that of the 5% solids enzymatic hydrolysis.

2.3.4. Ethanol Fermentation

Saccharomyces cerevisiae D5A obtained from American Type Culture Collection (ATCC, Manassas, VA) was used in the yeast fermentation. Inoculum was cultured for 15 h at 30°C in a 250mL flask on ATCC Medium No. 1245 (10 g/L yeast extract, 20 g/L Bacto peptone, and 20 g/L glucose). The culture broth for inoculum was centrifuged to collect yeast biomass as inoculum. The inoculum was mixed with an autoclaved nutrition solution (10 g/L of peptone, 5 g/L of yeast extract, and glucose in the hydrolysates). The inoculum-to-solution ratio of 1:10 was used to conduct the fermentation. Samples were taken at the beginning and end of a 24-h fermentation process for glucose and ethanol analysis.

2.3.5. Analytical Methods

Samples were diluted to 1% dry matter for alkalinity analysis using HACH method (Loveland, CO). Neutral detergent fiber (NDF), acid detergent fiber (ADF), acid

detergent lignin (ADL), cellulose, hemicellulose and lignin of the raw samples were analyzed using Van Soest fiber analysis system (Goering and Van Soest, 1970). Fiber analysis of the pretreated samples was conducted using the National Renewable Energy Laboratory's, Laboratory Analytical Procedure, Determination of Structural Carbohydrates and Lignin in Biomass (Sluiter et al. 2008). Mono-sugar concentrations including cellobiose, glucose, xylose, galactose, arabinose and mannose were determined using a Shimadzu Prominence 2010 with a Bio-rad Aminex HPX-87P analytical column (300×7.8mm, catalog number 125-0098) and a refractive index detector. The mobile phase was Millipore water with a flow rate of 0.6 mL /min and column temperature of 60°C. Ethanol concentrations were determined using an Agilent 1100 HPLC system equipped with a Bio-rad Aminex HPX-87H analytical column (300×7.8mm, catalog number 125-0140) and a refractive index detector. The mobile phase used was 0.005M sulfuric acid with a flow rate of 0.6 mL /min and column temperature of 55°C.

2.3.6. Statistical Analysis

A pair-wise comparison using the Statistical Analysis System program 8.0 (SAS Institute, Inc., Cary, NC) was conducted to evaluate the effects of reaction conditions and different feedstocks (CSTR AD fiber, switchgrass, and corn stover) on glucose concentration and cellulose utilization efficiency.

2.4. Results

2.4.1. Biomass Characterization

Compared to switchgrass and corn stover, AD fiber has half of the hemicellulose content (15.9% dry basis) while having only slightly reduced cellulose content (33.9% dry basis). Lower hemicellulose content reduces the problem of pentose fermentation that

biorefineries encounter. Considering the integrated process of anaerobic digestion and bioethanol production, the bacterial consortia within the anaerobic digester consume the majority of the C-5 sugars producing methane and carbon dioxide. The particle size of manure fibers is significantly reduced during the AD process as well. Ninety-two percent of the CSTR AD fiber has a particle size smaller than 1 mm, compared to only seventy-five percent in washed raw manure (Fig. 2.1.). Corn stover and switchgrass necessitate energy intensive grinding to reach this particle size.

2.4.2. Effect of Alkali Pretreatment

2.4.2.1. Fiber Components

Dilute alkali pretreatment caused substantially increased cellulose content, while it caused only a slight change in hemicellulose and lignin contents. In most cases, as the concentration of sodium hydroxide increased, the lignin content decreased. For the CSTR AD fiber, the greatest cellulose content (53.6%, dry basis) was attained with the pretreatment conditions of 130°C, 3 hours, and 3% NaOH. The lowest lignin content (18.3%, dry basis) was achieved with the pretreatment conditions of 130°C, 3 hours, and 3% NaOH (Table 2.2.).

Switchgrass achieved the greatest cellulose content (62.3%, dry basis) with the pretreatment conditions of 120°C, 2 hours, and 3% NaOH. The lowest lignin content (9.1%, dry basis) was reached with the conditions of 130°C, 2 hours, and 2% NaOH (Table 2.3.). The greatest cellulose content for corn stover (55.1%, dry basis) was achieved with the pretreatment conditions of 120°C, 1 hour, and 3% NaOH. The lowest lignin content (5.5%, dry basis) was attained with the conditions of 130°C, 2 hours, and 2% NaOH (Table 2.4.).

2.4.2.2. Cellulose Utilization Efficiency

The cellulose utilization efficiency (the ratio of cellulose used to produce glucose with the total cellulose in the original sample) was used to investigate the most effective conditions for the sodium hydroxide pretreatment of the CSTR AD fiber, corn stover, and switchgrass. The pretreatment conditions of 120°C and 3% NaOH for 3 hours produced the highest utilization efficiency (71.4%) of AD fiber (Fig. 2.2.A.). A least square means for effect was conducted to assess the interaction between time and temperature (Fig. B.6.); it showed no significant ($p>0.05$) difference between temperatures of 120 and 130°C for a reaction time of 2 hours but there was significant ($p<0.05$) difference between temperatures of 120 and 130° for a reaction time of 3 hours. However, there was a significant ($p<0.05$) difference for all cases between reaction times of 2 and 3 hours. Least square means comparisons of reaction time and alkali concentration (Fig. B.5.) showed significant ($p<0.05$) difference between reaction times of 2 and 3 hours on cellulose utilization efficiency. However, for alkali concentration, the only significant ($p<0.05$) difference was that 3% NaOH concentrations were significantly ($p<0.05$) greater than 1% NaOH concentrations. Least square means for effect of alkali concentration and temperature (Fig. B.4.) showed no significant ($p>0.05$) difference between conditions except that the condition of 1% NaOH and 130°C was significantly ($p<0.05$) less than all other conditions. This leads to the conclusion that for CSTR AD fiber, reaction time has the greatest effect on cellulose utilization efficiency.

Corn stover reached the greatest cellulose utilization efficiency of 70.6% under the reaction conditions of 120°C, 1% NaOH and 2 hours (Fig. 2.2B). A least square means comparison of reaction time and alkali concentration (Fig. C.5.) revealed that 2

hour reaction times produced significantly ($p<0.05$) greater cellulose utilization than 1 hour reaction times. In addition, the conditions of 1% NaOH and 2 hours produced the best cellulose utilization and were significantly ($p<0.05$) greater than all other conditions except for 2% NaOH and 2 hours. Least square means comparison of reaction time and temperature (Fig. C.6.) also showed that reaction times of 2 hours produced significantly ($p<0.05$) greater cellulose utilization than 1 hour reaction times. There was no significant ($p>0.05$) difference between reaction temperatures of 120 and 130°.Reaction conditions of 120°C and 2 hours produced the best cellulose utilization and was significantly ($p<0.05$) greater than all other conditions except for 130°C and 2 hours. Reaction time had the greatest effect of cellulose utilization efficiency for corn stover.

For switchgrass, the greatest cellulose utilization efficiency of 66.6% was obtained at 130°C, 1% NaOH and 2 hours, and was significantly ($p<0.05$) greater than all other conditions (Fig. 2.2.C.). There was much less dependence on reaction time for the utilization of cellulose in switchgrass. A least square means comparison of alkali concentration and reaction time (Fig. D.5.) revealed that for 0.5% NaOH, the cellulose utilization was significantly ($p<0.05$) greater for a 1 hour reaction time than for 2 hours. For 1% NaOH the cellulose utilization was significantly ($p<0.05$) greater for a 2 hour reaction time than for 1 hour. With a 2% NaOH concentration, there was no significant ($p>0.05$) difference between 1 and 2 hour reaction times. In a least square means comparison between reaction time and temperature (Fig. D.6.) there was no significant ($p>0.05$) difference between any times or temperatures.

2.4.2.3. Glucose Concentration

Glucose concentrations from enzymatic hydrolysis of pretreated samples were presented in Fig. 2.2. Reaction time was again the most important factor for the AD fiber. The highest glucose yield was 29.8 g/L for the reaction conditions of 130°C and 3% NaOH for 3 hours (Fig. 2.3.A.). This was significantly ($p<0.05$) greater than all other reaction conditions except for 130°C and 2% NaOH for 3 hours (Fig. B.4.), which had a glucose yield of 29.7 g/L. In addition, reaction times of 3 hours produced significantly ($p<0.05$) greater glucose concentrations than reaction times of 2 hours in all cases. Sodium hydroxide concentration was also an important factor of glucose concentration. In a least square means comparison of alkali concentration and reaction time (Fig. B.2.), increased alkali concentration resulted in significantly ($p<0.05$) greater glucose concentrations in all cases except for at 2 hours and 2 or 3% NaOH. Temperature was found to be of much lesser importance. In a least square means comparison for the effect of reaction time and temperature (Fig. B.3.), there was no significant ($p>0.05$) difference between temperatures of 120 and 130°C.

Corn stover had the largest glucose concentration of 30.5 g/L with the pretreatment reaction conditions of 120°C, 1 hour, and 2% NaOH (Fig. 2.3.B.). Alkali concentration of 1% NaOH produced significantly ($p<0.05$) increased glucose concentration as compared to 0.5% NaOH, however there was no significant ($p>0.05$) difference between 1 and 2% NaOH concentration, as revealed by least square means comparisons of the effect of alkali concentration and reaction time (Fig. C.2.), and alkali concentration and reaction temperature (Fig. C.1.). The additional reaction condition of 120°C, 1 hour, and 3% NaOH, produced an increased glucose concentration of 32.2 g/L.

Least square means comparison of the effect of reaction time and temperature (Fig. C.3.) revealed no significant ($p>0.05$) difference in times or temperatures except that the condition of 130°C and 1 hour produced significantly ($p<0.05$) lower glucose concentrations than all other reaction condition combinations.

The largest glucose concentration for switchgrass, 25.1 g/L, was produced from the pretreatment reaction conditions of 130°C, 2 hours, and 1% NaOH (Fig. 2.3.C.). However, after additional pretreatments were conducted with 3% NaOH, the largest glucose concentration was increased to 28.5 g/L, with reaction conditions of 120°C, 2 hours, and 3% NaOH. Sodium hydroxide concentration caused the greatest effects on glucose concentration. In a least square means comparison of the effect of alkali concentration and reaction temperature (Fig. D.1.), increased concentration resulted in significantly ($p<0.05$) greater glucose concentrations in all but one case, 130°C between 1 and 2% NaOH. Similarly, a least square means comparison of alkali concentration and reaction time (Fig. D.2.) revealed that increased concentration produced significantly ($p<0.05$) greater glucose concentrations in all but one scenario, 2 hours between 1 and 2% NaOH. Reaction time and temperature had much less effect on glucose concentration. A least square means comparison of reaction time and temperature (Fig. D.3.) revealed no significant ($p>0.05$) difference for times or temperatures.

2.4.3. Most Effective Pretreatment Conditions

The reaction conditions that had highest cellulose utilization efficiency and glucose concentration were chosen as the most effective dilute alkali pretreatment conditions. For the CSTR AD fiber, the best reaction conditions were 130°C and 2% NaOH for 3 hours, and 130°C, 3% NaOH, and 3 h. These conditions produced glucose

concentrations of 29.7 g/L and 29.8 g/L, with efficiencies of cellulose utilization of 68.2 % and 68.1%, respectively. Due to the fact that low concentration of sodium hydroxide reduced the chemical loading of the pretreatment, the conditions of 130°C and 2% NaOH for 3 hours was selected as the best conditions to treat AD fiber.

The most effective reaction conditions for switchgrass and corn stover were both determined to be 130°C, 1% NaOH, and 2 hours. For switchgrass, this condition produced a utilization efficiency and glucose concentration of 66.6% and 25.1 g/L respectively. Efficiency and glucose production for corn stover were 67.6% and 28.9 g/L respectively. Even though conditions with 3% NaOH produced the greatest glucose concentrations, the conditions were not chosen because of reduced cellulose utilization efficiencies and to reduce the chemical loading of pretreatment.

High solids enzymatic hydrolysis (10% dry basis) was then conducted on the pretreated samples that performed most effectively. Glucose concentrations of 49.8, 53.6, and 55.4 g/L were produced for the CSTR AD fiber, switchgrass, and corn stover, with cellulose utilization efficiencies of 62.6, 61.1, and 60.3 % respectively (Fig. 2.4.).

2.4.4. Ethanol Production

Ethanol fermentation was conducted on the hydrolysates from the high solids enzymatic hydrolysis of the most effectively pretreated feedstocks. An 80.3% ethanol yield (ethanol yield [%] = ethanol produced [g/L] / (0.51* glucose consumed [g/L])*100) was obtained from CSTR AD fiber, which was consistent with switchgrass (78.0%) and corn stover (83.0%) hydrolysates, and significantly ($p<0.05$) greater than pure glucose (59.5%). Ethanol concentrations of 14.7, 16.6, 18.1, and 18.9 g/L were produced from

CSTR AD fiber, corn stover, switchgrass, and pure glucose, with initial fermentation glucose concentrations of 36.6, 38.8, 45.8, and 59.4 g/L respectively (Fig. 2.5.).

2.5. Discussion

Due to the abundant quantity and year round availability of cattle manure, it serves as a large potential feedstock for ethanol production without the logistical storage problems associated with annual crops. The integrated process of anaerobic digestion and bioethanol production is able to utilize the main components of the biomass in a robust manner. The hemicellulose is consumed at a higher rate than cellulose in the AD process, producing methane that is combusted to generate heat and electricity. Therefore the problems associated with pentose fermentation are avoided and the glucose is utilized in the biorefinery for ethanol production with a robust commercial yeast strain, *Saccharomyces cerevisiae*. The remaining lignin from the biorefinery can be combusted to produce electricity due to its higher heating value of 21.2 MJ/Kg, dry basis (Domalski, 1987).

Reduced particle size is another benefit of manure fibers after the AD process. Ninety-two percent of the CSTR AD fiber has a particle size smaller than 1 mm, compared to seventy-five percent in washed raw cattle manure. Corn stover and switchgrass necessitate energy intensive grinding to reach this particle size. Removing the size reduction unit from the bioethanol process will remove 22% of the capital investment on feedstock storage and handling within the production facility, greatly improving the efficiency of cellulosic ethanol production (Aden et al., 2002).

To determine if CSTR AD fiber was a suitable feedstock for lignocellulosic ethanol production, dilute alkali pretreatment was used in a comparison experiment with

switchgrass and corn stover. Glucose concentration after enzymatic hydrolysis, cellulose utilization efficiency, and the changes in fiber composition were all used to compare the three feedstocks.

The statistical analysis on cellulose utilization efficiency elucidated that the most important reaction condition for CSTR AD fiber and corn stover was reaction time. The effects of temperature and sodium hydroxide concentration were less noticeable. However, switchgrass was not as dependent on reaction time and showed much less variability overall, except for the condition of 130°C, 2 hours, and 1% NaOH, which was significantly ($p<0.05$) greater than all other conditions. Similarly, the statistical analysis on glucose concentration revealed that the most significant reaction condition for CSTR AD fiber and corn stover was reaction time. However for switchgrass, the most significant reaction condition was alkali concentration.

The most effective conditions for CSTR AD fiber were determined to be 130°C, 3 hours, and 2% NaOH, while for corn stover and switchgrass the most effective conditions were 130°C, 2 hours, and 1% NaOH. The increased severity required for CSTR AD fiber to be as effectively pretreated as corn stover and switchgrass is likely caused by greater lignin content. The increased lignin content of the AD fiber gives extra structural support to the fiber, causing resistance to degradation. The results shown indicate that this recalcitrance is able to be overcome through increased severity. This research was limited to a maximum temperature of 130°C, but further increasing the temperature will likely reduce the requirements on reaction time and alkali concentration. Future research utilizing steam explosion pretreatment, with reaction temperatures as large as 230°C, will further assess the digestibility of AD fiber (results not included). Increased severity, with

decreased chemical loadings will allow for further comparisons of AD fiber to switchgrass and corn stover.

In the current research, the conversion efficiencies of CSTR AD fiber for glucose and ethanol (62.6 and 80.3%) are consistent with those of switchgrass (61.1 and 78.0%) and corn stover (60.3 and 83.0%) from enzymatic hydrolysis (10% solids) and fermentation respectively. Glucose concentrations of 49.8, 53.6, and 55.4 g/L were produced for the CSTR AD fiber, switchgrass, and corn stover respectively. The lower glucose concentration from the CSTR AD fiber is due to the lower initial cellulose concentration of CSTR AD fiber as compared to switchgrass and corn stover. The cellulose utilization efficiencies determined that the cellulose in CSTR AD fiber was able to be converted better than in switchgrass and corn stover.

2.6. Conclusions

This study showed that sodium hydroxide pretreatment was effective in improving the digestibility of anaerobically digested fiber to enhance the glucose and ethanol yields from enzymatic hydrolysis and fermentation. Removal of lignin from the AD fiber assisted the digestibility of the cellulose. The glucose concentration and cellulose conversion efficiency of CSTR AD fiber was consistent to that of switchgrass and corn stover. The lower monomeric glucose concentration from the CSTR AD fiber after enzymatic hydrolysis was due to lower initial cellulose concentration and not the conversion efficiency. The CSTR AD fiber had the best cellulose conversion efficiency. However, the study of other pretreatment methods, enzyme loading tests, scale-up, economic analysis, and life-cycle analysis of the overall conversion processes are needed for further conclusions.

Tables

Table 2.1. Fiber characteristics of raw feedstocks.

	CSTR AD fiber	Switchgrass	Corn Stover
Total Solids (TS) (%)	28.1±0.0	92.0±0.1	95.5±0.1
Cellulose (%TS)	33.9±0.5	37.1±0.5	39.7±1.0
Hemicellulose (%TS)	15.9±1.9	29.9±1.6	29.9±3.4
Lignin (%TS)	21.1±1.0	17.6±0.5	8.9±1.2
Alkalinity (mg $CaCO_3$/L)	740±40	90±0	30±10

Table 2.2. Fiber characteristics of pretreated CSTR AD fiber.

Temperature (C)	Time (hr)	Alkali (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)
120	2	1.0	43.4 ± 1.3	19.8 ± 0.1	23.3 ± 3.0
120	2	2.0	45.8 ± 0.3	18.7 ± 0.6	22.0 ± 3.3
120	2	3.0	50.3 ± 2.1	19.4 ± 0.7	20.8 ± 0.5
130	2	1.0	41.6 ± 0.6	20.8 ± 1.5	21.8 ± 0.1
130	2	2.0	45.9 ± 0.8	18.2 ± 0.4	18.9 ± 0.6
130	2	3.0	45.3 ± 2.4	17.6 ± 1.3	20.5 ± 0.7
120	3	1.0	42.8 ± 1.1	20.6 ± 0.4	23.5 ± 1.3
120	3	2.0	48.0 ± 2.5	21.4 ± 2.5	23.0 ± 1.5
120	3	3.0	48.6 ± 1.1	18.2 ± 0.6	22.1 ± 0.7
130	3	1.0	47.2 ± 0.0	22.0 ± 0.2	19.7 ± 1.4
130	3	2.0	48.2 ± 2.2	18.9 ± 0.0	18.3 ± 0.1
130	3	3.0	53.6 ± 0.7	16.2 ± 0.0	21.8 ± 1.7

Table 2.3. Fiber characteristics of pretreated switchgrass.

Temperature (C)	Time (hr)	Alkali (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)
120	1	0.5	47.3 ± 0.7	28.1 ± 0.2	16.4 ± 1.8
120	1	1.0	45.3 ± 0.1	26.9 ± 0.1	16.7 ± 1.3
120	1	2.0	53.4 ± 0.8	25.6 ± 0.3	11.2 ± 0.1
120	1	3.0	55.8 ± 0.0	20.7 ± 0.1	9.5 ± 4.1
130	1	0.5	47.7 ± 0.2	27.2 ± 0.1	13.1 ± 0.3
130	1	1.0	49.5 ± 1.0	27.5 ± 0.4	13.9 ± 0.6
120	1	2.0	54.1 ± 0.2	24.9 ± 0.6	11.8 ± 0.8
130	1	3.0	58.9 ± 0.1	22.7 ± 0.5	9.8 ± 0.8
120	2	0.5	46.5 ± 1.4	27.9 ± 0.1	16.9 ± 2.5
120	2	1.0	47.4 ± 1.1	27.2 ± 0.4	15.1 ± 1.4
120	2	2.0	55.7 ± 0.2	25.8 ± 0.4	11.5 ± 1.1
120	2	3.0	62.3 ± 1.5	21.6 ± 0.1	9.2 ± 1.3
130	2	0.5	46.5 ± 0.8	26.8 ± 0.0	15.1 ± 1.7
130	2	1.0	52.7 ± 1.7	27.6 ± 0.1	11.4 ± 0.3
130	2	2.0	56.2 ± 0.7	25.7 ± 0.3	9.1 ± 0.5

Table 2.4. Fiber characteristics of pretreated corn stover.

Temperature (C)	Time (hr)	Alkali (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)
120	1	0.5	44.6 ± 1.3	28.0 ± 1.3	14.1 ± 0.2
120	1	1.0	50.6 ± 0.3	28.7 ± 0.6	10.6 ± 0.1
120	1	2.0	47.0 ± 2.2	26.5 ± 0.3	13.8 ± 3.0
120	1	3.0	55.1 ± 0.4	23.8 ± 0.2	10.0 ± 0.1
130	1	0.5	43.5 ± 5.2	29.0 ± 1.2	15.2 ± 4.0
130	1	1.0	50.8 ± 0.2	29.3 ± 0.2	10.4 ± 0.2
120	1	2.0	47.6 ± 0.4	26.5 ± 0.4	10.4 ± 1.0
120	2	0.5	48.2 ± 1.2	29.3 ± 1.5	15.5 ± 0.6
120	2	1.0	49.8 ± 1.6	29.8 ± 1.3	10.4 ± 0.1
120	2	2.0	50.2 ± 1.8	27.0 ± 0.5	8.7 ± 0.7
130	2	0.5	45.8 ± 4.7	27.7 ± 0.6	14.9 ± 3.1
130	2	1.0	48.3 ± 1.2	28.2 ± 0.1	11.3 ± 0.5
130	2	2.0	53.6 ± 0.1	25.7 ± 0.6	5.5 ± 0.5

Figures

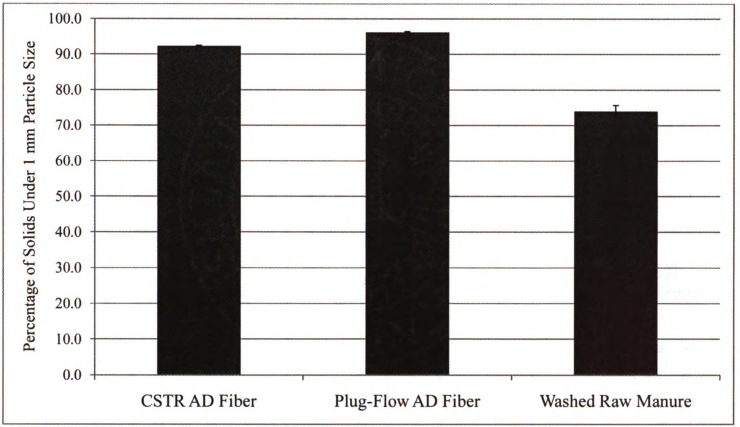

Figure 2.1. Particle size comparison of CSTR AD fiber, plug-flow AD fiber, and washed raw manure.

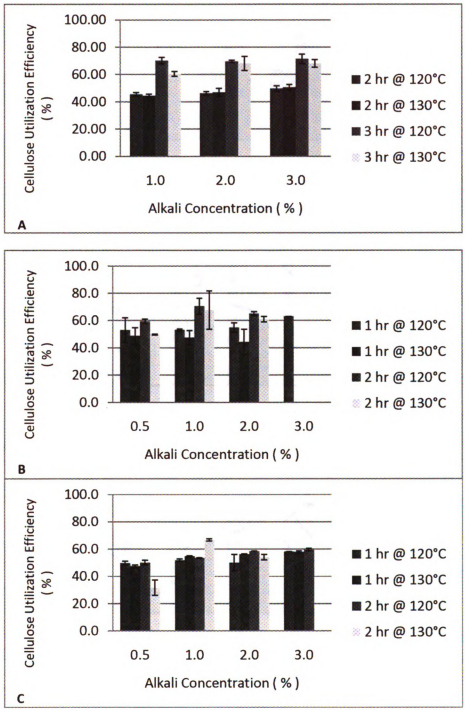

Figure 2.2. Cellulose utilization efficiency. A: CSTR AD Fiber. B: Corn Stover. C: Switchgrass.

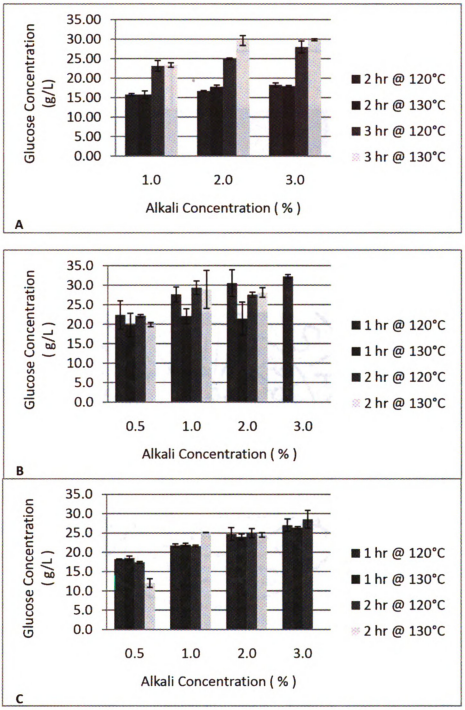

Figure 2.3. Glucose Concentration. A: CSTR AD Fiber. B: Corn Stover. C: Switchgrass.

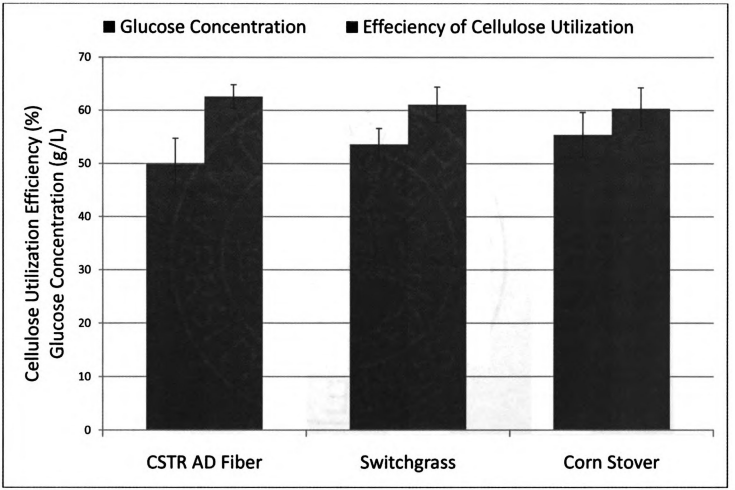

Figure 2.4. Glucose concentrations (g/L) and efficiencies of cellulose utilization (%) after 10% solids enzymatic hydrolysis of most effectively pretreated CSTR AD fiber, switchgrass, and corn stover.

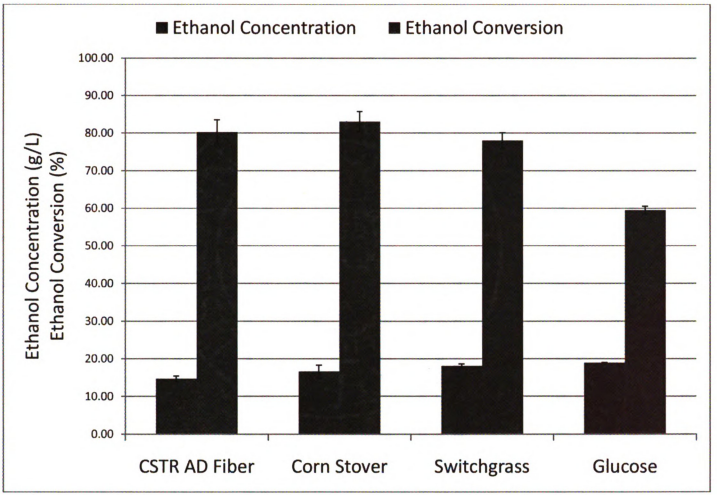

Figure 2.5. Ethanol concentration and conversion efficiency.

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Chapter Three

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CHAPTER 3

3.1. Abstract

Anaerobic digestion (AD) of animal manure is traditionally classified as a treatment to reduce the environmental impacts of odor, pathogens, and excess nutrients associated with animal manure. This report shows that AD also changes the composition of manure fiber and makes it suitable as a cellulosic feedstock for ethanol production. Anaerobically digested manure fiber (AD fiber) contains less hemicellulose (11%) and more cellulose (32%) than raw manure, and has better enzymatic digestibility than switchgrass. Using the most effective dilute alkali pretreatment (2% sodium hydroxide, 130°C, and 2 h), enzymatic hydrolysis of 10% (dry basis) pretreated AD fiber produces 51 g/L glucose at a conversion rate of 90%. The ethanol fermentation on the hydrolysate has a 72% ethanol yield. The results indicate that 120 million dry tons of cattle manure available annually in the U.S. can generate 63 million dry tons of AD fiber that can produce more than 1.67 billion gallons of ethanol. Integrating AD with biorefining will make significant contribution to the cellulosic ethanol production.

3.2. Introduction

Anaerobic digestion (AD) is a biological conversion process that has been widely used to convert organic residues into renewable energy, while alleviating environmental concerns associated with the waste, such as odor, greenhouse gas (GHG) emissions, and subsurface contamination (Speece, 1996). A number of microorganisms, including Clostridia spp. and Archaeobacteria spp., are involved in the AD process. The microorganisms work synergistically through four biological steps (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) to degrade the organic matter in residues (Chynoweth and Isaacson, 1987). There are three output streams of AD: biogas, liquid effluent, and solid digestate (AD fiber). Methane is the major component in the biogas; when combusted it produces heat and electricity. Liquid effluent contains nitrogen and phosphorous; it can be used as a nutrient source to culture algae and can provide non-food feedstock for biorefineries (Wilkie and Mulbry, 2002). As for the AD fiber, cellulose and lignin are the major components, which undergo relatively little changes during conventional AD processes (Table 3.1.). It has been widely accepted by the scientific community that AD fiber is not suitable to be further converted to other useful energy/chemical products due to its "recalcitrant" structure and low nutrient value (Tambone et al., 2009). Thus, it is currently used by the agricultural industry as soil amendment or animal bedding (Johnson et al., 2006). However, there is a lack of research on AD fiber to answer how "recalcitrant" it is, or in another words, is it really "recalcitrant" compared to other cellulosic residues? This study conducted on one of the major lignocellulosic residues, cattle manure, presents interesting findings of

implementing AD to treat cattle manure and generate large quantities of cellulosic feedstock. This will make significant contribution to the cellulosic ethanol production.

Cattle manure rich in carbohydrates and protein is a potential source of feedstock for production of renewable bio-based energy. It has been estimated that 120 million dry tons of cattle manure are produced annually in the United States on 67,000 dairy and 956,500 beef cattle farms (USDA Economic Research Service, 1997; USDA National Agricultural Statistics Service, 2009). It can generate 63 million dry tons of AD fiber that can produce more than 1.67 billion gallons of ethanol. This will make a significant contribution to the goal of generating 16 billion gallons of cellulosic fuel in the U.S. by 2022. Most cattle farms have large storage space, and operate year round, while the cellulosic bioethanol industry is concerned about an insufficient supply of feedstock for producing bioethanol as a fossil fuel substitute (Perlack et al., 2005). If the majority of cattle farms in the U.S. apply AD technology, the combination of animal operation and AD will not only generate a cellulosic feedstock with improved quality, but also provide an excellent supply system for biomass distribution; this will significantly alleviate the barrier of feedstock logistics. Besides cellulosic feedstock production, extensive application of AD technology on 120 million dry tons of cattle manure will capture 14 million tons of methane (equivalent to the Global Warming Potential (GWP) of 302 million tons of CO2) that is capable of generating 756 PJ of heat. An integrated solution of cattle manure treatment and bioethanol production will turn an environmental (soil, water, and air pollution) and economic liability into a public and private asset.

3.3. Materials and Methods

3.3.1. Fiber Samples

Raw manure and AD fiber samples were collected from a private dairy farm with 3,000 cows. Washed raw manure and AD fiber samples were obtained by washing 1 kg of raw sample six separate times with 6 kg of de-ionized water each and then separating out the solid using 20 and 60 mesh screens respectively. Switchgrass was collected from the Michigan State University Crop and Soil Science Teaching and Research Field Facility, and samples were dried and grinded on-site using a grinder (Willey Mill, Standard Model No.3, Arthur H. Thomas, Philadelphia, PA) with 4mm size opening.

3.3.2. Dilute Sulfuric Acid Treatment

Different fibers were treated in flasks using autoclave (Brinkmann 2540M, Tuttnauer USA Co. Ltd, Hauppauge, NY) at various acid concentration (1%, 2%, 3 wt%), retention time (0.5, 1, 2 h) and temperature (110, 120, 130°C) using a complete random design (CRD). Fiber concentration was fixed at 6% based on dry matter. Treated mixture solutions were neutralized to pH values of 4.0–5.0 using a 20% sodium hydroxide solution. After filtering with Whatman (#1) filter paper and washing the contents using 300mL 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.

3.3.3. Alkali Treatment

The alkali treatments were also carried out by a CRD with two replications of 54 treatment combinations. Three sodium hydroxide concentrations (0.5%, 1%, 2 wt%) with three reaction durations (0.5, 1, 2 h) were investigated at three different temperatures

(110, 120, 130°C). Fiber concentration was fixed at 6% dry matter. The treatment was fulfilled in the same autoclave described above. Treated mixture solutions were neutralized to pH values of 4.0–5.0 using 20% sulfuric acid solution. 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.

3.3.4. Enzymatic Hydrolysis Process

Wet solid samples (1 and 2 g dry matter) and de-ionized water were mixed with a total mass of 20 g into a 125mL shake flask, which makes the solid concentrations of 5% and 10% (w/w). All mixed samples were autoclaved before adding enzymes. Cellulase (ACCELLERASETM 1000, Genencor, Rochester, NY) at loading of 26 FPU/g dry substrate at 5% solid concentration and 52 FPU/g dry substrate at 10% solid concentration were used to fulfill the enzymatic hydrolysis. The flasks were shook at 140 rpm, and the reaction temperature was 50°C. After 72 h, aliquots were boiled for 5 min and filtered with Whatman (#1) filter paper. The filtrates were filtered into HPLC vials with Millex-GS 0.22 mm membrane for analysis of glucose and other monomeric sugars such as xylose, arabinose, and galactose.

3.3.5. Ethanol Fermentation

Saccharomyces cerevisiae D5A obtained from American Type Culture Collection (ATCC, Manassas, VA) was used in the yeast fermentation. Inoculum was cultured for 15 h at 30°C in a 250mL flask on ATCC Medium No. 1245 (10 g/L yeast extract, 20 g/L Bacto peptone, and 20 g/L glucose). The culture broth for inoculum was centrifuged to collect yeast biomass as inoculum. The inoculum was mixed with an autoclaved nutrition

solution (10 g/L of peptone, 5 g/L of yeast extract, and glucose in the hydrolysates). The inoculum-to-solution ratio of 1:10 was used to conduct the fermentation. Samples were taken at the beginning and end of a 24-h fermentation process for glucose and ethanol analysis.

3.3.6. Analytical Methods

Samples were diluted to 5% dry matter for ammonium and total Kjedahl nitrogen (TKN), and to 1% dry matter for alkalinity analysis using HACH method (Loveland, CO). Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) of samples were analyzed using Van Soest Fiber Analysis System (Goering and Van Soest, 1970). NDF, ADF, and ADL were used to calculate cellulose, hemicellulose, and lignin contents. Cellulose and hemicellulose can be determined by the differences of %ADF–%ADL and %NDF–%ADF, respectively. Lignin content was expressed by ADL. Glucose, ethanol and other mono-sugars were determined using an Agilent 1100 HPLC system equipped with a Bio-rad Aminex HPX-87H analytical column and a refractive index detector. The mobile phase was 0.005M sulfuric acid with a flow rate of 0.6 mL/min. Column temperatures were 65 and 55°C for sugar and ethanol, respectively (Ruiz and Ehrman, 1996). High purity standards including glucose (Catalog Number: 49158), xylose (Catalog Number: 95729), galactose (Catalog Number: 48259), arabinose (Catalog Number: 10840), and ethanol (Catalog Number: 459828) were purchased from Sigma-Aldrich, St. Louis, MO.

3.3.7. Statistical Analysis

Pair-wise comparison using the Statistical Analysis System program 8.0 (SAS Institute, Inc., Cary, NC) was conducted to evaluate the effects of reaction conditions and

different feedstocks (raw manure, AD fiber, and switchgrass) on glucose conversion and ethanol production.

3.4 Results

3.4.1. Fiber Quality

To better quantify the effects of AD, the composition changes of manure fibers during the course of AD were investigated. The hemicellulose and protein contents in manure are significantly reduced, while the cellulose and lignin contents are greatly increased (Table 3.1.). Compared to switchgrass, AD fiber contains lower hemicellulose content (11.6%, dry basis) and similar cellulose content (32.3%, dry basis). Lower hemicellulose content eliminates the problem of pentose utilization that cellulosic biorefineries encounter. Considering the integrated process (AD and bioethanol production), the majority of C-5 sugars was utilized by AD to generate methane. The higher heating value of methane is larger than ethanol (approximately 52.5 MJ/kg). Methane from hemicellulose generates more energy (electricity energy) than ethanol from hemicellulose due to the relatively low conversion rate (80%) of C-5 sugars to ethanol (Aden et al., 2002). Meanwhile, a non-recombinant, industrially robust fermenting strain, S. Cerevisiae, can be used to efficiently perform the hexose (C-6 sugar) fermentation on AD fiber for ethanol production. Thus, in terms of system efficiency, the integrated process is better than ethanol production on both C-5 and C-6 sugars from raw manure. Additionally, the particle size is reduced during AD. Eighty-eight percent (dry basis) of plug-flow AD fiber has a particle size smaller than 1 mm, while the original manure fiber has 75% (dry basis) (Fig. 2.1.). Since 22% of capital investment on feedstock storage and handling of a cellulosic ethanol production process is for size

reduction unit (Aden et al., 2002), removing the size reduction unit from the bioethanol process, along with using AD fiber with lower hemicellulose content, will significantly reduce the production cost, and therefore greatly improve the efficiency of cellulosic ethanol production. Accordingly, AD fiber has more favorable chemical and physical properties than other cellulosic feedstocks such as switchgrass (Table 3.1). However, the degree to which AD fiber is "recalcitrant" still has not been answered.

3.4.2. Hydrolysis and Fermentation

In order to explore how "recalcitrant" it is, the AD fiber was used as a feedstock, along with raw manure, washed AD fiber (removing the alkalinity), and switchgrass, to compare enzymatic digestibility. Two pretreatment methods of dilute acid and dilute alkali treatments, followed by enzymatic hydrolysis, were selected to investigate the digestibility. The acid treatment experiments concluded that the most effective conditions were 1% of acid concentration, 130°C of reaction temperature, and 2 h of reaction time. Enzymatic hydrolysis of the acid treated AD fiber has a glucose conversion rate of 22%, which is higher than that of acid treated raw manure (12%) and lower than acid treated washed AD fiber (41%) (Fig. 3.1.A, Table 3.2.). Under the same reaction conditions (130°C of reaction temperature and 2 h of reaction time) with 1% of sodium hydroxide concentration the alkali-treated AD fiber has a 73% glucose conversion rate (glucose conversion rate [%] = glucose content [g]/ (1.1*cellulose in sample [g])*100), which is significantly ($P<0.05$) higher than that of raw manure (19%) and washed AD fiber (67%) (Fig. 3.1.B, Table 3.2.). The difference in glucose conversion rates between AD fiber and washed AD fiber are mainly caused by the alkalinity and ammonia content in the samples (Table 3.1.). During the acid treatments, the higher alkalinity in AD fiber consumed a

certain amount of acid and decreased the efficiency of the acid treatment; while in the alkali treatments the performance was enhanced due to the alkalinity and ammonia in AD fiber. Based on glucose conversion rates from two different treatments, the dilute alkali method is more effective than the dilute acid method to treat AD fiber. Furthermore, the optimization of dilute alkali treatment of AD fiber concluded that, under the most effective dilute alkali treatment (2% of alkali concentration, 130°C of reaction temperature, and 2 h of reaction time), the treated AD fiber generates 51 g/L glucose at a 90% glucose conversion rate (Table 3.2., Fig. 3.2.).

A comparison experiment was conducted with switchgrass using optimized dilute alkali treatment (Fig. 3.1.C., Table 3.2.). The alkali treated AD fiber has a glucose conversion rate of 90%, significantly ($P<0.05$) higher than switchgrass (62%). The data demonstrate that the alkali treated AD fiber has better enzymatic digestibility than alkali-treated switchgrass. In order to further evaluate the ethanol production yield from AD fiber, an enzymatic hydrolysis of dilute alkali-treated fiber at high solid contents (10% dry basis) followed by ethanol fermentation was conducted (Fig. 3.1.D.). Alkali treated AD fiber and switchgrass were compared using a C-6 fermentation strain S. cerevisiae D5A. A 72% ethanol yield (ethanol yield [%] = ethanol produced [g]/(0.51* 1.11*cellulose in sample [g]*100)) was obtained from AD fiber, which has no significant ($P>0.05$) difference between pure glucose and switchgrass hydrolysate. These results, combined with low hemicellulose content and reduced size, confirm that AD can act as an environmentally friendly biological pretreatment method to develop a desirable feedstock (AD fiber) for biorefineries.

3.5. Discussion

3.5.1. Mass Balance

Based on the experimental results, a mass balance analysis was conducted on a cow to discover the impacts of AD fiber on ethanol production. Approximately 55 kg manure per day at 84.5% moisture content was excreted from one cow. After mixing with recycled AD liquid effluent, one kilogram of AD influent contains 0.12 kg of total solid, 0.132 kg of COD, 0.026 kg of cellulose, 0.020 kg of hemicellulose, 0.017 kg of lignin, and 0.025 kg of crude protein. The detailed mass balance is presented in Figure 3.3. After 20 days of AD, 40% of chemical oxygen demand (COD) was converted into biogas, 77% of protein and 56% of hemicellulose was consumed, while both cellulose and lignin were only slightly changed (7% and10% reductions for cellulose and lignin, respectively). After liquid/solid separation of AD effluent, 4.5 kg/day of AD fiber with 32.3% cellulose, 11.6% hemicellulose, and 25.1% lignin was produced. The data from the experiments of enzymatic hydrolysis and fermentation were applied in the calculation of the mass balance. The 8.5 kg of dry manure per day from a cow can produce 0.347 kg ethanol/day. Since approximately 120 million dry tons of cattle manure is available in the U.S. (USDA Economic Research Service, 1997), it can produce 63 million tons of AD fiber as cellulosic feedstock via AD technology. A potential ethanol production of 1.67 billion gallons per year can be produced from this amount of biomass, which accounts for approximately 10% of the 16 billion gallons of cellulosic biofuel by 2022 (The Energy Independence Act, 2007). In addition, the optimal carbon/nitrogen (C/N) ratio of AD is 25–32:1 and cattle manure has a C/N ratio of 15:1 (Table I), which means that there is a potential of mixing other high C/N ratio agricultural residues such as corn stover and

switchgrass with cattle manure to improve the performance of the digestion. This will greatly increase the amount of AD fiber, and lead to production of more ethanol from integrated animal operations and ethanol production.

3.5.2. Water Balance

Integrating AD and ethanol fermentation addresses the concern of water demand by cellulosic ethanol production. Two to six gallons of water are needed to produce a gallon of ethanol from cellulosic feedstocks such as switchgrass and corn stover (Aden, 2007). Reducing the total amount of water use for cellulosic ethanol production is one of the keys towards a sustainable bioenergy solution. A mass balance on water shows a positive water demand for AD fiber ethanol production (Fig. 3.4.). The moisture in the manure provides enough water for the process. The AD and ethanol production in the integrated system generate 35 kg/cow/ day of liquid effluent with less nutrients and 8 kg/cow/day of distilled water, respectively. The water can be recycled for dilution and other uses during the AD and ethanol production. Thus, additional fresh water is not necessary for the process.

3.5.3. Environmental Impacts

Implementation of AD to confine the methane production will alleviate the GHG emissions associated with the animal industry. Current disposal practices for manure cause methane to be released through natural processes. Up to 7% of total GHG emissions are from methane generated directly by animal-related agricultural operations (Steinfeld et al., 2006). If the 120 million dry tons of cattle manure available annually in the U.S. is treated by AD, 14.4 million tons of methane (based on 1.02 kg of methane per 8.5 kg dry cattle manure in Fig. 3.3.) is captured each year (equivalent to the GWP of

302.4 million tons of CO2); burning the methane will generate 756 PJ of heat (the heating value of methane is 52.5 MJ/kg). Considering both methane and ethanol from the integrated AD/ethanol system on cattle manure, 13.4 million tons of carbon (10.8 million tons from 14.4 million tons of methane, and 2.6 million tons from 1.67 billion gallons of ethanol) will be sequestrated annually in the United States.

3.5.4. New Model of Ethanol Production

The new model of ethanol production can be established based upon these results (Fig. 3.5.). A regional bioethanol production plant could be centralized within cattle/dairy farmland. A 20 million gallon (60 million kg) ethanol production needs 688 tons of dry manure cellulose per day (1 ton of dry manure cellulose produces 240 kg of ethanol based on the experimental result presented in Fig. 3.3.) as feedstock; medium size cattle/dairy farms with 1,000 cows generate 8.5 ton dry manure per day. Using AD to treat this manure, each farm can produce 1.45 tons of dry AD cellulose per day. Four hundred seventy-five medium size cattle/dairy farms can produce 400 tons of dry AD cellulose per day for 20 million gallons of ethanol production. Implementation of AD on a national scale with 1 million cattle farms will yield approximately 63 million dry tons of AD fiber annually for ethanol production. Eighty-two 20 million gallon cellulosic ethanol plants can be established using the AD fiber as feedstock (Table 3.3.). The year-round operation, compared with seasonal grain-based feedstocks, plus large available space on cattle and dairy farms, provide a local supply system for biomass distribution, significantly reducing the transportation and storage cost for the bioethanol production. The waste streams from ethanol production such as stillage can be transported back to the farm as animal feed or AD influent. In addition, the sustainability of cattle production

systems will be improved by reducing the GHG emissions, potential surface and ground water pollution, and noxious odor, while at the same time generating electricity and AD fiber that will greatly enhance farm income. The integration of AD and cellulosic ethanol production will create a win-win-win solution for fuel ethanol production, cattle operations, and the environment.

Tables

Table 3.1. Characteristics of raw dairy manure, AD fiber, and switchgrass.

	Raw dairy manure	AD fiber	Switchgrass
Dry matter, %	15.5 ± 0.6	28.1 ± 0.7	91.5 ± 0.3
Cellulose, % dry basis	21.7 ± 2.4	32.3 ± 0.8	37.1 ± 0.5
Hemicellulose, % dry basis	17.2 ± 2.2	11.6 ± 0.6	29.9 ± 1.6
Lignin, % dry basis	14.5 ± 3.2	25.1 ± 1.2	17.6 ± 0.5
Crude protein, % dry basis	16.3 ± 0.1	7.5 ± 0.6	6.9 ± 0.7
C, % dry basis	45.49 ± 0.5	48.4 ± 1.9	47.8 ± 1.2
N, % dry basis	2.6 ± 0.02	1.2 ± 0.13	1.1 ± 0.2
Ammonia-N, % dry basis	0.17 ± 0.02	0.36 ± 0.03	-
C:N ratio	17.3	40.3	43.5
pH	8.5 ± 0.1	9.2 ± 0.0	5.8 ± 0.2
Total alkalinity (mg $CaCO_3$/L)[b]	1370 ± 10	400 ± 0.0	30 ± 0.2

*Raw dairy manure and AD fiber samples were taken from a commercial plug-flow anaerobic digester on a 3,000 cow dairy farm. The temperature was maintained at 40°C; the retention time was 20 days.
[a] The end-point pH for total alkalinity measurement was 4.5.

Table 3.2. Characteristics of treated samples.

	Cellulose, % dry basis	Hemicellulose, % dry basis	Lignin, % dry basis
Acid treated raw manure [a]	30.9 ± 0.6	3.2 ± 0.3	20.8 ± 0.7
Acid treated AD fiber [a]	43.2 ± 0.7	0	40.1 ± 2.3
Acid treated washed AD fiber [a]	48.9 ± 2.4	0	41.2 ± 2.4
Alkali treated raw manure [b]	34.6 ± 1.3	14.4 ± 1.7	14.9 ± 0.8
Alkali treated AD fiber I [b]	49.3 ± 0.5	8.7 ± 0.6	23.5 ± 1.0
Alkali treated washed AD fiber [b]	49.6 ± 0.7	10.9 ± 1.1	23.8 ± 0.9
Alkali treated AD fiber II [c]	48.2 ± 0.4	3.9 ± 0.5	23.8 ± 0.2
Alkali treated switchgrass [c]	69.5 ± 4.0	11.6 ± 0.1	8.6 ± 1.9

[a] The treatment conditions were: 1% of acid concentration, 130°C of reaction temperature, and 2 h of reaction time.
[b] The treatment conditions were: 1% of alkali concentration, 130°C of reaction temperature, and 2 h of reaction time.
[c] The treatment conditions were: 2% of alkali concentration, 130°C of reaction temperature, and 2 h of reaction time.

Table 3.3. Potential integrated AD/ethanol production in the United States.*,**,†

	State	Cattle farms	Total number of cattle (AU)	manure (ton/year, dry base)	Methane Production (ton/year)	Electricity from methane (GWh/year)	Ethanol Production (Gallon/year)	Number of 20 Mgal ethanol plants
1	Alabama	29,008	1,228,360	1,679,683	138,574	44,436	23,381,192	**1**
2	Alaska	147	6,184	8,336	688	221	116,043	**0**
3	Arizona	2,950	733,991	1,122,175	92,579	29,687	15,620,678	**1**
4	Arkansas	31,880	1,422,080	1,964,492	162,071	51,971	27,345,733	**1**
5	California	19,154	4,371,169	7,524,698	620,788	199,068	104,743,790	**5**
6	Colorado	16,943	2,654,937	3,601,839	297,152	95,288	50,137,594	**3**
7	Connecticut	1,531	63,125	118,240	9,755	3,128	1,645,907	**0**
8	Delaware	580	10,459	14,369	1,185	380	200,013	**0**
9	Florida	15,851	1,607,330	2,342,430	193,250	61,970	32,606,624	**2**
10	Georgia	22,769	1,039,263	1,510,559	124,621	39,962	21,026,985	**1**
11	Hawaii	731	151,954	213,559	17,619	5,650	2,972,748	**0**
12	Idaho	13,355	1,627,748	2,486,646	205,148	65,785	34,614,108	**2**
13	Illinois	32,920	1,128,536	1,656,954	136,699	43,835	23,064,798	**1**
14	Indiana	30,635	768,415	1,185,228	97,781	31,356	16,498,375	**1**
15	Iowa	54,694	2,777,748	3,956,897	326,444	104,681	55,080,007	**3**
16	Kansas	39,760	4,994,471	6,681,080	551,189	176,750	93,000,636	**5**
17	Kentucky	51,715	1,946,689	2,783,535	229,642	73,639	38,746,810	**2**
18	Louisiana	15,294	768,676	1,106,646	91,298	29,277	15,404,511	**1**
19	Maine	2,587	94,925	176,093	14,528	4,659	2,451,219	**0**
20	Maryland	5,923	242,532	426,858	35,216	11,293	5,941,864	**0**
21	Massachusetts	1,754	22,701	31,395	2,590	831	437,013	**0**
22	Michigan	22,416	879,375	1,537,269	126,825	40,669	21,398,785	**1**
23	Minnesota	47,991	1,975,774	3,292,747	271,652	87,111	45,835,042	**2**
24	Mississippi	20,108	913,194	1,278,113	105,444	33,813	17,791,328	**1**
25	Missouri	74,084	3,430,067	4,805,597	396,462	127,133	66,893,903	**3**

Table 3.3. Continued. Potential integrated AD/ethanol production in the United States.*,**,†

26	Montana	15,107	2,366,673	3,194,075	263,511	84,500	44,461,520	2
27	Nebraska	35,823	5,294,691	7,073,313	583,548	187,126	98,460,524	5
28	Nevada	1,784	447,361	628,590	51,859	16,630	8,749,972	0
29	N. Hampshire	1,197	42,204	79,985	6,599	2,116	1,113,390	0
30	New Jersey	1,954	51,332	90,343	7,453	2,390	1,257,578	0
31	New Mexico	8,731	1,410,703	2,143,299	176,822	56,702	29,834,719	1
32	New York	25,532	1,442,648	2,773,273	228,795	73,368	38,603,956	2
33	N. Carolina	23,358	768,955	1,124,256	92,751	29,743	15,649,647	1
34	North Dakota	16,458	1,512,710	2,092,159	172,603	55,349	29,122,849	1
35	Ohio	38,662	1,049,247	1,718,492	141,776	45,463	23,921,409	1
36	Oklahoma	59,694	3,777,056	5,154,913	425,280	136,375	71,756,395	4
37	Oregon	17,803	1,280,226	1,818,107	149,994	48,099	25,308,052	1
38	Pennsylvania	40,914	1,529,059	2,793,328	230,450	73,898	38,883,119	2
39	Rhode Island	237	2,339	3,198	264	85	44,517	0
40	S. Carolina	10,011	368,957	523,890	43,221	13,860	7,292,551	0
41	South Dakota	25,075	2,976,280	4,096,062	337,925	108,362	57,017,179	3
42	Tennessee	52,607	1,718,607	2,437,754	201,115	64,491	33,933,536	2
43	Texas	144,843	11,369,854	15,558,533	1,283,579	411,605	216,574,781	11
44	Utah	8,930	774,665	1,148,729	94,770	30,390	15,990,306	1
45	Vermont	5,041	316,605	619,234	51,087	16,382	8,619,733	0
46	Virginia	28,645	1,293,281	1,880,364	155,130	49,746	26,174,673	1
47	Washington	12,921	1,107,595	1,772,278	146,213	46,886	24,670,116	1
48	West Virginia	12,868	342,223	480,911	39,675	12,723	6,694,276	0
49	Wisconsin	68,902	3,137,060	5,812,235	479,509	153,764	80,906,316	4
50	Wyoming	6,856	1,367,915	1,839,912	151,793	48,675	25,611,569	1
	All States	**1,218,733**	**80,664,721**	**118,484,288**	**9,774,954**	**3,134,534**	**1,649,301,294**	**82**

*The calculation is based on the experimental results.

**AU is short for animal unit.

† Efficiency of generator is set at 40%.

Figures

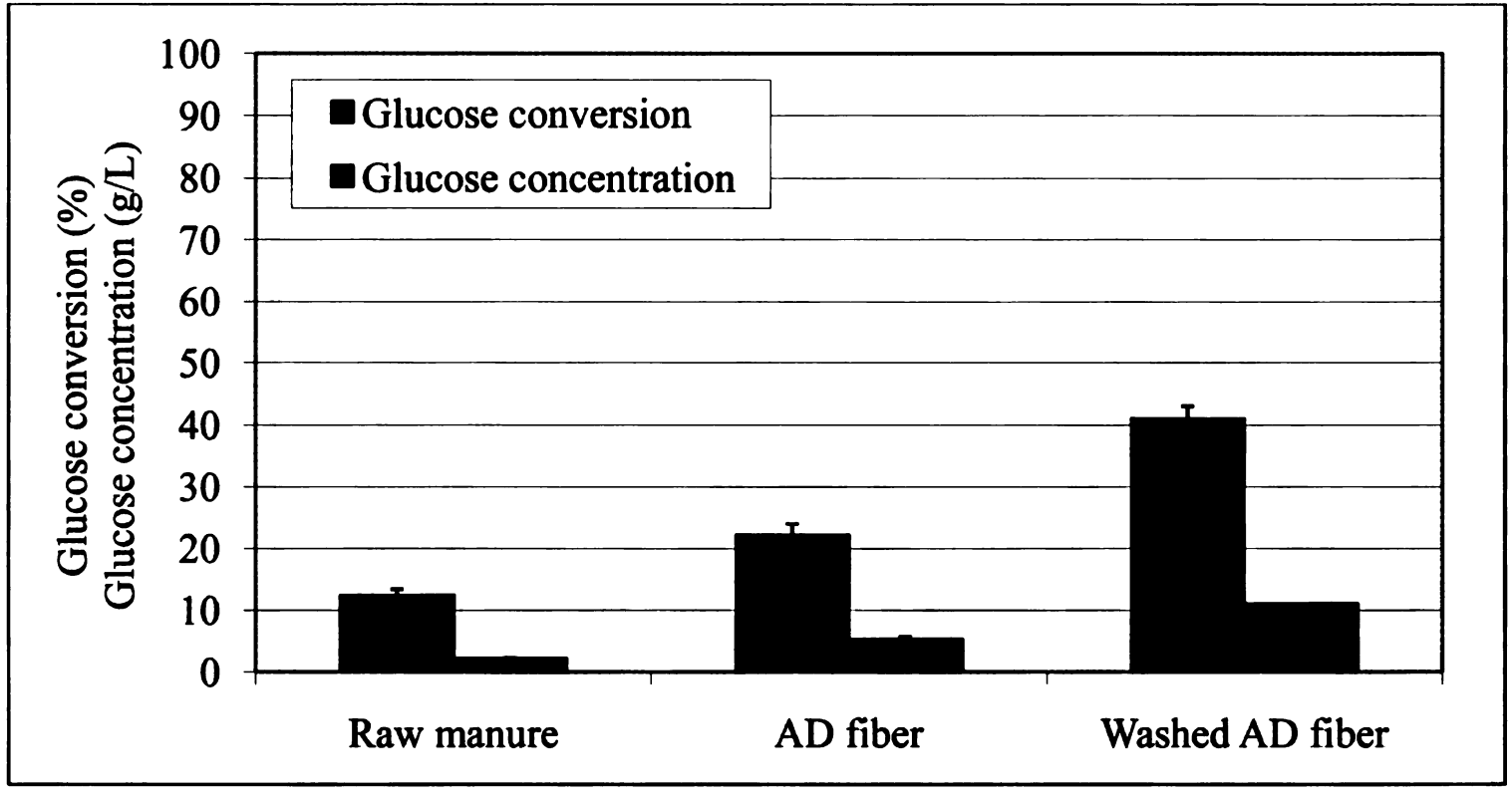


Figure 3.1.A. Glucose production from acid treated samples.

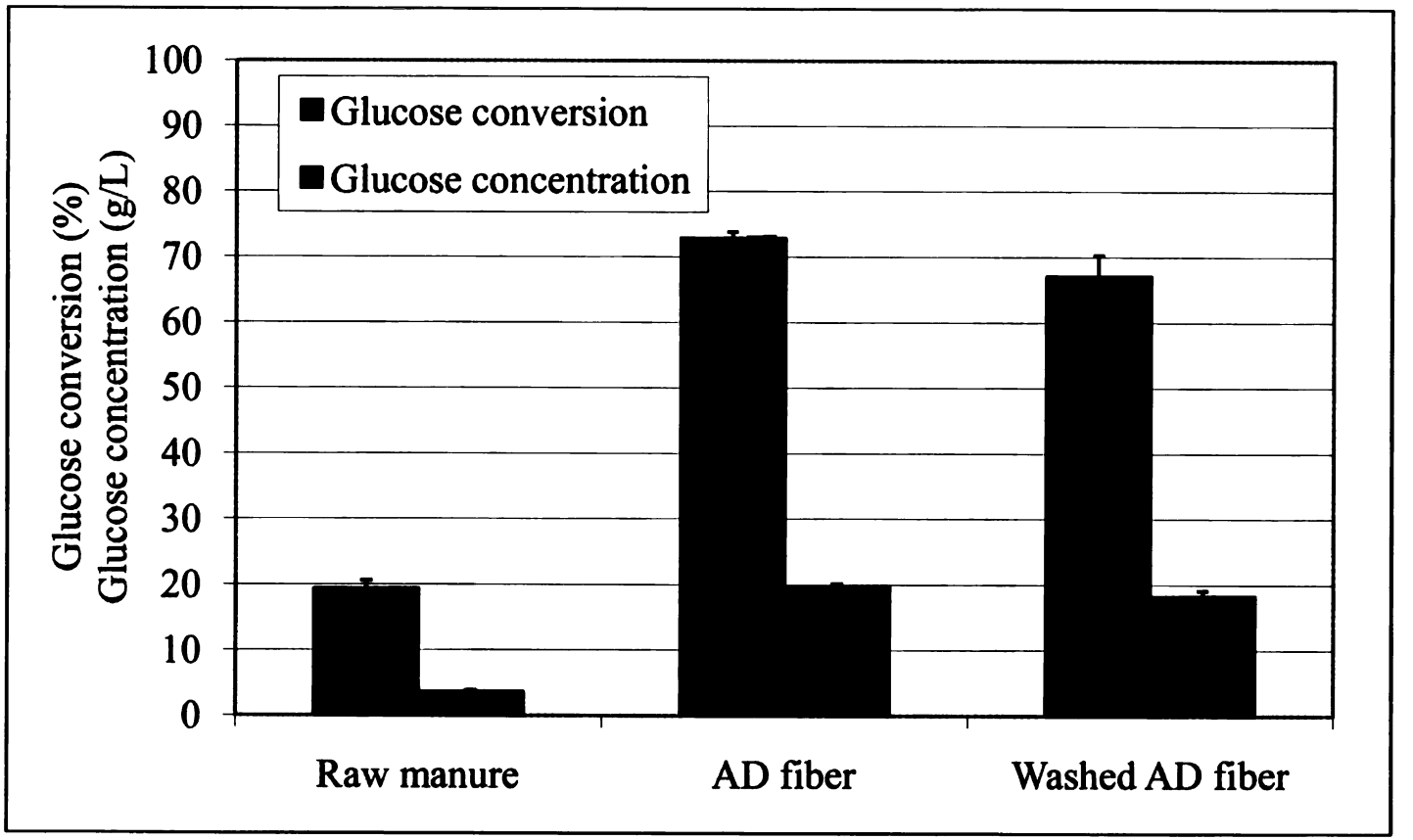


Figure 3.1.B. Glucose production from alkali treated samples.

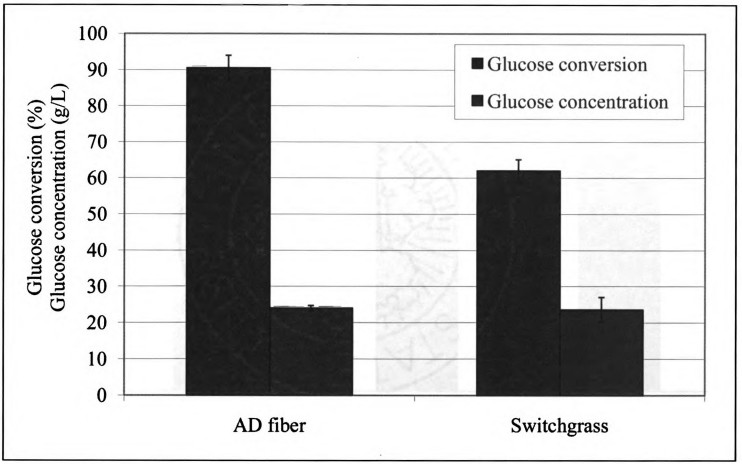


Figure 3.1.C. Comparison of glucose production from alkali treated AD fiber and switchgrass.

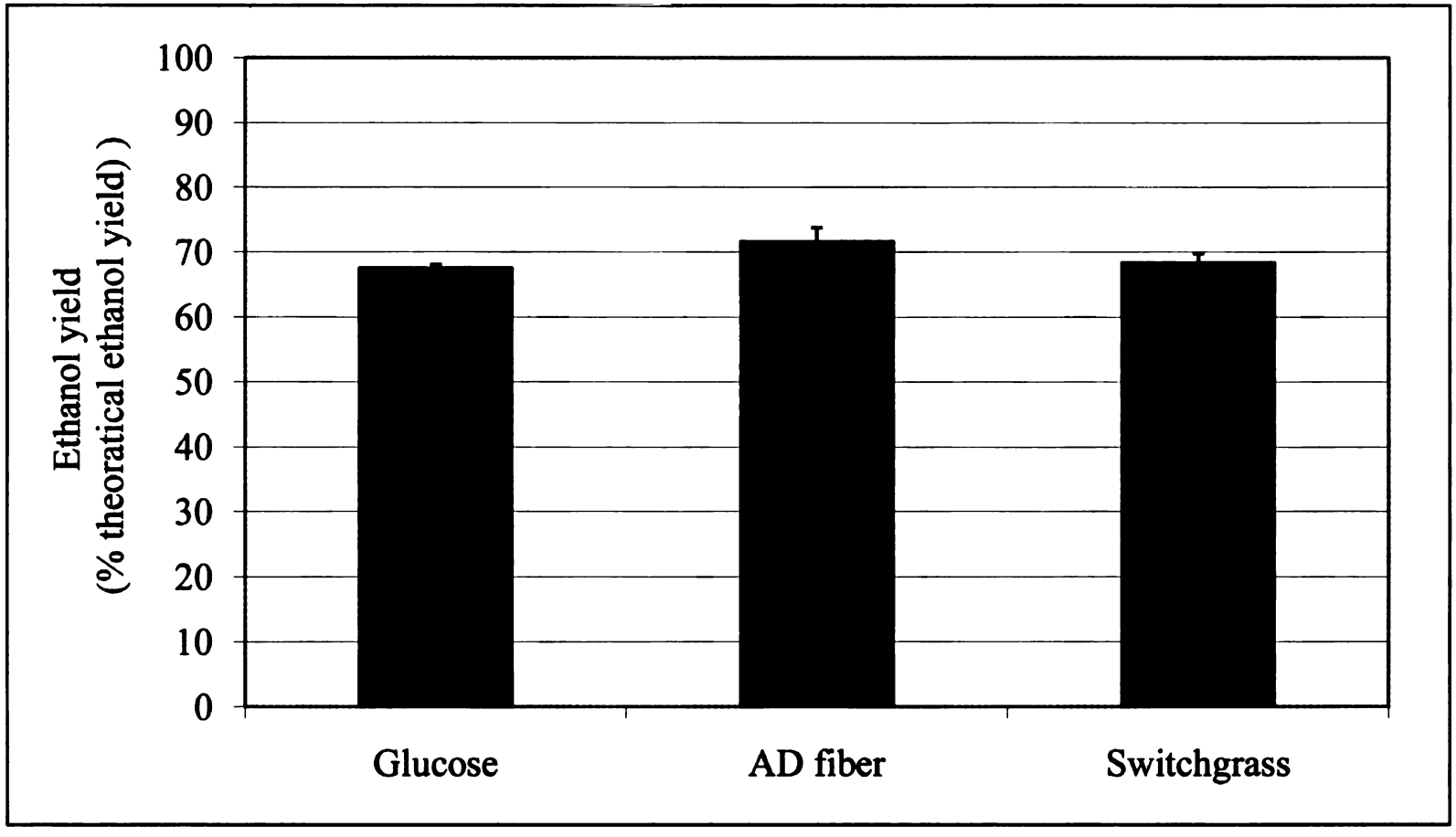


Figure 3.1.D. Ethanol yield from different hydrolysates. Glucose concentrations of glucose, AD fiber hydrolysate, and switchgrass hydrolysate are 54.44±0.43 g/L, 51.13±0.51 g/L, and 48.75±0.59 g/L, respectively.

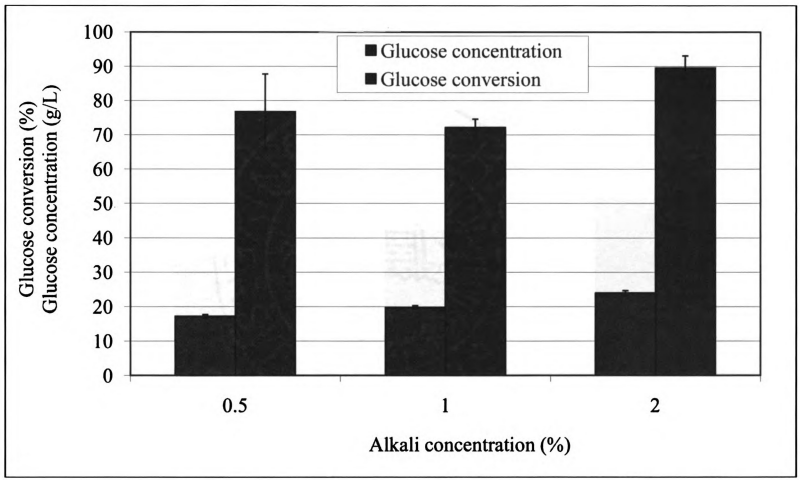


Figure 3.2.A. Optimization of alkali treatment of AD fiber. Glucose conversion from enzymatic hydrolysis on 5% (dry basis) of alkali treated AD fiber.

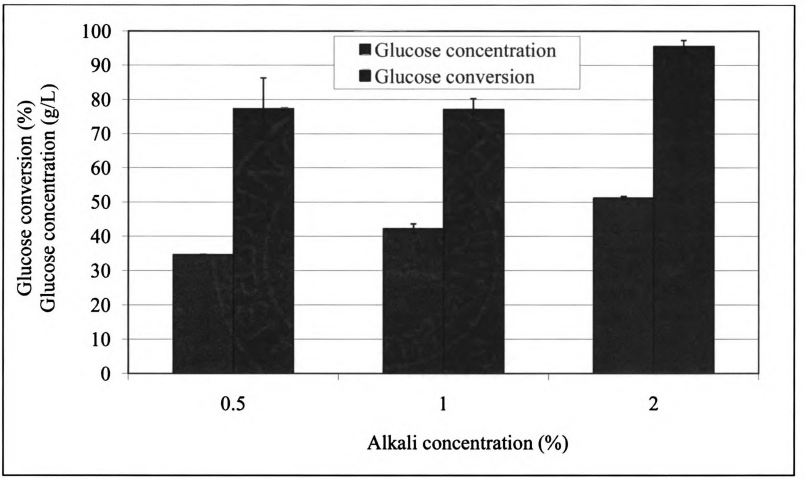


Figure 3.2.B. Optimization of alkali treatment of AD fiber. Glucose conversion from enzymatic hydrolysis on 10% (dry basis) of alkali-treated AD fiber.

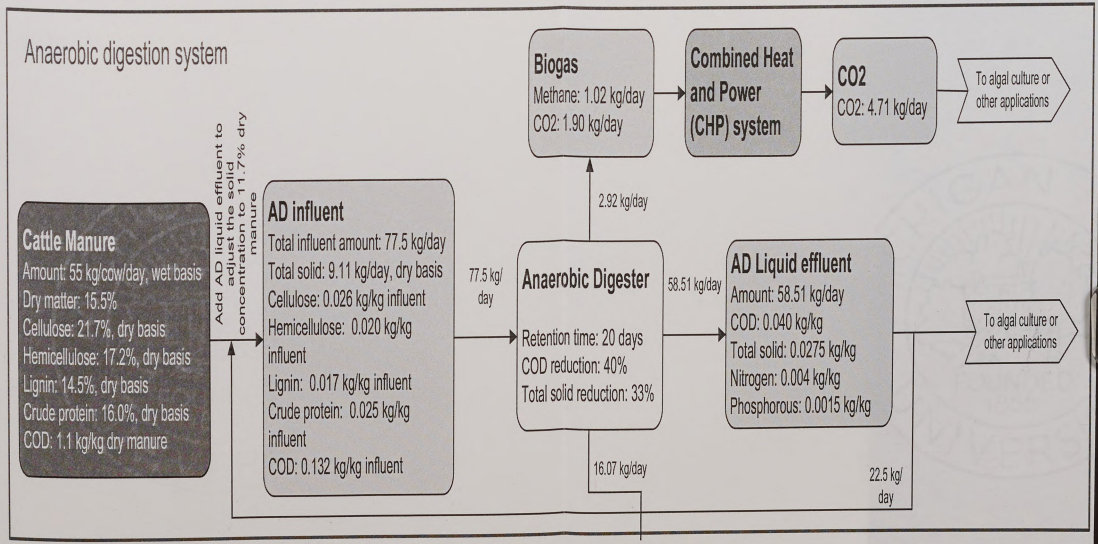


Figure 3.3. Mass balance of integrated anaerobic digestion and ethanol production system per dairy cow. a: All data used in the mass balance calculation (except CO2 from CHP system) were obtained from lab experiments and digester operation. b: Carbon dioxide generated from CHP was calculated based on the stoichiometric relationship of methane and carbon dioxide. One kilogram of methane is theoretically capable of generating 2.75 kg of CO2.

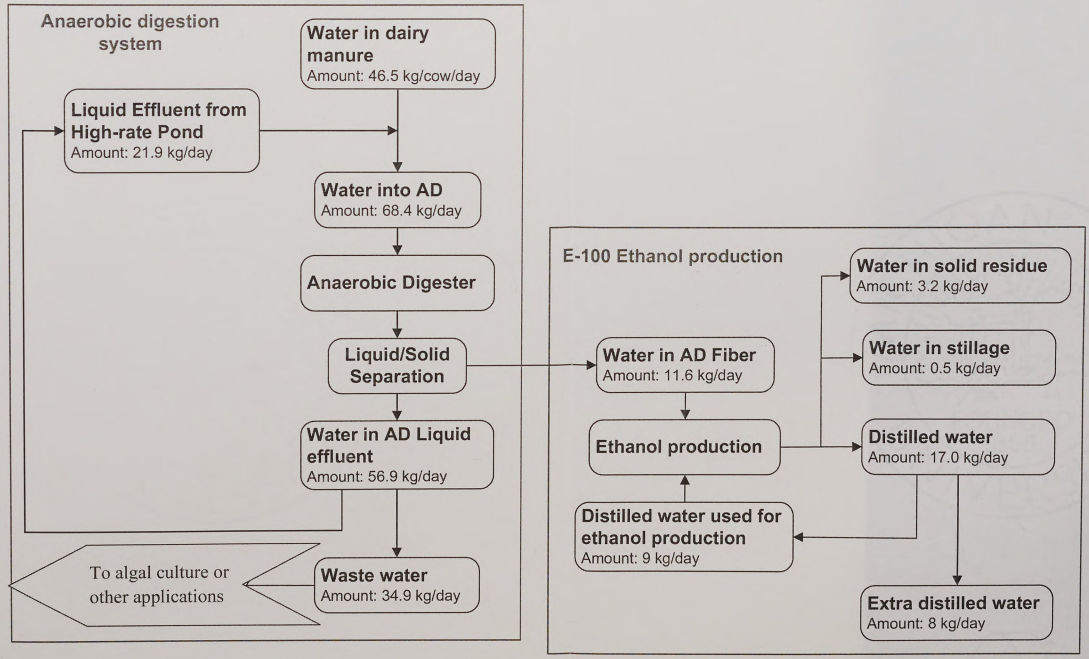


Figure 3.4. Water balance of integrated anaerobic digestion and ethanol production.

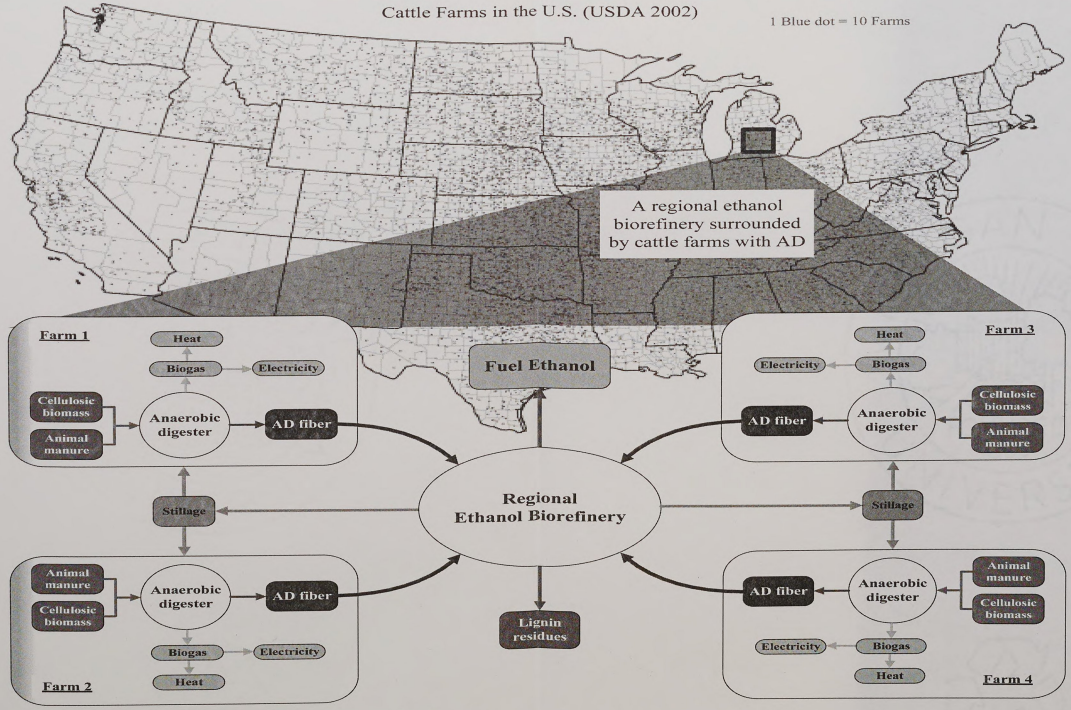
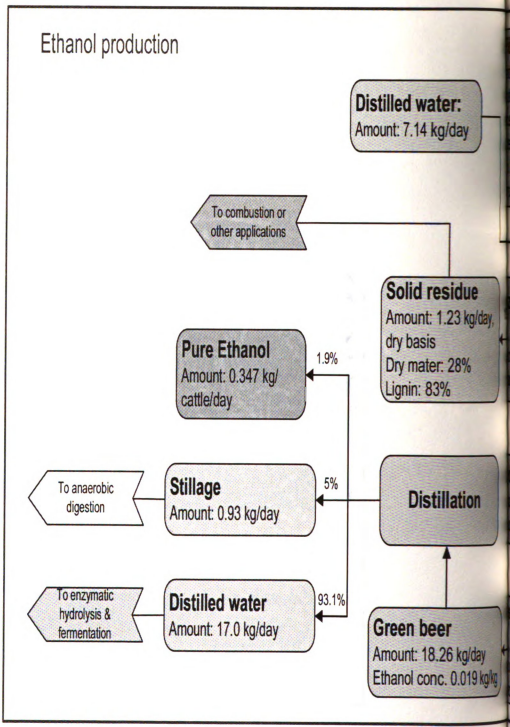
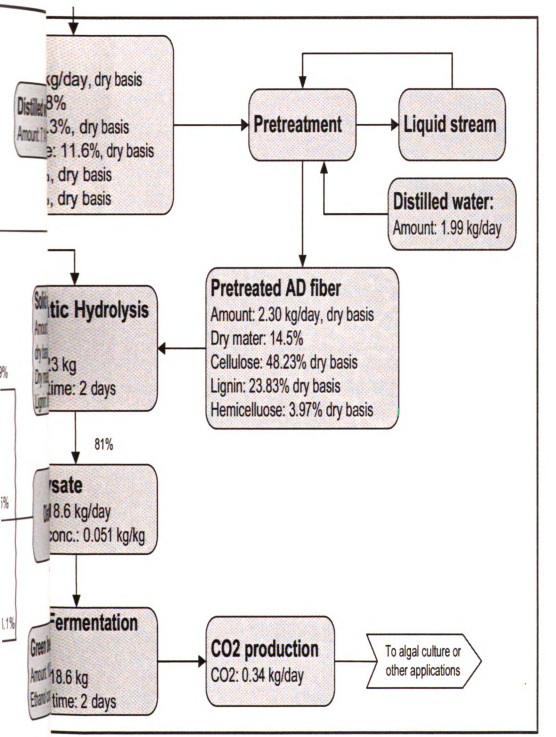


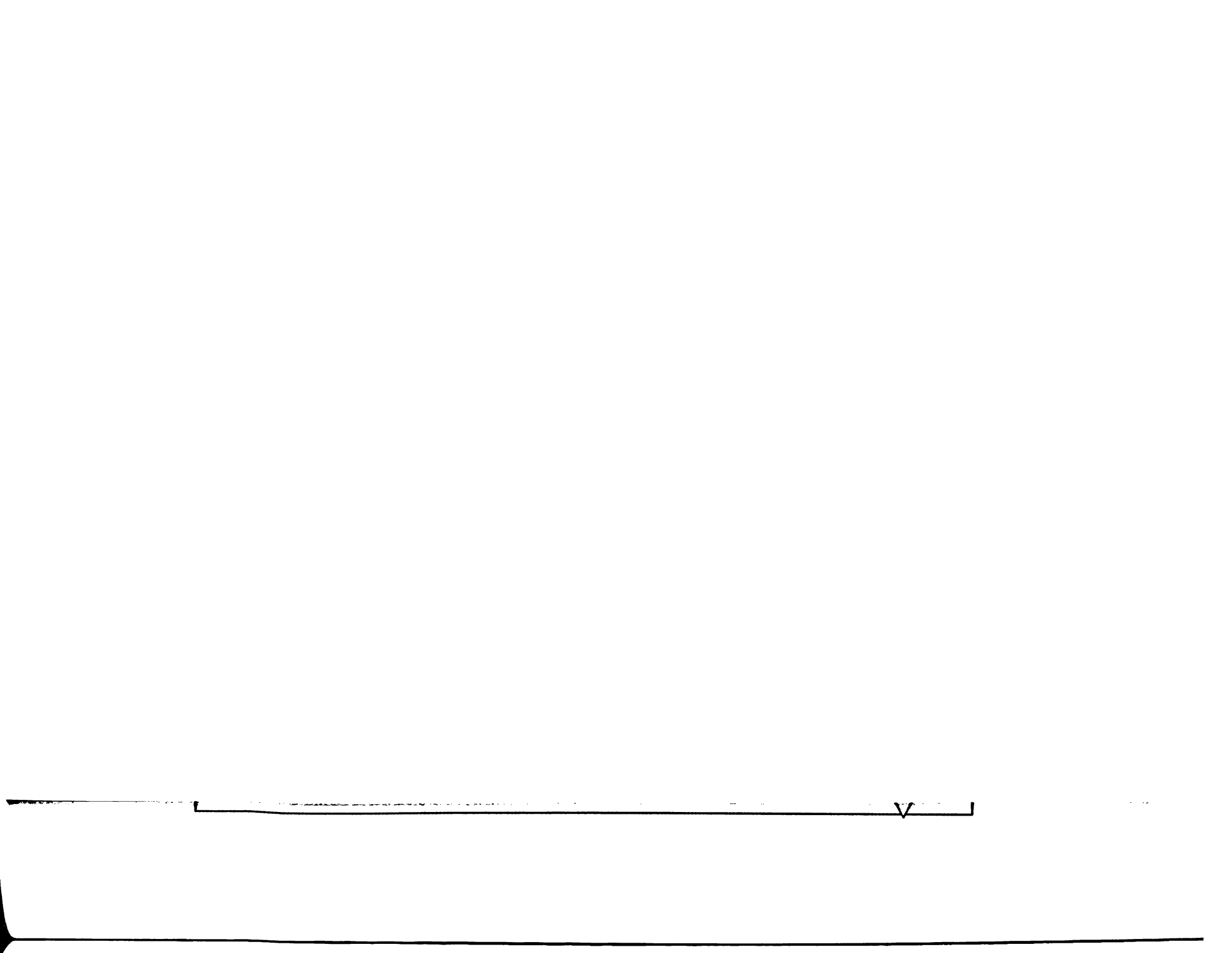
Figure 3.5. Operational model of anaerobic digestion systems and regional ethanol production.



3.3. Continued. Mass balance of integrated anaero and eth



and ethanol production system per dairy cow.



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Conclusions

Due to the abundant quantity and year round availability of cattle manure, it serves as a large potential feedstock for ethanol production without the logistical storage problems associated with annual crops. Each cattle produces 8.5 kg of dry manure per day, which can be converted to 0.347 kg ethanol/day. Since approximately 120 million dry tons of cattle manure is available in the United States, it can produce 63 million tons of AD fiber as cellulosic feedstock via AD technology. A potential ethanol production of 1.67 billion gallons per year can be produced from this amount of biomass, which accounts for approximately 10% of the 16 billion gallons of cellulosic biofuel by 2022. The integrated process of anaerobic digestion and bioethanol production is able to utilize the main components of the biomass in a robust manner. The hemicellulose reducing sugars are consumed at higher rates than cellulose in the AD process, producing methane and carbon dioxide, which are combusted to generate heat and electricity. Therefore the problems associated with pentose fermentation are avoided and the cellulose is utilized in the biorefinery for ethanol production with a robust hexose fermenting commercial yeast strain, *Saccharomyces cerevisiae*.

In addition, the optimal carbon/nitrogen (C/N) ratio of AD is 25–32:1 and cattle manure has a C/N ratio of 15:1 (Table 3.1.), which means that there is a potential of mixing other high C/N ratio agricultural residues such as corn stover and switchgrass with cattle manure to improve the performance of the digestion. This will greatly increase the amount of AD fiber, and lead to production of more ethanol from integrated animal operations and ethanol production.

The main reason AD fiber is a suitable feedstock for lignocellulosic ethanol production is based on the results from enzymatic hydrolysis and fermentation. The conversion efficiencies for raw cellulose to glucose and glucose to ethanol for CSTR AD fiber (62.6 and 80.3%) were consistent with that of switchgrass (61.1 and 78.0%) and corn stover (60.3 and 83.0%). The glucose and ethanol concentrations after enzymatic hydrolysis and fermentation for CSTR AD fiber (49.84 and 14.69 g/L) were also consistent with switchgrass (53.63 and 18.10 g/L) and corn stover (55.42 and 16.61 g/L). The CSTR AD fiber had lower glucose and ethanol concentrations due to the lower initial cellulose concentration and not the conversion efficiency.

Reduced particle size is another benefit of manure fibers after the AD process. Ninety-two percent of the CSTR AD fiber and ninety-six percent of the PFR AD fiber have a particle size smaller than 1 mm, compared to only seventy-five percent for washed raw manure. Corn stover and switchgrass necessitate energy intensive grinding to reach this particle size. Removing the size reduction unit from the bioethanol process will remove 22% of the capital investment on feedstock storage and handling within the production facility, greatly improving the efficiency of cellulosic ethanol production (Aden et al., 2002).

Reducing the total amount of water use for cellulosic ethanol production is also one of the keys towards a sustainable bioenergy solution. Two to six gallons of water are needed to produce a gallon of ethanol from cellulosic feedstocks such as switchgrass and corn stover (Aden, 2007). The mass balance on water showed a positive water demand for AD fiber ethanol production, which meant that the moisture in the manure provided enough water for the process.

The new model of ethanol production can be established based upon these results. A regional bioethanol production plant could be centralized within cattle/dairy farmland. In order to produce 20 million gallons of ethanol, 688 dry tons of manure cellulose is required per day as feedstock. Medium sized cattle or dairy farms with 1,000 cows generate 8.5 dry tons of manure per day. Using AD to treat this manure, each farm can produce 1.45 tons of dry AD cellulose per day. Four hundred seventy-five medium size cattle/dairy farms can produce 688 dry tons of AD cellulose per day for 20 million gallons of ethanol production.

Nationwide, eighty-two 20 million gallon cellulosic ethanol plants can be established using the AD fiber as feedstock. The year-round operation, compared with seasonal grain-based feedstocks, plus large available space on cattle and dairy farms, provide a local supply system for biomass distribution, significantly reducing the transportation and storage cost for the bioethanol production. The waste streams from ethanol production such as stillage can be transported back to the farm as animal feed or AD influent. In addition, the sustainability of cattle production systems will be improved by reducing the GHG emissions, potential surface and ground water pollution, and noxious odor, while at the same time generating electricity and AD fiber that will greatly enhance farm income. The integration of AD and cellulosic ethanol production will create a win-win-win solution for fuel ethanol production, cattle operations, and the environment.

Recommendations for further analysis include; addition of corn stover and switchgrass to the AD, the study of other pretreatment methods including steam explosion, enzyme loading tests, fermentation optimization, scale-up, economic analysis,

and life-cycle analysis of the overall conversion processes. All these areas must be addressed to reach further conclusions about the integrated process of anaerobic digestion and biorefining.

Appendices

Appendix A

Dilute Sodium Hydroxide Pretreatment Conditions and Hydrolysis Results

Table A.1. CSTR AD Fiber Pretreatment Conditions and Hydrolysis Results

Temperature (C)	Time (hr)	Alkali (%)	Glucose Concentration (g/L)	Cellulose Utilization Efficiency (%)
120	2	1	15.95	46.45
120	2	1	15.55	44.49
120	2	2	16.57	45.31
120	2	2	16.76	47.18
120	2	3	17.85	48.27
120	2	3	18.63	51.11
130	2	1	15.04	43.76
130	2	1	16.44	45.20
130	2	2	18.05	48.99
130	2	2	17.45	44.81
130	2	3	18.03	49.18
130	2	3	17.73	51.96
120	3	1	24.07	71.93
120	3	1	22.10	68.51
120	3	2	25.11	70.14
120	3	2	24.79	68.94
120	3	3	26.91	68.92
120	3	3	29.04	73.83
130	3	1	22.99	59.16
130	3	1	23.78	61.73
130	3	2	30.56	71.84
130	3	2	28.76	64.50
130	3	3	30.02	70.14
130	3	3	29.67	66.06

Table A.2. Switchgrass Pretreatment Conditions and Hydrolysis Results

Temperature (C)	Time (hr)	Alkali (%)	Glucose Concentration (g/L)	Cellulose Utilization Efficiency (%)
120	1	0.5	18.15	48.67
120	1	0.5	18.25	50.69
120	1	1.0	22.01	52.51
120	1	1.0	21.43	51.27
120	1	2.0	23.55	54.22
120	1	2.0	25.85	45.81
130	1	0.5	18.06	48.08
130	1	0.5	18.86	46.89
130	1	1.0	22.19	55.00
130	1	1.0	21.68	54.66
130	1	2.0	23.45	56.45
130	1	2.0	24.52	56.18
120	2	0.5	17.52	48.77
120	2	0.5	17.19	51.24
120	2	1.0	21.43	53.30
120	2	1.0	21.71	53.68
120	2	2.0	25.78	58.59
120	2	2.0	24.07	58.54
130	2	0.5	12.79	35.69
130	2	0.5	11.24	27.67
130	2	1.0	25.04	66.01
130	2	1.0	25.10	67.26
130	2	2.0	24.87	55.54
130	2	2.0	24.08	52.73
120	1	3.0	25.86	58.12
120	1	3.0	28.10	58.16
130	1	3.0	26.12	57.40
130	1	3.0	26.54	58.47
120	2	3.0	30.15	60.27
120	2	3.0	26.85	58.81

Table A.3. Corn Stover Pretreatment Conditions and Hydrolysis Results

Temperature (C)	Time (hr)	NaOH (%)	Glucose Concentration (g/L)	Cellulose Utilization Efficiency (%)
120	1	0.5	24.95	59.31
120	1	0.5	19.74	46.67
120	1	1.0	26.23	53.60
120	1	1.0	28.99	52.60
120	1	2.0	32.93	57.22
120	1	2.0	28.10	52.39
120	1	3.0	31.88	63.02
120	1	3.0	32.55	63.00
130	1	0.5	17.70	44.59
130	1	0.5	21.92	53.07
130	1	1.0	20.66	43.90
130	1	1.0	23.41	51.08
130	1	2.0	24.40	50.78
130	1	2.0	18.36	37.73
120	2	0.5	22.35	60.55
120	2	0.5	21.84	58.32
120	2	1.0	28.12	66.42
120	2	1.0	30.55	74.71
120	2	2.0	27.07	66.10
120	2	2.0	28.03	64.04
130	2	0.5	20.29	49.50
130	2	0.5	19.51	50.04
130	2	1.0	25.46	57.64
130	2	1.0	32.39	77.51
130	2	2.0	28.99	62.44
130	2	2.0	27.26	59.50

Appendix B

Statistical Analysis Software (SAS) Least Squares Means results for effect on glucose concentration and cellulose utilization efficiency for CSTR AD fiber

alkaline	temp	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	120	19.4175000	0.4046771	<.0001	1
1	130	19.5625000	0.4046771	<.0001	2
2	120	20.8075000	0.4046771	<.0001	3
2	130	23.7050000	0.4046771	<.0001	4
3	120	23.1075000	0.4046771	<.0001	5
3	130	23.8625000	0.4046771	<.0001	6

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The GLM Procedure
Least Squares Means

Least Squares Means for effect alkaline*temp
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4	5	6
1	.	0.8043	0.0318	<.0001	<.0001	<.0001
2	0.8043	.	0.0503	<.0001	<.0001	<.0001
3	0.0318	0.0503	.	0.0003	0.0017	0.0002
4	<.0001	<.0001	0.0003	.	0.3170	0.7878
5	<.0001	<.0001	0.0017	0.3170	.	0.2117
6	<.0001	<.0001	0.0002	0.7878	0.2117	.

Figure B.1. Least squares means results for effect of pretreatment temperature and alkali concentration, on glucose concentration of alkali pretreated CSTR AD fiber after enzymatic hydrolysis (5% solids).

alkaline	time	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	2	15.7450000	0.4046771	<.0001	1
1	3	23.2350000	0.4046771	<.0001	2
2	2	17.2075000	0.4046771	<.0001	3
2	3	27.3050000	0.4046771	<.0001	4
3	2	18.0600000	0.4046771	<.0001	5
3	3	28.9100000	0.4046771	<.0001	6

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The GLM Procedure
Least Squares Means

Least Squares Means for effect alkaline*time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4	5	6
1		<.0001	0.0252	<.0001	0.0016	<.0001
2	<.0001		<.0001	<.0001	<.0001	<.0001
3	0.0252	<.0001		<.0001	0.1621	<.0001
4	<.0001	<.0001	<.0001		<.0001	0.0159
5	0.0016	<.0001	0.1621	<.0001		<.0001
6	<.0001	<.0001	<.0001	0.0159	<.0001	

Figure B.2. Least squares means results for effect of pretreatment time and alkali concentration, on glucose concentration of alkali pretreated CSTR AD fiber after enzymatic hydrolysis (5% solids).

temp	time	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
120	2	16.8850000	0.3304175	<.0001	1
120	3	25.3366667	0.3304175	<.0001	2
130	2	17.1233333	0.3304175	<.0001	3
130	3	27.6300000	0.3304175	<.0001	4

Least Squares Means for effect temp*time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4
1		<.0001	0.6193	<.0001
2	<.0001		<.0001	0.0004
3	0.6193	<.0001		<.0001
4	<.0001	0.0004	<.0001	

Figure B.3. Least squares means results for effect of pretreatment time and temperature, on glucose concentration of alkali pretreated CSTR AD fiber after enzymatic hydrolysis (5% solids).

alkaline	time	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	2	44.9750000	1.2790373	<.0001	1
1	3	65.3325000	1.2790373	<.0001	2
2	2	46.5725000	1.2790373	<.0001	3
2	3	68.8550000	1.2790373	<.0001	4
3	2	50.1300000	1.2790373	<.0001	5
3	3	69.7375000	1.2790373	<.0001	6

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The GLM Procedure
Least Squares Means

Least Squares Means for effect alkaline*time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4	5	6
1		<.0001	0.3945	<.0001	0.0146	<.0001
2	<.0001		<.0001	0.0753	<.0001	0.0314
3	0.3945	<.0001		<.0001	0.0728	<.0001
4	<.0001	0.0753	<.0001		<.0001	0.6344
5	0.0146	<.0001	0.0728	<.0001		<.0001
6	<.0001	0.0314	<.0001	0.6344	<.0001	

Figure B.5. Least squares means results for effect of pretreatment time and alkali concentration, on cellulose utilization efficiency of alkali pretreated CSTR AD fiber after enzymatic hydrolysis (5% solids).

alkaline	temp	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	120	57.8450000	1.2790373	<.0001	1
1	130	52.4625000	1.2790373	<.0001	2
2	120	57.8925000	1.2790373	<.0001	3
2	130	57.5350000	1.2790373	<.0001	4
3	120	60.5325000	1.2790373	<.0001	5
3	130	59.3350000	1.2790373	<.0001	6

The GLM Procedure
Least Squares Means

Least Squares Means for effect alkaline*temp
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4	5	6
1		0.0116	0.9795	0.8668	0.1631	0.4262
2	0.0116		0.0110	0.0159	0.0008	0.0025
3	0.9795	0.0110		0.8466	0.1701	0.4407
4	0.8668	0.0159	0.8466		0.1234	0.3393
5	0.1631	0.0008	0.1701	0.1234		0.5205
6	0.4262	0.0025	0.4407	0.3393	0.5205	

Figure B.4. Least squares means results for effect of pretreatment temperature and alkali concentration, on cellulose utilization efficiency of alkali pretreated CSTR AD fiber after enzymatic hydrolysis (5% solids).

temp	time	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
120	2	47.1350000	1.0443296	<.0001	1
120	3	70.3783333	1.0443296	<.0001	2
130	2	47.3166667	1.0443296	<.0001	3
130	3	65.5716667	1.0443296	<.0001	4

Least Squares Means for effect temp*time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4
1		<.0001	0.9041	<.0001
2	<.0001		<.0001	0.0069
3	0.9041	<.0001		<.0001
4	<.0001	0.0069	<.0001	

Figure B.6. Least squares means results for effect of pretreatment time and temperature, on cellulose utilization efficiency of alkali pretreated CSTR AD fiber after enzymatic hydrolysis (5% solids).

Appendix C

Statistical Analysis Software (SAS) Least Squares Means results for effect on glucose concentration and cellulose utilization efficiency of alkali pretreated corn stover

alkaline	temp	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0.5	120	22.2200000	1.3641844	<.0001	1
0.5	130	19.8550000	1.3641844	<.0001	2
1	120	28.4725000	1.3641844	<.0001	3
1	130	25.4800000	1.3641844	<.0001	4
2	120	29.0325000	1.3641844	<.0001	5
2	130	24.7525000	1.3641844	<.0001	6

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The GLM Procedure
Least Squares Means

Least Squares Means for effect alkaline*temp
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4	5	6
1		0.2438	0.0071	0.1169	0.0041	0.2138
2	0.2438		0.0008	0.0129	0.0005	0.0260
3	0.0071	0.0008		0.1468	0.7766	0.0778
4	0.1169	0.0129	0.1468		0.0904	0.7127
5	0.0041	0.0005	0.7766	0.0904		0.0466
6	0.2138	0.0260	0.0778	0.7127	0.0466	

Figure C.1. Least squares means results for effect of pretreatment temperature and alkali concentration, on glucose concentration of alkali pretreated corn stover after enzymatic hydrolysis (5% solids).

alkaline	time	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0.5	1	21.0775000	1.3641844	<.0001	1
0.5	2	20.9975000	1.3641844	<.0001	2
1	1	24.8225000	1.3641844	<.0001	3
1	2	29.1300000	1.3641844	<.0001	4
2	1	25.9475000	1.3641844	<.0001	5
2	2	27.8375000	1.3641844	<.0001	6

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The GLM Procedure
Least Squares Means

Least Squares Means for effect alkaline*time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4	5	6
1		0.9676	0.0761	0.0013	0.0267	0.0044
2	0.9676		0.0708	0.0012	0.0247	0.0040
3	0.0761	0.0708		0.0454	0.5706	0.1441
4	0.0013	0.0012	0.0454		0.1249	0.5156
5	0.0267	0.0247	0.5706	0.1249		0.3466
6	0.0044	0.0040	0.1441	0.5156	0.3466	

Figure C.2. Least squares means results for effect of pretreatment time and alkali concentration, on glucose concentration of alkali pretreated corn stover after enzymatic hydrolysis (5% solids).

temp	time	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
120	1	26.8233333	1.1138519	<.0001	1
120	2	26.3266667	1.1138519	<.0001	2
130	1	21.0750000	1.1138519	<.0001	3
130	2	25.6500000	1.1138519	<.0001	4

Least Squares Means for effect temp*time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4
1		0.7580	0.0033	0.4707
2	0.7580		0.0060	0.6751
3	0.0033	0.0060		0.0132
4	0.4707	0.6751	0.0132	

Figure C.3. Least squares means results for effect of pretreatment time and temperature, on glucose concentration of alkali pretreated corn stover after enzymatic hydrolysis (5% solids).

alkaline	temp	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0.5	120	56.2125000	3.1615471	<.0001	1
0.5	130	49.3000000	3.1615471	<.0001	2
1	120	61.8325000	3.1615471	<.0001	3
1	130	57.5325000	3.1615471	<.0001	4
2	120	59.9375000	3.1615471	<.0001	5
2	130	52.6125000	3.1615471	<.0001	6

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The GLM Procedure
Least Squares Means

Least Squares Means for effect alkaline*temp
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4	5	6
1		0.1480	0.2327	0.7729	0.4210	0.4364
2	0.1480		0.0160	0.0904	0.0348	0.4730
3	0.2327	0.0160		0.3552	0.6792	0.0615
4	0.7729	0.0904	0.3552		0.6005	0.2927
5	0.4210	0.0348	0.6792	0.6005		0.1273
6	0.4364	0.4730	0.0615	0.2927	0.1273	

Figure C.4. Least squares means results for effect of pretreatment temperature and alkali concentration, on cellulose utilization efficiency of alkali pretreated corn stover after enzymatic hydrolysis (5% solids).

alkaline	time	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0.5	1	50.9100000	3.1615471	<.0001	1
0.5	2	54.6025000	3.1615471	<.0001	2
1	1	50.2950000	3.1615471	<.0001	3
1	2	69.0700000	3.1615471	<.0001	4
2	1	49.5300000	3.1615471	<.0001	5
2	2	63.0200000	3.1615471	<.0001	6

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The GLM Procedure
Least Squares Means

Least Squares Means for effect alkaline*time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4	5	6
1		0.4250	0.8929	0.0016	0.7629	0.0190
2	0.4250		0.3544	0.0071	0.2787	0.0842
3	0.8929	0.3544		0.0012	0.8670	0.0147
4	0.0016	0.0071	0.0012		0.0009	0.2010
5	0.7629	0.2787	0.8670	0.0009		0.0107
6	0.0190	0.0842	0.0147	0.2010	0.0107	

Figure C.5. Least squares means results for effect of pretreatment time and alkali concentration, on cellulose utilization efficiency of alkali pretreated corn stover after enzymatic hydrolysis (5% solids).

temp	time	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
120	1	53.6316667	2.5813924	<.0001	1
120	2	65.0233333	2.5813924	<.0001	2
130	1	46.8583333	2.5813924	<.0001	3
130	2	59.4383333	2.5813924	<.0001	4

Least Squares Means for effect temp*time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4
1		0.0088	0.0883	0.1377
2	0.0088		0.0003	0.1520
3	0.0883	0.0003		0.0048
4	0.1377	0.1520	0.0048	

Figure C.6. Least squares means results for effect of pretreatment time and temperature, on cellulose utilization efficiency of alkali pretreated corn stover after enzymatic hydrolysis (5% solids).

Appendix D

Statistical Analysis Software (SAS) Least Squares Means results for effect on glucose concentration and cellulose utilization efficiency of alkali pretreated switchgrass

alkaline	temp	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0.5	120	17.7775000	0.3794596	<.0001	1
0.5	130	15.2375000	0.3794596	<.0001	2
1	120	21.6450000	0.3794596	<.0001	3
1	130	23.5025000	0.3794596	<.0001	4
2	120	24.8125000	0.3794596	<.0001	5
2	130	24.2300000	0.3794596	<.0001	6

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The GLM Procedure
Least Squares Means

Least Squares Means for effect alkaline*temp
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4	5	6
1		0.0005	<.0001	<.0001	<.0001	<.0001
2	0.0005		<.0001	<.0001	<.0001	<.0001
3	<.0001	<.0001		0.0047	<.0001	0.0004
4	<.0001	<.0001	0.0047		0.0311	0.2002
5	<.0001	<.0001	<.0001	0.0311		0.2990
6	<.0001	<.0001	0.0004	0.2002	0.2990	

Figure D.1. Least squares means results for effect of pretreatment temperature and alkali concentration, on glucose concentration of alkali pretreated switchgrass after enzymatic hydrolysis (5% solids).

alkaline	time	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0.5	1	18.3300000	0.3794596	<.0001	1
0.5	2	14.6850000	0.3794596	<.0001	2
1	1	21.8275000	0.3794596	<.0001	3
1	2	23.3200000	0.3794596	<.0001	4
2	1	24.3425000	0.3794596	<.0001	5
2	2	24.7000000	0.3794596	<.0001	6

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The GLM Procedure
Least Squares Means

Least Squares Means for effect alkaline*time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4	5	6
1		<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001		<.0001	<.0001	<.0001	<.0001
3	<.0001	<.0001		0.0166	0.0005	0.0002
4	<.0001	<.0001	0.0166		0.0810	0.0245
5	<.0001	<.0001	0.0005	0.0810		0.5179
6	<.0001	<.0001	0.0002	0.0245	0.5179	

Figure D.2. Least squares means results for effect of pretreatment time and alkali concentration, on glucose concentration of alkali pretreated switchgrass after enzymatic hydrolysis (5% solids).

temp	time	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
120	1	21.5400000	0.3098275	<.0001	1
120	2	21.2833333	0.3098275	<.0001	2
130	1	21.4600000	0.3098275	<.0001	3
130	2	20.5200000	0.3098275	<.0001	4

Least Squares Means for effect temp*time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4
1		0.5689	0.8582	0.0382
2	0.5689		0.6939	0.1070
3	0.8582	0.6939		0.0531
4	0.0382	0.1070	0.0531	

Figure D.3. Least squares means results for effect of pretreatment time and temperature, on glucose concentration of alkali pretreated switchgrass after enzymatic hydrolysis (5% solids).

alkaline	temp	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0.5	120	49.8425000	1.2828090	<.0001	1
0.5	130	39.5825000	1.2828090	<.0001	2
1	120	52.6900000	1.2828090	<.0001	3
1	130	60.7325000	1.2828090	<.0001	4
2	120	54.2900000	1.2828090	<.0001	5
2	130	55.2250000	1.2828090	<.0001	6

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The GLM Procedure
Least Squares Means

Least Squares Means for effect alkaline*temp
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4	5	6
1		0.0001	0.1425	<.0001	0.0305	0.0118
2	0.0001		<.0001	<.0001	<.0001	<.0001
3	0.1425	<.0001		0.0008	0.3951	0.1876
4	<.0001	<.0001	0.0008		0.0040	0.0104
5	0.0305	<.0001	0.3951	0.0040		0.6156
6	0.0118	<.0001	0.1876	0.0104	0.6156	

Figure D.4. Least squares means results for effect of pretreatment temperature and alkali concentration, on cellulose utilization efficiency of alkali pretreated switchgrass after enzymatic hydrolysis (5% solids).

alkaline	time	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0.5	1	48.5825000	1.2828090	<.0001	1
0.5	2	40.8425000	1.2828090	<.0001	2
1	1	53.3600000	1.2828090	<.0001	3
1	2	60.0625000	1.2828090	<.0001	4
2	1	53.1650000	1.2828090	<.0001	5
2	2	56.3500000	1.2828090	<.0001	6

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The GLM Procedure
Least Squares Means

Least Squares Means for effect alkaline*time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4	5	6
1		0.0011	0.0218	<.0001	0.0266	0.0011
2	0.0011		<.0001	<.0001	<.0001	<.0001
3	0.0218	<.0001		0.0031	0.9162	0.1252
4	<.0001	<.0001	0.0031		0.0025	0.0633
5	0.0266	<.0001	0.9162	0.0025		0.1046
6	0.0011	<.0001	0.1252	0.0633	0.1046	

Figure D.5. Least squares means results for effect of pretreatment time and alkali concentration, on cellulose utilization efficiency of alkali pretreated switchgrass after enzymatic hydrolysis (5% solids).

temp	time	C6 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
120	1	50.5283333	1.0474092	<.0001	1
120	2	54.0200000	1.0474092	<.0001	2
130	1	52.8766667	1.0474092	<.0001	3
130	2	50.8166667	1.0474092	<.0001	4

Least Squares Means for effect temp*time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: C6

i/j	1	2	3	4
1		0.0362	0.1389	0.8489
2	0.0362		0.4551	0.0515
3	0.1389	0.4551		0.1896
4	0.8489	0.0515	0.1896	

Figure D.6. Least squares means results for effect of pretreatment time and temperature, on cellulose utilization efficiency of alkali pretreated switchgrass after enzymatic hydrolysis (5% solids).